THE ROLE OF TRANSITION METALS IN PARTICLE TOXICITY

JANE DELAMERE McNEILLY

A thesis submitted in partial fulfilment of the requirements of the Degree of Doctor of Philosophy awarded by the University of Edinburgh

MRC Centre for Inflammation Research
University of Edinburgh

November 2004
I hereby declare that this thesis was written by me and that all of the work contained herein was performed by myself except where otherwise indicated.

Jane Delamere McNeilly
Abstract

Exposure to particulate air pollution is known to induce a spectrum of adverse pulmonary effects in both occupational and ambient environments. There is considerable evidence to suggest that oxidative stress-mediated mechanisms may be fundamental in the initiation and development of many of these effects, including the induction of an inflammatory response. The pathogenicity of different particle types depends on their inherent physical and chemical characteristics. Current research investigating the molecular mechanisms involved in environmental particulate-induced inflammation have consistently highlighted particle size, endotoxins, organics and transition metal content as key features driving the inflammatory process. The aim of this thesis was to determine the importance of transition metals in the pathogenicity of both an occupational and ambient air pollution particulate. Firstly, the contribution of soluble transition metals in the pro-inflammatory effects of two different particle types, welding fumes and diesel exhaust particles was examined. In addition, the molecular signalling mechanisms by which welding fumes induce inflammation were investigated. As basic models of pulmonary inflammation, the expression of the chemokine IL-8 in a type II alveolar epithelial cell line (A549) and the ability to cause inflammation as assessed by bronchoalveolar lavage of rats instilled with particles were studied.

Exposure of A549 cells to various fractions of welding fumes identified that the increased expression of IL-8 was entirely attributable to soluble components. The “washed” welding fume particulates had no IL-8 inducing activity. Further analysis, by chelation and Inductively Coupled Plasma-Mass Spectrometry, established that soluble transition metals were responsible for the enhanced expression of IL-8. The depletion of intracellular glutathione, concomitant with increased intracellular reactive oxygen species generation and the attenuated IL-8 production in the presence of the antioxidant N-acetyl-L-cysteine, suggested the involvement of an oxidant-mediated mechanism. In addition, the soluble fraction of welding fumes enhanced DNA-binding of NF-κB and AP-1 via phosphorylation of p38 MAPK. An acute inflammatory response was observed in rats following instillation of the soluble components of welding fumes. This was completely abrogated by pretreatment of fumes with a transition metal chelator and so confirmed the role of soluble transition metals in welding fume-induced inflammation. In contrast to welding fumes, both the particulate and soluble fractions of diesel exhaust particles enhanced IL-8 expression in alveolar epithelial cells. Furthermore, removal of either the organic or soluble transition metal components reduced, but did not abolish, IL-8 production suggesting synergism between the organic and metals components of diesel may drive the pro-inflammatory effects.

Overall, in terms of morphology, composition and potency, welding fumes and diesel exhaust particulates differ considerably. However, soluble transition metals play an important role in the pro-inflammatory effects of both particle types by acting via oxidant-mediated signalling mechanisms culminating in inflammatory gene expression.
Acknowledgements

I would like to extend my gratitude towards Professors Ken Donaldson and William MacNee for their guidance, encouragement and support. I would also like to thank Dr Iain Beverland for his initial input in obtaining my funding and Dr Mat Heal for his unrelenting patience and invaluable advice with regards to metals. I am indebted to the staff, past and present at the ELEGI laboratory, in particular Drs Al Jiménez, Hazel Jardine, Julie Wickenden, Ellen Drost, Peter Gilmour, Rodger Duffin and Irfan Rahman for their scientific input, advice, suggestions and continuous support. I would like to thank Drs David Anderson and Al Jiménez for reading the many versions of this thesis. I extend my thanks to Eileen Neal for her help and encouragement and Spike Clay and Dr David Brown for their invaluable assistance with the in vivo study. I would like to acknowledge Dr Leon Hibbs and Dr Mark Gibson for the metal analysis, Dr Alan Howe for providing the welding fumes, Cathy Simpson and Shona MacCall for their assistance with the flow cytometry, Stephen Mitchell for his technical assistance with the transmission electron microscopy, Dr John Cherrie and Steve Clark for the particle size distribution analysis and all the staff at the MFAA. In addition I would like to thank the COLT foundation for their financial support.

Finally I am forever grateful to my friends and family for their encouragement, in particular Perry for his continued patience and belief in me, my parents for their unrelenting support, encouragement and never-ending faith and my sister Ali who has been with me through all the ups and downs of this thesis.

I dedicate this thesis to Nana and Grandad Delamere.
I only wish you were here today.
Table of Contents

Chapter 1

Introduction

1.1 The Respiratory system ........................................... 2
1.2 The Alveolar Regions .............................................. 4
1.2.1 The Alveolar Macrophage ....................................... 6
1.3 Inflammation ....................................................... 7
1.4 Reactive Oxygen Species and the Lungs ......................... 11
1.4.1 Production of Reactive Oxygen Species by Endogenous Sources .......... 12
1.4.2 Production of Reactive Oxygen Species by Exogenous Sources ............ 14
1.5 Detrimental Effects of Reactive Oxygen and Nitrogen Species ............... 14
1.6 Antioxidant Defences in the Lungs ................................ 16
1.6.1 Glutathione ..................................................... 21

1.7 Cell Signalling ..................................................... 24
1.7.1 Extracellular Receptor Regulated Kinase 1/2 ...................... 27
1.7.2 C-Jun N-Terminal Kinases ...................................... 28
1.7.3 p38 MAPK ....................................................... 29
1.7.4 Redox Sensitive Transcription Factors ................................ 30
1.7.5 NF-κB ........................................................... 31
1.7.6 AP-1 ............................................................... 34

1.8 Particle Interaction with the Lungs ................................. 37
1.8.1 Particle Clearance Mechanisms in the Lungs ...................... 38
1.9 Hypothesized Mechanisms of Particle Induced Adverse Health Effects ....... 39
1.9.1 Ultra-fine Particles ............................................. 39
1.9.2 Transition Metals ............................................... 40
1.10 PM10 .............................................................. 44
1.11 Asbestos ........................................................... 46
1.12 Crystalline Silica (α-Quartz) ..................................... 47

1.13 Welding Fumes ....................................................... 49
1.13.1 The History of Welding ......................................... 49
1.13.2 Welding Processes ............................................. 49
1.13.3 Welding Fume Characteristics: Composition & Morphology .............. 52
1.13.4 Adverse Health Effects of Welding Fumes: Human Studies .............. 53
1.13.5 Metal Fume Fever .............................................. 55
1.13.6 Chronic Bronchitis 56
1.13.7 Respiratory Infection and Immunity 56
1.13.8 Lung Cancer 57
1.13.9 Toxicological Effects of Welding Fumes *In Vivo* 58
1.13.10 Toxicological Effects of Welding Fumes *In Vitro* 59

1.14 Diesel Exhaust Particles 61
1.14.1 Morphological & Chemical Characteristics of Diesel Exhaust Particles 61
1.14.2 Organics 61
1.14.3 Quinones 63
1.14.4 Epidemiological Evidence for the Effects of Diesel Exhaust Particles In Occupational Environments 65
1.14.5 Inflammatory Effects of Diesel Exhaust Particles 65
1.14.6 Adjuvant Effects of Diesel Exhaust Particles 67

1.15 Summary and Aims 69

Chapter 2

Materials & Methods

2.1 Culture of Human Type II Alveolar Epithelial Cells A549 72
2.2.1 Welding Fume Particles 72
2.2.2 Diesel Exhaust Particles (DEP) 73
2.3 Particle Characterisation: Transmission Electron Microscopy 73
2.4 Treatment of A549 cells with Particles 74
2.5 Cell Morphology 74
2.6 MTT assay for Cellular Metabolic Activity 75
2.7 Assessment of Cytotoxicity: LDH release 76
2.8 Determination of Protein concentration: Bicinchoninic Acid Protein Assay 77
2.9 φX174 RF1 Plasmid DNA Assay 78
2.10 Assessment of Total Glutathione Concentration (GSH + GSSG) 79
2.11 Isolation of Total Cellular RNA 81
2.12 Preparation of complementary DNA (cDNA) 82
2.13 Amplification of DNA by Polymerase Chain Reaction (PCR) 83
2.14 Assessment of Intracellular ROS 84
2.15 Enzyme-Linked Immunosorbent Assay for Interleukin-8 (IL-8) 86
2.16 Preparation of Nuclear and Cytosolic Extracts 87
2.17 Electrophoretic Mobility Shift Assay (EMSA) 88
2.18 Determination of Lipid Peroxidation by Thiobarbituric Acid
Reactive Substances (TBARS) ........................................... 89
2.19 Western Blotting ...................................................... 90
2.19.1 Preparation of Cell Lysates ........................................ 90
2.19.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) & Western Blotting ............................. 90
2.20 In Vivo Model .......................................................... 92
2.20.1 Intra-tracheal instillation and Bronchiolar Alveolar Lavage ................................................................. 93
2.20.2 Preparation of Cytospins & Differential Cell Counts ................................................................. 93
2.20.3 Enzyme-Linked Immunosorbent Assay (ELISA): MIP-2 ................................................................. 93
2.21 Statistical Analysis ...................................................... 94

Chapter 3
The Pro-Inflammatory Effects of Welding Fumes In Vitro

3.1 Introduction .............................................................. 97
3.2 Welding Fume Particle Morphology .................................. 99
3.3 The Effect of Welding Fumes on Metabolic Activity of Alveolar Epithelial Cells ................................................................. 101
3.4 Cytotoxicity of Welding Fumes ........................................ 103
3.5 The Effect of Welding Fumes on Alveolar Epithelial Cell Morphology ................................................................. 107
3.6 The Effect of Welding Fumes on IL-8 Gene Expression in Alveolar Epithelial Cells ................................................................. 109
3.7 The Role of the Soluble Fraction of Welding Fumes on IL-8 Gene Expression ................................................................. 111
3.8 The Effect of Welding Fumes on IL-8 Protein Production in Alveolar Epithelial Cells ................................................................. 112
3.9 Time Course of Release of Soluble Fractions of Welding Fumes ................................................................. 114
3.10 The Role of Soluble Transition Metals in Welding Fume Induced IL-8 production ................................................................. 115
3.11 The Effect of Welding Fumes on Intracellular GSH levels in Alveolar Epithelial Cells ................................................................. 116
3.12 Acellular Production of Reactive Oxygen Species by Welding Fumes ................................................................. 118
3.13 The Induction of Intracellular Reactive Oxygen Species by Welding Fumes ................................................................. 120
3.14 Lipid Peroxidation following Exposure to Welding Fumes ................................................................. 123
3.15 The Effect of Antioxidants on IL-8 production by Alveolar Epithelial Cells ................................................................. 126
3.16 Discussion ........................................................................ 129
### Chapter 4

**Metal Composition of Welding Fumes**

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Introduction</td>
<td>141</td>
</tr>
<tr>
<td>4.2</td>
<td>Metal Analysis of Welding Fumes</td>
<td>142</td>
</tr>
<tr>
<td>4.3</td>
<td>Correlations between Metal Concentration of Welding Fumes and IL-8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Production in Alveolar Epithelial Cells</td>
<td></td>
</tr>
<tr>
<td>4.3.1</td>
<td>Ability of Chromium to Mimic the Pro-Inflammatory Effects of Welding Fumes</td>
<td>144</td>
</tr>
<tr>
<td>4.3.2</td>
<td>Ability of Various Metal Combinations to Mimic the Pro-Inflammatory Effects of Welding Fumes</td>
<td>147</td>
</tr>
<tr>
<td>4.4</td>
<td>Discussion</td>
<td>150</td>
</tr>
</tbody>
</table>

### Chapter 5

**Signalling Mechanisms Involved in the Pro-Inflammatory Effects of Welding Fumes**

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>Introduction</td>
<td>157</td>
</tr>
<tr>
<td>5.2</td>
<td>Activation of NF-κB by Welding Fume in Alveolar Epithelial Cells</td>
<td>159</td>
</tr>
<tr>
<td>5.3</td>
<td>The Role of Welding Fume Fractions in NF-κ B-DNA Binding in Alveolar Epithelial Cells</td>
<td>161</td>
</tr>
<tr>
<td>5.4</td>
<td>The Effect of Welding Fumes on AP-1 in Alveolar Epithelial Cells</td>
<td>164</td>
</tr>
<tr>
<td>5.5</td>
<td>The Role of Reactive Oxygen Species in NF-κB and AP-1-DNA Binding in Alveolar Epithelial Cells</td>
<td>166</td>
</tr>
<tr>
<td>5.6</td>
<td>The Effect of Welding Fumes on the Activation of p38 MAPK</td>
<td>170</td>
</tr>
<tr>
<td>5.7</td>
<td>The Effect of a p38 Inhibitor on Welding Fume induced NF-κB &amp; AP-1 DNA binding</td>
<td>173</td>
</tr>
<tr>
<td>5.8</td>
<td>The Role of p38 in Welding Fume induced IL-8 Expression</td>
<td>176</td>
</tr>
<tr>
<td>5.9</td>
<td>Discussion</td>
<td>177</td>
</tr>
</tbody>
</table>

### Chapter 6

**The Inflammatory Effects of Welding Fumes In Vivo**

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1</td>
<td>Introduction</td>
<td>186</td>
</tr>
<tr>
<td>6.2</td>
<td>Analysis of Bronchoalveolar Lavage Fluid</td>
<td>187</td>
</tr>
<tr>
<td>6.2.1</td>
<td>Cellular Parameters of Lung Inflammation</td>
<td>187</td>
</tr>
</tbody>
</table>
Chapter 7

The Pro-Inflammatory Effects of Diesel Exhaust Fumes: A Comparative Study

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.1 Introduction</td>
<td>207</td>
</tr>
<tr>
<td>7.2 Diesel Exhaust Particle Morphology</td>
<td>209</td>
</tr>
<tr>
<td>7.3 The Effect of Diesel Exhaust Particle on Metabolic Activity of Alveolar Epithelial Cells</td>
<td>210</td>
</tr>
<tr>
<td>7.4 Cytotoxicity of Diesel Exhaust Particle</td>
<td>212</td>
</tr>
<tr>
<td>7.5 The Effect of Diesel Exhaust Particle on IL-8 Gene Expression in Alveolar Epithelial Cells</td>
<td>214</td>
</tr>
<tr>
<td>7.6 The Effect of Diesel Exhaust Particle on IL-8 Protein release by Alveolar Epithelial Cells</td>
<td>216</td>
</tr>
<tr>
<td>7.7 The Inflammatory Effects of Diesel Exhaust Particle In Vivo</td>
<td>220</td>
</tr>
<tr>
<td>7.8 The Role of Organics in the Pro-Inflammatory Effects of Diesel Exhaust Particles in Alveolar Epithelial Cells</td>
<td>223</td>
</tr>
<tr>
<td>7.9 The Role of Soluble Transition Metals in the Pro-Inflammatory Effects of Diesel Exhaust Particles in Alveolar Epithelial Cells</td>
<td>225</td>
</tr>
<tr>
<td>7.10 Production of Free Radicals by Diesel Exhaust Particles</td>
<td>227</td>
</tr>
<tr>
<td>7.11 The Role of Reactive Oxygen Species in the Pro-Inflammatory Effects of Diesel Exhaust Particles</td>
<td>229</td>
</tr>
<tr>
<td>7.12 Comparative Effects of Welding Fumes and Diesel Exhaust Particles on Alveolar Epithelial Cells</td>
<td>232</td>
</tr>
<tr>
<td>7.13 Discussion</td>
<td>235</td>
</tr>
</tbody>
</table>

Chapter 8

Summary and Future Work

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.1  Summary and Future Work</td>
<td>245</td>
</tr>
<tr>
<td>8.2  Summary</td>
<td>250</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
</tr>
<tr>
<td>------------------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>References</td>
<td></td>
</tr>
<tr>
<td>Appendices</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Reference Concentrations for Selected PAHs in SRM 2975</td>
</tr>
<tr>
<td>II</td>
<td>Reagents and Buffers</td>
</tr>
<tr>
<td>III</td>
<td>Size Distribution of Welding Fume Particles</td>
</tr>
<tr>
<td>IV</td>
<td>IL-8 Gene Expression in Alveolar Epithelial Cells</td>
</tr>
<tr>
<td>V</td>
<td>IL-8 Standard Curve</td>
</tr>
<tr>
<td>VI</td>
<td>IL-10 Protein Production in Alveolar Epithelial Cells</td>
</tr>
<tr>
<td>Publication</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AhR</td>
<td>aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>Al</td>
<td>aluminium</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulphate</td>
</tr>
<tr>
<td>ARDS</td>
<td>acute respiratory distress syndrome</td>
</tr>
<tr>
<td>ARE</td>
<td>antioxidant response element</td>
</tr>
<tr>
<td>Arnt</td>
<td>AhR nuclear translocator</td>
</tr>
<tr>
<td>As</td>
<td>arsenic</td>
</tr>
<tr>
<td>ATF-2</td>
<td>activating transcription factor-2</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BALF</td>
<td>bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BHT</td>
<td>butylated hydroxytoluene</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>bZIP</td>
<td>basic region leucine zipper</td>
</tr>
<tr>
<td>CA$^{2+}$</td>
<td>calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAPs</td>
<td>concentrated ambient particles</td>
</tr>
<tr>
<td>CB</td>
<td>carbon black</td>
</tr>
<tr>
<td>cc</td>
<td>cold competitor</td>
</tr>
<tr>
<td>Cd</td>
<td>cadmium</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>Co</td>
<td>cobalt</td>
</tr>
<tr>
<td>CO</td>
<td>carbon monoxide</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>Cr</td>
<td>chromium</td>
</tr>
<tr>
<td>CRE</td>
<td>cyclic AMP responsive element</td>
</tr>
<tr>
<td>CREB</td>
<td>CRE binding protein</td>
</tr>
<tr>
<td>Cu</td>
<td>copper</td>
</tr>
<tr>
<td>Cu,Zn-SOD</td>
<td>copper-zinc superoxide dismutase</td>
</tr>
<tr>
<td>CX</td>
<td>chelex treatment</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>cytochrome P450 1A1</td>
</tr>
<tr>
<td>DCF</td>
<td>2',7'-dichlorofluorescein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>DCFH-DA</td>
<td>dichlorodihydrofluorescein diacetate</td>
</tr>
<tr>
<td>DEP</td>
<td>diesel exhaust particulates</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DMTU</td>
<td>dimethylthiourea</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>dinucleotide triphosphates</td>
</tr>
<tr>
<td>DTNB</td>
<td>dithiobisnitrobenzoic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>DTPA</td>
<td>diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EC-SOD</td>
<td>extracellular superoxide dismutase</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ELF</td>
<td>epithelial lining fluid</td>
</tr>
<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular-signal regulated kinase</td>
</tr>
<tr>
<td>ETC</td>
<td>electron transport chain</td>
</tr>
<tr>
<td>F</td>
<td>fluoride</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorter</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
</tr>
<tr>
<td>FCAW</td>
<td>flux cored arc welding</td>
</tr>
<tr>
<td>Fe</td>
<td>iron</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescence immuno tagged cell sort</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehydes-3-phosphate</td>
</tr>
<tr>
<td>γGCS</td>
<td>γ-glutamycysteine synthetase</td>
</tr>
<tr>
<td>GMAW</td>
<td>gas shielded metal arc welding</td>
</tr>
<tr>
<td>GMAW-SS</td>
<td>gas shielded metal arc welding-stainless steel</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>GR</td>
<td>glutathione reductase</td>
</tr>
<tr>
<td>GRO-α</td>
<td>growth regulated oncogene-alpha</td>
</tr>
<tr>
<td>GS</td>
<td>glutathione synthetase</td>
</tr>
<tr>
<td>GSH</td>
<td>L-γ-glutamyl-L-cysteinylglycine, reduced form</td>
</tr>
<tr>
<td>GSHme</td>
<td>glutathione monoethyl ester</td>
</tr>
<tr>
<td>GPx</td>
<td>glutathione peroxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GSSG</td>
<td>L-γ-glutamyl-L-cysteinylglycine, oxidized form</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>4-HNE</td>
<td>4-hydroxy-2-nonenal</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HOCl</td>
<td>hypochlorous acid</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intracellular adhesion molecule-1</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>inductively coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>γ-IFN</td>
<td>γ-interferon</td>
</tr>
<tr>
<td>IgE</td>
<td>immunoglobulin E</td>
</tr>
<tr>
<td>IκB</td>
<td>inhibitory kappa B</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IL-1β</td>
<td>interleukin -1 beta</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>IL-8</td>
<td>interleukin-8</td>
</tr>
<tr>
<td>IPF</td>
<td>idiopathic pulmonary fibrosis</td>
</tr>
<tr>
<td>JAK</td>
<td>janus protein kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-jun N-terminal kinase</td>
</tr>
<tr>
<td>KPE</td>
<td>potassium phosphate-EDTA buffer</td>
</tr>
<tr>
<td>L'</td>
<td>lipid radical</td>
</tr>
<tr>
<td>LBT$_4$</td>
<td>leukotriene B4</td>
</tr>
<tr>
<td>LD</td>
<td>lethal dose</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>LOO'</td>
<td>peroxyl radical</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activate protein kinases</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK kinase</td>
</tr>
<tr>
<td>MEKK</td>
<td>MAPK kinase kinase</td>
</tr>
<tr>
<td>MFF</td>
<td>metal fume fever</td>
</tr>
<tr>
<td>MIP-1</td>
<td>macrophage inflammatory protein-1</td>
</tr>
<tr>
<td>MIP-2</td>
<td>macrophage inflammatory protein-2</td>
</tr>
<tr>
<td>MMAW</td>
<td>manual metal arc welding</td>
</tr>
<tr>
<td>MMAW-SS</td>
<td>manual metal arc welding-stainless steel</td>
</tr>
<tr>
<td>M-MLV</td>
<td>moloney murine leukaemia virus</td>
</tr>
<tr>
<td>Mn</td>
<td>manganese</td>
</tr>
<tr>
<td>Mn,SOD</td>
<td>manganese superoxide dismutase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MT</td>
<td>metallothionein</td>
</tr>
<tr>
<td>MTT</td>
<td>(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide</td>
</tr>
<tr>
<td>MS</td>
<td>mild steel</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl-L-cysteine</td>
</tr>
<tr>
<td>NADPH</td>
<td>( \beta )-nicotinamide adenine dinucleotide phosphate, reduced form</td>
</tr>
<tr>
<td>NADP+</td>
<td>( \beta )-nicotinamide adenine dinucleotide phosphate, oxidised form</td>
</tr>
<tr>
<td>nc</td>
<td>non-competitor</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-kappa B</td>
</tr>
<tr>
<td>NFAT</td>
<td>nuclear factor of activated T cells</td>
</tr>
<tr>
<td>Ni</td>
<td>nickel</td>
</tr>
<tr>
<td>NIK</td>
<td>NF-κB-inducing kinase</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization sequence</td>
</tr>
<tr>
<td>NP-40</td>
<td>nonident P40</td>
</tr>
<tr>
<td>NO'</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NO₂</td>
<td>nitrogen dioxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>( ^1\text{O}_2 )</td>
<td>singlet oxygen</td>
</tr>
<tr>
<td>( \text{O}_2^- )</td>
<td>superoxide anion</td>
</tr>
<tr>
<td>( \text{O}_3 )</td>
<td>ozone</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OEL</td>
<td>occupational exposure limit</td>
</tr>
<tr>
<td>'OH</td>
<td>hydroxyl radical</td>
</tr>
<tr>
<td>ONOO'</td>
<td>peroxynitrite</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>8-oxo-dG</td>
<td>8-oxo-7,8-dihydro-2'-deoxyguanosine</td>
</tr>
<tr>
<td>PAH</td>
<td>polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>PBS-CMF</td>
<td>( \text{Ca}^{2+}/\text{Mg}^{2+} )-free phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDTC</td>
<td>pyrrolidine dithiocarbamate</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>Phox</td>
<td>phagocyte NADPH oxidase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PM</td>
<td>particulate matter</td>
</tr>
<tr>
<td>PM(_{10})</td>
<td>PM with a mean aerodynamic diameter &lt; 10 microns</td>
</tr>
<tr>
<td>PM(_{2.5})</td>
<td>PM with a mean aerodynamic diameter &lt; 2.5 microns</td>
</tr>
<tr>
<td>PM(_{0.1})</td>
<td>PM with a mean aerodynamic diameter &lt; 0.1 microns</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear leukocytes</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethyl sulfonyl fluoride</td>
</tr>
<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acids</td>
</tr>
<tr>
<td>RANTES</td>
<td>regulated-on-activation normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RHD</td>
<td>Rel homology domain</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROFA</td>
<td>residual oil fly ash</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>S</td>
<td>soluble fractions</td>
</tr>
<tr>
<td>-'S</td>
<td>thyl radical</td>
</tr>
<tr>
<td>SAPK</td>
<td>stress activated protein kinase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>SH</td>
<td>thiol</td>
</tr>
<tr>
<td>Si</td>
<td>silicon</td>
</tr>
<tr>
<td>SIE</td>
<td>sis-inducible enhancer/element</td>
</tr>
<tr>
<td>SO_{2}</td>
<td>sulphur dioxide</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>Sol</td>
<td>soluble fractions</td>
</tr>
<tr>
<td>SP</td>
<td>surfactant protein</td>
</tr>
<tr>
<td>SRE</td>
<td>serum response element</td>
</tr>
<tr>
<td>SRF</td>
<td>serum response factor</td>
</tr>
<tr>
<td>SS</td>
<td>stainless steel</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducers and activators of the transcription</td>
</tr>
<tr>
<td>SV40</td>
<td>simian virus 40</td>
</tr>
<tr>
<td>TBA</td>
<td>thiobarbituric acid</td>
</tr>
<tr>
<td>TBARS</td>
<td>thiobarbituric acid reactive substances</td>
</tr>
<tr>
<td>TBE</td>
<td>tris-borate-EDTA</td>
</tr>
<tr>
<td>TCA</td>
<td>tricholoracetic acid</td>
</tr>
<tr>
<td>TCF</td>
<td>ternary complex factor</td>
</tr>
<tr>
<td>TD</td>
<td>toxic dose</td>
</tr>
<tr>
<td>TE</td>
<td>tris-EDTA</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TEMED</td>
<td>(N,N,N'-tetramethylethylenediamine)</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>TGF-α</td>
<td>transforming growth factor-alpha</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-beta</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>Ti</td>
<td>tin</td>
</tr>
<tr>
<td>TMP</td>
<td>1,1,3,3-tetramethoxypropane</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>Toc'</td>
<td>α-tocopheroyl radical</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoyl-13-acetate</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor associated factor</td>
</tr>
<tr>
<td>TRE</td>
<td>TPA-response element</td>
</tr>
<tr>
<td>TWI</td>
<td>the welding institute</td>
</tr>
<tr>
<td>Tyr</td>
<td>tyrosine</td>
</tr>
<tr>
<td>uf</td>
<td>ultra-fine (less than 100 nm)</td>
</tr>
<tr>
<td>ufCB</td>
<td>ultra-fine carbon black</td>
</tr>
<tr>
<td>ufTiO₂</td>
<td>ultra-fine titanium dioxide</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>vanadium</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cellular adhesion molecule-1</td>
</tr>
<tr>
<td>W</td>
<td>whole</td>
</tr>
<tr>
<td>Wa</td>
<td>washed particulate fractions</td>
</tr>
<tr>
<td>Zn</td>
<td>zinc</td>
</tr>
</tbody>
</table>

XIV
Chapter 1

Introduction
The Respiratory System

The respiratory system is a complex structure extending from the mouth and nose and terminating at the alveolus, where gas exchange between the blood and atmosphere occurs. The airways can be divided anatomically into two main regions, the upper and lower respiratory tracts. The upper respiratory tract which includes the nose and nasal cavities, the pharynx and the larynx, functions as a crude filter to remove large particles and equilibrate air with the pulmonary environment (Berne et al., 2000). Air enters the lower respiratory tract via the trachea where it is conducted into the two primary bronchi (Figure 1.1). The trachea and bronchi are supported by cartilage in the form of irregular sometimes crescentic rings, which progressively decrease in number with subsequent branching (Vander et al., 1994). The bronchi themselves branch sequentially into smaller airways initially sub-dividing into secondary, then tertiary bronchi leading to the terminal respiratory bronchioles which are devoid of any cartilage (Jeffery, 1995). Further branching of the bronchioles into the alveoli air ducts and alveolar sacs provide the large surface area, approximately 85 m², which facilitates maximum gas exchange. A terminal bronchiole plus its associated alveoli are termed a respiratory acinus (Berne et al., 2000; Jeffery, 1995). The walls of the alveolar ducts contain thin spiral bands of elastin and collagen and are surrounded by smooth muscle which enables the ducts to expand and contract upon inhalation and exhalation respectively and prevents the lungs from collapsing.

Progression from the bronchioles to the alveolar regions results in a change in epithelial cell population. The bronchioles are predominantly comprised of Clara cells, ciliated cells, goblet cells, serous cells and endocrine cells, which are critical for lung function (Jeffery et al., 1975). Clara cells are usually restricted to the terminal bronchi and act as progenitors of ciliated, mucus and brush cells, proliferating in response to injury of the bronchial epithelium (Hong et al., 2001). The mucous, serous and ciliated cells in this region form the mucociliary escalator. Mucus secreted from goblet cells traps airborne pollutants such as particles or microorganisms, and is then transported to the trachea via the actively beating cilia, where it is eliminated from the body by either expectoration or swallowing. In the alveolar regions, the epithelium becomes thinner and more cuboidal and mucus-secreting
cells are replaced by flattened type I epithelial cells, capable of gas exchange (Plopper et al., 1997).

Figure 1.1 Anatomy of the Respiratory System. (Adapted from Raabe, 1999; Kierszenbaum, 2002; Martini, 2004). A. Alveolar duct; B. Alveolar sac; C. Alveoli
1.2 The Alveolar Region

The gas-exchange region of the lungs is comprised of numerous alveoli, each having an approximate volume of 187 mm$^3$. The alveoli are lined by a complete simple squamous epithelium which is separated from the interstitium by basement membrane and consists of two morphologically distinct cell types, Type I and Type II pneumocytes or squamous and granular cells respectively (Jeffery, 1995). The adult lung contains ~ 300 million alveoli, each measuring approximately 250 µm in diameter when expanded (Jeffery, 1995). Type I pneumocytes form approximately 90-95 % of the alveolar epithelial surface, yet account for only 4-5 % of the total lung cell population (Crapo et al., 1983). These terminally differentiated flattened cells, 0.2 µm in thickness, have few cytoplasmic organelles, as their main function is to provide a complete barrier suitable for gas exchange. Their delicate nature, due to their large surface area and low metabolic activity make type I cells highly susceptible to damage from agents such as oxygen radicals, proteases and particles (Jeffery, 1995). Interspersed between these cells are thicker, cuboidal shaped type II pneumocytes, generally situated at the “corners” of the alveoli (Figure 1.2 A). Although more numerous than type I cells, due to their cuboidal shape they only cover approximately 5-10 % of the alveolar surface (Crapo et al., 1982; 1983).

In contrast to their type I counterparts, type II pneumonocytes, are highly metabolically active, covered at their apex by microvilli and contain numerous cytoplasmic organelles, including large, well-developed mitochondria and osmophilic lamellar inclusion bodies of surfactant (Figure 1.2 B). The primary metabolic function of type II cells is the synthesis and secretion of surfactant, which is predominantly composed of phospholipids, although ~ 10 % is comprised of serum proteins, apoproteins, SP-A to D. Pulmonary surfactant lines the alveoli, lowering the air-liquid surface tension and reduces the elastic recoil of the lung tissue, thereby preventing lung collapse. In addition, surfactant plays a role in host defences, containing antioxidant activity and bacteria opsonising proteins (Pison et al., 1994), which promote alveolar macrophage activity especially phagocytosis. In addition,
type II pneumocytes play a key role as progenitors of type I cells during normal tissue turnover and in response to cell injury (Finkelstein, 1990).

Figure 1.2.A. Terminal Bronchiole and Alveolus B. TEM of a type II alveolar epithelial cell (Kierszenbaum, 2002).
1.2.1 The Alveolar Macrophage

A further cell type present within the lungs is the macrophage which is classified according to its location; interstitial, intravascular or the predominant sub-population, the alveolar macrophage. Alveolar macrophages are "professional" phagocytes that play a critical role in pulmonary defence, guarding the lower airways by engulfing any deposited microbes or particles (Crapo et al., 1982). Following ingestion of these substances, the macrophages migrate towards the mucociliary escalator where the cell and its content are propelled out of the lungs, normally to be swallowed. The progenitors of alveolar macrophages are proliferating macrophage precursors in the interstitium of the lung and peripheral blood monocytes which migrate from the bloodstream into lung tissue, where they differentiate into macrophages (Adamson et al., 1980). The average lifespan of a macrophage is 21-28 days, with ~1% of the macrophage population undergoing division at any one time (Bitterman et al., 1984). These macrophages comprise > 80% of the total cell population in the bronchoalveolar lavage fluid of healthy lungs (Thomas et al., 1999). Under normal physiological conditions, the alveolar macrophages represent one of the principal defence mechanisms in the lung, protecting the respiratory epithelium from damaging agents. However, when activated, they may release harmful substances such as lysosomal, proteolytic enzymes and radical species that can damage lung tissue.
1.3 Inflammation

Inflammation within the lungs is a critical defence mechanism protecting the host from injury, by destroying and removing potentially harmful agents such as particles. It is characterized by initial increased vascular permeability, granulocyte accumulation, followed by infiltration of mononuclear phagocytes and is generally a self-limiting process and a return to normal tissue homeostasis (Larsen et al., 2000). The specific histology of an inflammatory response is determined by a combination of mediators including cytokines, chemokines and growth factors produced by a variety of cell types such as macrophages and epithelial cells.

Macrophages

Macrophages are a major cellular component of the innate immune system. In addition to their phagocytic activity in the removal of foreign organisms in response to various stimuli, they release a number of inflammatory mediators including cytokines, a group of soluble polypeptides and glycoproteins that act primarily in the local milieu in an autocrine and paracrine fashion (Strieter et al., 1996; Chung, 2002). These include tumor necrosis factor-alpha (TNF-α), and interleukin -1 beta (IL-1β), growth factors (Chung, et al., 1999); transforming growth factor-beta (TGF-β), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) and chemokines; interleukin-8 (IL-8) and growth regulated oncogene-alpha (GRO-α). The continued expression of these mediators plays a major role in chronic lung disease (Shapiro, 1999). The release of cytokines, such as TNF-α, IL-1β, up-regulates the expression of adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1) and endothelial-leukocyte adhesion molecule-1 (E-selectin) on the vascular endothelium (Tosi et al., 1992; Lazaar et al., 2002). These adhesion molecules tether leukocytes thus enabling extravasation into the tissue, an essential process involved in leukocyte recruitment. In addition, the production of the potent chemoattractant IL-8 results in the rapid recruitment of neutrophils into the lungs following insult.
Due to their proximity to the epithelium, macrophage-derived cytokines such as TNF-α and IL-1β stimulate the production of pro-inflammatory cytokines from epithelial cells, thus amplifying the inflammatory response (Strieter et al., 1989). Studies by Standiford et al. (1990), and Jiménez et al. (2002), demonstrated that cytokines released by alveolar macrophages in response to treatment of lipopolysaccharides (LPS) or particulate matter < 10 μm diameter (PM₁₀) stimulated the release of the pro-inflammatory cytokine IL-8 from alveolar epithelial cells (A549), demonstrating this paracrine amplifying action. A biphasic cytokine response to metal-rich welding fumes, was reported in the bronchoalveolar lavage fluid (BALF) of welders with an initial peak of TNF-α, 3 hrs post inhalation followed by enhanced levels IL-8 and IL-6, after 8 and 24 hrs respectively (Blanc et al., 1993). These studies illustrate the dynamic cytokine signalling network within the milieu of the lungs, whereby the release of mediators from one cell modulates the synthesis of receptor molecules and other cytokines in neighbouring cell types thus further stimulating the release of cytokines and enhancing inflammation. In addition to their involvement in cytokine synthesis, macrophages are also potent producers of lipid mediators such the leukotriene, LTB₄. LTB₄ is a potent chemoattractant for neutrophils which is thought to account for the majority of neutrophil chemotactic activity by macrophages immediately following stimulation (Martin et al., 1987; 1989).

Epithelial Cells

Epithelial cells also play an important role in the inflammatory response, either through the production of inflammatory mediators in responses to stimuli such as growth factors and cytokines or via the direct interaction with inflammatory cells through cell-cell adhesion molecules such as ICAM-1 (Tosi et al., 1992; Canonica et al., 1995). Alveolar and bronchial epithelial cells have been shown to release a plethora of inflammatory cytokines including IL-6 and 8, TNF-α, RANTES, MCP-1 and MIP-1 which influence the activation and function of numerous inflammatory cells. RANTES, a potent eosinophil attractant and the monocyte and basophil chemo-
attractant, MCP-1, are released by epithelial cells in response to mediators such as TNF-α and gamma-interferon (γ-IFN). In addition, the colony-stimulating factor GM-CSF, also an eosinophil and neutrophil chemokine, potentiates the differentiation and survival of these cells, thus demonstrating a pivotal role for these inflammatory cytokines in the pathophysiology of allergic diseases such as asthma (Borish et al., 1996). Indeed, diesel exhaust particles, which have an adjuvant effect, are known to cause increased release of both IL-8 and GM-CSF in bronchial and alveolar epithelial cells in vitro (Juvin et al., 2002; Baulig et al., 2003) and to induce airway hyper-responsiveness in both atopic and non-atopic mice via GM-CSF synthesis (Ohta et al., 1999). Epithelial cells also regulate inflammation via the production of reactive oxygen and nitrogen species, and lipid mediators such as leukotrienes, that can lead to bronchoconstriction and prostaglandins, which are enhanced during both acute and chronic inflammation (Adler et al., 1994; Gutteridge et al., 2000).

**Neutrophils**

A classical characteristic of the acute inflammatory response is an airway neutrophilia. The recruitment of polymorphonuclear cells (PMNs) into the airways involves the release of chemotactic factors such as IL-8 from activated macrophages and epithelial cells. Due to its function in gas exchange, the lung has two distinct circulations, the pulmonary and the bronchial (systemic) circulations, and the total leukocyte pools can be divided into the circulating and the non-circulating (marginated or sequestered) pools (Drost et al., 2002). Emigration of marginated neutrophils involves initial margination, following capture by rolling adhesive interactions mediated predominantly by selectins; P, E and L-selectins and adhesion to the endothelium via surface adhesion molecules VCAM-1 and ICAM-1,2 (Von Andrian et al., 1992). These adhesion molecules are up-regulated by the release of cytokines such as TNF-α and IL-1β from activated macrophages and epithelial cells. Emigration into the tissue from the pulmonary vasculature is the result of neutrophils' ability to deform in response to stimuli, thus enabling them to squeeze
between the endothelial cells and move up the chemotactic gradient to the inflamed region (Snyderman et al., 1981; Stockley, 1995). Once at the site of inflammation, activated neutrophils phagocytose and destroy invading pathogens via proteolytic enzymes, such as elastase and matrix metalloproteases present in cytoplasmic granules and lysosomes (Drost et al., 2002) and reactive oxygen species (ROS) generated by the NADPH oxide system (Forman et al., 2002; Ambruso et al., 2004).

Inflammation is a critical defence mechanism in the lungs but it can be harmful when it is uncontrolled and/or chronic. This arises from prolonged exposure to damaging agents, oxidative stress or additive interactions between these factors. The chronic inflammatory response may be pathogenic, leading to tissue destruction or fibrosis. During chronic inflammation, a secondary or reparative phase may occur in which polypeptide growth factors stimulate the recruitment and proliferation of mesenchymal cells and re-epithelialization of injured tissue. In response to epithelial cell injury, type II cells proliferate and differentiate into type I cells. Tissue repair involving deposition of extra-cellular matrix (collagens I and III, fibronectin and proteoglycans) is also increased in response to cytokines such as TGF-β. This sequence of events is essential in the reparative process to maintain the structural/functional status of the lungs, but if this process is disrupted or continually stimulated, it can lead to the replacement of functional tissue with fibrosis (Limper et al., 1992). For example, up-regulation of TGF-α, a potent mitogen for epithelial and mesenchymal cells and type II pneumonocytes, is associated with pulmonary fibrosis in rats exposed to both asbestos fibres and silica particles (Liu et al., 1996; Absher et al., 1993).

Pulmonary damage may occur as a result of increased release of toxic substances including oxygen radicals, proteases and defensins from inflammatory cells such as neutrophils, causing oxidative stress and a protease/antiprotease imbalance and creating a highly damaging environment in the lung (Kuhn, 1986; Rahman et al., 1996). It is hypothesized that particle-induced inflammation may add to underlying airway inflammation, a characteristic of conditions such as Chronic Obstructive Pulmonary Disease (COPD) and asthma. This may result in the
increased morbidity and mortality associated with increased air pollution (Donaldson et al., 2000; MacNee et al., 2000). Indeed, inflammation has been demonstrated to play a key role in the adverse health effects of numerous particle types (Donaldson et al., 2003), and it is suggested that oxidative stress, resulting from an antioxidan-oxidant imbalance may be a key mechanism involved in this process.

1.4 Reactive Oxygen Species and the Lungs

By virtue of its large surface area and position, the airspace epithelium is continually exposed to an array of potentially damaging agents, such as particulates, gases or microbes, many of which induce an oxidative burden. In addition, the oxygen tension within the airways and alveolar spaces is almost three times greater than in other tissues, thus favouring oxidative reactions (Jeffery, 1995). Free radicals are compounds which contain one or more unpaired electrons in their outer orbitals (Aust, 2004). Due to this configuration, free radicals are highly reactive, and when generated in intra-cellular environments they have the potential to attack and modify key cellular constituents such as proteins, lipids and DNA (Galaris et al., 2002). There are a number of endogenous mechanisms of free radical formation such as the release of superoxide molecules from the mitochondrial electron transport chain, (ETC) (Turrens, 2003) and the NADPH oxidase enzyme system (Frei, 1994), which can be increased during inflammation thus making the lung epithelium a target for oxidative damage. Although antioxidants are present within the lungs, free radicals produced in excess may not be removed effectively and the redox-balance may be tipped towards oxidation. This oxidative stress can have severe consequences, leading to cellular and tissue damage, inflammation and DNA damage and has been implicated in a number of human respiratory diseases including COPD (MacNee, 2001), acute respiratory distress syndrome (ARDS), emphysema (Cross et al., 1994), asthma (Henricks et al., 2001) and pneumoconiosis (Fubini et al., 2003).
1.4.1 Production of Reactive Oxygen Species by Endogenous Sources

As with any tissue, the cells of the respiratory system respire aerobically. Cellular respiration occurs within the mitochondria and involves the electron transport chain (ETC), culminating in the production of metabolic energy in the form of ATP. However, a small percentage of all oxygen that is reduced along the ETC is converted to superoxide anions (O$_2^-$) or singlet oxygen (1O$_2$) within the mitochondria (Freeman et al., 1982). In defence, superoxide anions undergo rapid dismutation to form hydrogen peroxide, H$_2$O$_2$, in the presence of one of the three isoforms of superoxide dismutase (SOD); MnSOD, present in the mitochondria, Cu,ZnSOD found in the cytoplasm and an extra-cellular form of Cu,ZnSOD, EC-SOD (Mates et al., 1999). In the presence of transition metals such as iron, H$_2$O$_2$ is involved in Fenton-chemistry resulting in the production of highly toxic hydroxyl radicals (OH$^-$) (Halliwell et al., 1995). The importance of this mechanism with regard to particulate air pollution is discussed in full in subsequent chapters of this manuscript.

Other sources of reactive oxygen species (ROS) include the NADPH-cytochrome P450 enzyme system present in the endoplasmic reticulum (Goeptar et al., 1995; Thannickal et al., 2000). This particular mechanism has been implicated in the harmful effects of diesel exhaust particles as discussed in detail later. Likewise, enzymatic pathways such as xanthine oxidase, cyclooxygenase, lipoxygenase and oxidation of glyceraldehydes, thiols and haem proteins all produce oxidants (Slater, 1984; Halliwell et al., 1985). Activated inflammatory cells, including macrophages, neutrophils and eosinophils generate ROS and reactive nitrogen species (RNS), in response to a variety of stimuli, including bacteria, particles and cytokines. This “respiratory burst” results in the accelerated uptake of oxygen which is converted to O$_2^-$ by the membrane NADPH oxidase system. The catalytic moiety of phagocyte NAPDH oxidase (Phox) is the membrane-associated flavocytochrome gp91phox, which is inactive in resting phagocytes (Lambeth, 2004). Upon activation, the cytosolic components (Rac2, p47$^{phox}$ and p67$^{phox}$) of the oxidase system assemble with membrane-associated flavocytochrome b$_{558}$ (cyt b), comprised of two subunits,
an NADPH binding site, two heme groups and bound FAD, which together form the active enzyme (Babior, 1999). The formation of this complex enables the flow of electrons via a two step mechanism, initially from NADPH to FAD (step 1) then to the heme of cyt b and finally molecular oxygen (step 2). The one-electron reduction of molecular oxygen results in the formation of superoxide anions (Bokoch et al., 2003; reviews by Babior, 1999 and Lambeth, 2004). Superoxide anions undergo rapid dismutation to H₂O₂ and in concert with O₂⁻ provide micro-molar concentrations of primary oxygen metabolites, which facilitate microbial killing (Laurent, 1995; Fantone et al., 1985). In addition, during the “respiratory burst” these activated phagocytes also produce nitric oxide and L-citrulline, by enzymatic conversion of L-arginine and O₂ by nitric oxidase synthase (NOS) (Reaction 1).

\[ \text{L-Arg} + \text{O}_2 + \text{NADPH} \xrightarrow{\text{NOS}} \text{NO}^\cdot + \text{L-citrulline} \]

The highly damaging molecule peroxynitrite (ONOO⁻) is then formed by the reaction of NO⁻ and O₂⁻. Neutrophils also contain numerous toxic intermediates within their cytoplasmic granules such as myeloperoxidase, lysosomal hydrolases, elastase and protease, (Styrt, 1989), which upon neutrophil degranulation, form the powerful antibacterial agent, hypochlorous acid (HOCl) via the interaction with H₂O₂ (Weiss, 1989; Reuben et al., 2004). However, if these oxidants are produced in excess, as would occur in chronic inflammation, they may cause severe damage to pulmonary tissue.
1.4.2 Production of Reactive Oxygen Species by Exogenous Sources

Many components present within inhaled air such as ozone, cigarette smoke and diesel exhaust particulates are potentially capable of causing oxidative stress in the lungs (Chen et al., 2004). They may act either directly by inducing oxidative stress involving mechanisms such as Fenton chemistry or quinone cycling or indirectly via the recruitment of inflammatory cells (Churg, 2003). In some cases a combination of both mechanisms occurs increasing the oxidative burden and enhancing the tissue damage. For example, cigarette smoke contains in excess of $10^{15}$ free radicals and oxidants per puff of which alkyl and peroxyl radicals are the predominant form (Pryor et al., 1983b). In addition quinones and hydroquinones which catalyze the oxidation of hydrocarbons are also present within cigarette tar (Pryor et al., 1983a). Consequently, cigarette smoke induces oxidative stress, causing pulmonary inflammation and resulting in an increased oxidant burden in the airways (Chow, 1993). The oxidative activity of a number of particle types including welding fumes and diesel exhaust particles are discussed in full in subsequent chapters.

1.5 Detrimental Effects of Reactive Oxygen and Nitrogen Species

Reactive oxygen species (ROS) are ubiquitous molecular entities generated during normal physiological processes (Gracy et al., 1999) and play an important role in signal transduction pathways regulating cell development, growth, survival and production of inflammatory mediators (Chen et al., 2004). However, when generated in excess the antioxidant defense mechanisms become overwhelmed and severe damage to cellular constituents such as lipids, nucleic acids and proteins may occur (Reuben et al., 2004). Oxidative stress is also a potent initiator of the inflammatory response and is described in detail in the following chapters of this thesis.

Damage to proteins may cause structural changes and alter enzyme activity as demonstrated by hydrogen peroxide-induced fragmentation of SOD (Choi et al., 1999). Oxidation of individual amino acids may lead to loss of function. Due to their
thiol or sulphhydryl groups, proteins containing cysteine and methionine are particularly susceptible to oxidation and nitration from transition metals resulting in the formation of disulfides, methion sulfoxide and sulfones (Aust, 2004). The formation of disulfide bonds can result in protein cross-linking which may inhibit proteolytic degradation of damaged proteins (Friguet et al., 1994). Likewise, peroxynitrite nitration of tyrosine residues may seriously alter signalling pathways involving tyrosine phosphorylation and cellular regulation via adenylation of tyrosine residues (Ischiropoulos et al., 1995; van, V et al., 1995). Oxidation of DNA may lead to cell death or mutations. DNA is particularly vulnerable to oxidation by hydroxyl radicals and lipid peroxidation by-products leading directly to DNA strand breakage and adduct formation (Doelman et al., 1990), or indirectly activating specific metabolic DNA strand-breaking pathways such as a calcium-dependent endonuclease (Birnboim, 1988). Although DNA repair mechanisms exist whereby damaged bases or nucleotides are excised, mis-repair can lead to mutations, modulating protein functions which can culminate in cancer. Increased formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), one of the most common oxidative DNA lesions is frequently observed following exposure to carcinogenic particles including crystalline silica and asbestos (Nehls et al., 1997; Schins et al., 2002; Yamaguchi et al., 1999; Schins, 2002).

As a result of their structure, lipids are highly susceptible to oxidative damage by a process termed lipid peroxidation. This process is initiated by the abstraction of a hydrogen atom from the methyl group on polyunsaturated fatty acids (PUFA), resulting in the formation of a carbon centered lipid radical (L·) (Figure 1.3) (Reaction 2), which forms a conjugated diene radical. In turn, this intermediate reacts with oxygen forming a peroxyl radical (LOO·) that can react with an adjacent PUFA side chain propagating a chain reaction (Reaction 3). This disruption of the lipid bilayer in the plasma membrane may lead to membrane blebbing and lysis. Lipid hydroperoxides can decompose, generating a number of potentially-damaging substances such as alkanes, ketones, hydroxy radicals and aldehydes such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) that can diffuse from their site of production causing further damage by oxidising proteins or DNA or by
inhibiting enzyme activity (Doelman et al., 1990; Esterbauer et al., 1991; Uchida et al., 1999). In addition, ROS can increase the release of arachidonic acids from phospholipids, resulting in enhanced levels of eicosanoids and isoprostanes, which are frequently measured as biomarkers of airway inflammation in diseases such as asthma and COPD (Wood et al., 2003).

\[ \text{[2]} \quad L-H + X' \rightarrow L' + X-H \]

\[ \text{[3]} \quad L' + O_2 \rightarrow \text{LOO}' \]

\[ \text{[4]} \quad \text{LOO}' + L-H \rightarrow L' + \text{LOOH} \]

Figure 1.3 Mechanism of Lipid Peroxidation

Oxidative stress, ROS/RNS and lipid peroxidation intermediates have numerous damaging effects within the cell such as inhibiting pro-inflammatory signalling enzymes, causing mutations, apoptosis and necrosis, and consequently have been implicated in a wide range of conditions including cancer, COPD, asthma, aging and Alzheimer's disease (review by Mates, 2000). It is therefore paramount that cells and tissues have effective defence mechanisms to protect against oxidant induced damage.

1.6 Antioxidant Defences in the Lung

The lungs possess complex antioxidant systems to minimize damage to biological molecules. This integrated system comprises various types of antioxidants, such as preventative antioxidants, which block the formation of new reactive oxygen and nitrogen species and scavenging antioxidants which effectively remove oxidants thus preventing or stopping free radical damage. The epithelial lining fluid (ELF) acts as the first line of defense against potentially damaging agents by forming the interface between the air-space and airway epithelium and the external environment.
The epithelial cells of the bronchial and alveolar regions of the lungs are bathed in a thin continuous film, 0.2-0.5 μm thick of ELF, which contains a number of low molecular weight antioxidants, metal-binding proteins, antioxidant enzymes and unsaturated lipids (Cross et al., 1994). Metal-binding proteins present in the ELF play an essential role in maintaining cellular homeostasis by binding transition metal ions, preventing autoxidation reactions such as oxidation of ascorbate and adrenalin, and inhibiting the formation of hydroxyl radicals via Fenton chemistry (Halliwell et al., 1990; van der V et al., 2000). Both transferrin and lactoferrin bind free ionic iron, thus preventing its participation in Fenton chemistry (Gutteridge, 1982). Lactoferrin is also released by activated neutrophils at sites of inflammation, and possesses a higher affinity for iron than transferrin at lower pHs (Halliwell et al., 1990). The principal copper-binding protein in human plasma is ceruloplasmin, which is thought to be important in the prevention of lipid peroxidation and α1-antitrypsin oxidation in blood (Vallyathan, 2004). In addition, ceruloplasmin also plays an essential role in iron metabolism as a ferroxidase enzyme, oxidizing iron from its reduced state (Fe2+) to the oxidized form (Fe3+) thus protecting against iron-driven free radical reactions such as lipid peroxidation (Gutteridge, 1983; Vallyathan, 2004). Non-enzymatic antioxidants such as ascorbic acid, α-tocopherol, uric acid and reduced glutathione, are also present within the ELF but their concentration varies. For example levels of GSH and ascorbate are more concentrated in the ELF than in the plasma, confirming their relative importance in protecting the airspaces (Halliwell et al., 1990). Table 1.1 describes the main antioxidants present within the lungs and specifies their antioxidant properties.
<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Extracellular</strong></td>
<td></td>
</tr>
<tr>
<td>Transferrin</td>
<td>Binds ferric ions (2 per mole protein)</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Binds ferric ions at lower pH (2 per mole protein)</td>
</tr>
<tr>
<td>Albumin</td>
<td>Binds copper, heme, scavenges HOCI</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>Ferroxidase activity, binds copper (non-specific), scavenger of O₂⁻ Reoxidation of copper by H₂O₂</td>
</tr>
<tr>
<td>EC-SOD</td>
<td>Catalytically removes O₂⁻ to H₂O₂ and O₂</td>
</tr>
<tr>
<td>EC-GSH Peroxidase</td>
<td>Catalytically removes H₂O₂ and hydroperoxides</td>
</tr>
<tr>
<td>Glutathione</td>
<td>Radical scavenger</td>
</tr>
<tr>
<td>Mucus</td>
<td>'OH radical scavenger</td>
</tr>
<tr>
<td>Uric acid</td>
<td>Radical scavenger and metal binder</td>
</tr>
<tr>
<td>Ascorbic acid (Vitamin C)</td>
<td>Radical scavenger (O₂⁻, 'OH, H₂O₂, ROO⁻)</td>
</tr>
<tr>
<td><strong>Intracellular</strong></td>
<td></td>
</tr>
<tr>
<td>SOD (Cu, Zn, Mn)</td>
<td>Catalytically removes O₂⁻ to H₂O₂ and O₂</td>
</tr>
<tr>
<td>Catalase</td>
<td>Removes H₂O₂ when present in high concentrations</td>
</tr>
<tr>
<td>Glutathione Peroxidase (Se)</td>
<td>Removes H₂O₂ when present in low steady-state concentrations. Removes organic hydroperoxidases (ONOO⁻)</td>
</tr>
<tr>
<td>Glutathione</td>
<td>Radical scavenger</td>
</tr>
<tr>
<td><strong>Membrane</strong></td>
<td></td>
</tr>
<tr>
<td>α-Tocopherol (Vitamin E)</td>
<td>Lipid soluble, Chain breaking antioxidant (ROO⁻)</td>
</tr>
<tr>
<td>β-carotene</td>
<td>Lipid soluble radical scavenger and O₂ quencher</td>
</tr>
</tbody>
</table>

*Table 1.1. Antioxidants present within the lungs. (Sies, 1993; Halliwell et al., 1995; 1999)*
Enzymatic Antioxidants

The major enzymatic antioxidants present in the lungs are superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx). The three main isoforms of SOD are classified according to their cellular distribution and associated metal ions; Cu,Zn-SOD is present in the cytoplasm, MnSOD is found in the mitochondria and extracellular (EC-SOD), which is expressed in the highest concentration in the human lung is associated with the plasma membrane (Sies, 1993; Vallyathan, 2004). Whilst Cu,Zn-SOD is constitutively expressed in the lung, a variety of stimuli including cytokines, particles and fibres enhance the expression of MnSOD (Janssen et al., 1994; Vallyathan, 2004). The main role of SOD is as a scavenging antioxidant, catalysing the dismutation of $\text{O}_2^-$ to hydrogen peroxide and oxygen. The resultant hydrogen peroxide is subsequently reduced to water, catalysed by the haem-containing protein, catalase. SOD and catalase thus function in tandem to protect the respiratory epithelium from oxidant damage. EC-SOD has been implicated as an important regulator of particle-induced oxidative stress and lung damage, as illustrated by the decrease in lung injury in EC-SOD over-expressing mice compared to wild type following instillation of residual oil fuel ash (ROFA) (Ghio et al., 2002b).

In addition to its role in the catalytic dismutation of $\text{H}_2\text{O}_2$, catalase also acts as a peroxidase at low $\text{H}_2\text{O}_2$ concentrations utilising co-substrates such as ascorbate to breakdown $\text{H}_2\text{O}_2$ to water (Halliwell et al., 1999). In mammalian cells, NADPH is bound to catalase, which may protect this enzyme from inactivation by $\text{H}_2\text{O}_2$ (Kirkman et al., 1987). The selenenzyme, glutathione peroxidase (GPx) also catalyses the breakdown of hydrogen peroxide to water, in addition to its role in the oxidation of GSH to GSSG via reduction of hydroperoxidases, thereby protecting cells from oxidative damage.
Non-enzymatic Antioxidants

Non-enzymatic antioxidants react directly with oxidants and so are essentially “sacrificed” either becoming oxidised or forming radicals. These small-molecule antioxidants include ascorbic acid (vitamin C), α-tocopherol (vitamin E), uric acid and glutathione. The lipid-soluble antioxidant vitamin E is principally involved in the prevention or interruption of peroxidation of polyunsaturated fatty acids present in the cell membrane (Halliwell et al., 1993). It does so by donating a phenolic hydrogen, resulting in the formation of an α-tocopheroxyl radical (Toc'), which still has oxidant properties, albeit to a lesser extent than hydroxyl radicals (Chaudiere et al., 1999; Brigelius-Flohe et al., 1999) (Reaction 5).

\[
\text{LOO}^\bullet + \text{TocH} \rightarrow \text{LOOH} + \text{Toc}^\bullet
\]

Synergism between vitamin C and vitamin E occurs whereby ascorbic acid reacts with the potentially harmful α-tocopheroxyl radical, thus removing it and regenerating vitamin E. In turn, the vitamin C radical is reduced back to ascorbic acid via the NADPH system (Packer et al., 1979). These synergistic interactions are an essential feature of the antioxidant network that effectively protects cells by destroying radical species. In addition, the regeneration of reducing co-factors primarily via the glutathione and/or NAD(P)H pathways, provides a dynamic protective mechanism within cells (Chaudiere et al., 1999).

A number of other antioxidants are present within airway epithelial cells including thioredoxins, carotenoids, ubiquinone and metallothioneins (Chaudiere et al., 1999). Due their high affinity for metals such as zinc, copper, cadmium and mercury, the low molecular weight, cysteine-rich, metal-binding metallothionein proteins (MTs), are fundamentally involved in maintaining metal ion homeostasis and detoxification. This results in protection against consequences of oxidative stress arising from the production of hydroxyl radicals via Fenton chemistry. (Deneke, 2000; Theocharis et al., 2003). In addition, due their high cysteine content (~30 %)
(Lansdown, 2002), MTs are thought to have direct antioxidant properties (Coyle et al., 2002), acting as scavengers of free hydroxyl radicals and superoxide anions via oxidation of their sulphhydryl groups. They also confer protection against oxidant damage to DNA (Sato et al., 1999; Chubatsu et al., 1993). The MTs may be particularly important in protecting the airways from the adverse effects of airborne particle-associated metals which are hypothesized to play an important role in initiating oxidative stress and pulmonary inflammation.

Although all of the above antioxidants are important constituents of the cellular defence mechanisms, glutathione is often considered the principal intra and extra-cellular antioxidant. It protects against oxidative stress but also has a major role in protein and DNA synthesis (Meister, 1983), signal transduction, enzyme activity (Rahman, 2003), apoptosis and immune responses (Droge et al., 1994).

1.6.1 Glutathione

The tri-peptide γ-glutamyl-L-cysteinylglycine, glutathione (GSH), is an essential intra- and extra-cellular polypeptide thiol antioxidant, ubiquitously expressed within all cells (Figure 1.4.1). GSH synthesis involves the sequential catalysis of the amino acids; glycine, cysteine and glutamine by γ-glutamylcysteine synthetase (γ-GCS) (1) and glutathione synthetase (GS) (2) where γ-GCS and cysteine represent the rate limiting enzyme and substrate involved in de novo GSH synthesis (Meister et al., 1983; van, V et al., 2000) (Figure 1.4.2).

In the lung, glutathione is synthesized by type II epithelial cells and exported to the extra-cellular environment where it is present in high concentrations in the ELF in its reduced state (GSH). These levels are maintained by the limited availability of the enzyme γ-glutamyl transpeptidase (GT), present in the plasma membrane of pulmonary epithelial cells, which breaks γ-glutamyl bonds, releasing the three constituent amino acids. Cysteine and glutamine are then imported with the subsequent production of GSH (Meister et al., 1983). If the extra-cellular
environment contains a high thiol concentration, increased release of GSH occurs, whereas the presence of high concentrations of disulfides, creating an oxidizing environment, prevents GSH release and increases cellular uptake and re-synthesis (Lu et al., 1993). GSH maintains the redox balance within the lungs by directly scavenging oxidants via the donation of a hydrogen atom from its sulfhydryl group, resulting in the formation of glutathione disulphide (GSSG). GSSG is then reduced back to GSH by glutathione reductase (GR) in a reaction utilizing the co-factor NADPH. This is a critical step in maintaining the intracellular GSH/GSSG homeostasis which is vital for normal cellular physiological processes. This is illustrated by the numerous inflammatory conditions such as cystic fibrosis (Roum et al., 1993), IPF (Cantin et al., 1989) and ARDS (Bunnell et al., 1993), which have decreased levels of GSH in ELF, whereas, enhanced levels of total GSH are present in conditions such as asthma (Smith et al., 1993). Previous studies have demonstrated that acute exposure to oxidants such as cigarette smoke which cause an initial decrease in GSH concentrations, increase the expression of γ-GCS via up-regulation of the redox-sensitive transcription factor activator protein-1 (AP-1). This results in the enhanced production of GSH illustrating the compensatory mechanisms involved in GSH homeostasis (Rahman et al., 1995a).

Figure 1.4.1 Chemical structure of γ-glutamyl-L-cysteinylglycine, glutathione (GSH).
Figure 1.4.2. Schematic diagram depicting the sequential catalytic reactions involved in glutathione or \( \gamma \)-glutamyl-L-cysteglycine synthesis. \( \gamma \)-GCS: \( \gamma \)-glutamyl-L-cysteinglycine synthetase; GS: glutathione synthetase.
1.7 Cell Signalling

Many extra-cellular stimuli, including particles, oxidative stress, cytokines and other inflammatory mediators induce gene expression and other responses via the activation of the mitogen-activated protein kinase (MAPK) signalling pathways. There is growing evidence for the involvement of oxidants in many intracellular signalling pathways, as shown by inhibition of downstream signalling events in the presence of antioxidants (reviews by Chen et al., 2001b; Kyriakis et al., 2001).

The MAPK are a family of proline-targeted serine-threonine kinases that transmit stimuli from the cell membrane to the nucleus via sequential phosphorylation and dephosphorylation events involving the transfer of a phosphate group from an ATP molecule (Chang et al., 2001; Hagemann et al., 2001). Three distinct MAPK pathways have been described in mammalian cells, which appear to be conserved from yeast to man (Kumar et al., 2003). The extra-cellular signal regulated kinases 1 and 2 (ERKs), were the first members of the MAPK to be discovered and are by far the best-characterised signalling cascade. Subsequently, the c-jun-N-terminal kinase 1 (JNK1) and the p38 MAPKs were identified. Currently over 20 different MAP kinase isoforms have been reported, which are thought to be important in substrate specificity and provide distinct functional outcomes (Chen et al., 2001b). These kinases contain a 60-70 % homology, differing in the sequence and size of their activation loop, in addition to their specific response to various stimuli (Kumar et al., 2003). The MAPKs are organised hierarchically into three-tiered modules, activated by phosphorylation of threonine and tyrosine residues at the Thr-Xaa-Tyr (T-X-Y) dual phosphorylation motif by a dual specificity MAPK Kinase (MEK/MKKs). The Xaa corresponds to a glutamate residue Glu (E) in ERK, proline Pro (P) in JNK and glycine Gly (G) in p38 MAPKs (Paul et al., 1997). This dual phosphorylation confers the relative specificity of each MAPK sub-group, enabling their independent regulation (Hagemann et al., 2001; Chen et al., 2001b). The MAPK kinases, in turn are phosphorylated by a serine/threonine kinase termed MAPK kinase kinase (MEKKs) (Figure 1.5) (Martindale et al., 2002). In general, the ERK pathways are activated by mitogenic and proliferative stimuli, whereas the
JNK and p38 MAPKs, classically referred to as the stress-activated protein kinases (SAPKs), respond to cellular stress often resulting in growth arrest or apoptosis (Ichijo, 1999; Kyriakis et al., 2001). Stimulus specificity, cell type specificity and the balance between the ERKs and SAPKs may determine whether malignant cells undergo differentiation or apoptosis. Signalling through the MAPK pathways culminates in the phosphorylation-dependent activation of a variety of transcription factors leading to cellular responses including proliferation, inflammation and apoptosis (Davis, 1994; Kyriakis et al., 2001; Zhang et al., 2002).
Figure 1.5 Mitogen Activated Protein Kinase (MAPK) Cascade illustrating the specific residues phosphorylated and the down stream transcription factors targeted. MEKK:MAPK kinase Kinase; MEK: MAPK kinase. T: threonine; Y: tyrosine; S: serine; E: glutamate; P: praline; G: glycine. P: phosphate group.
1.7.1 Extra-cellular receptor Regulated Kinases 1/2

The ERK signalling cascade was the first MAPK to be characterised and is identified as a vital mediator regulating a number of cellular fates including growth, proliferation and survival (review by Kyosseva, 2004). Numerous extra-cellular stimuli including growth factors, cytokines and carcinogens activate the ERK1/2 pathway via protein tyrosine kinases or G protein-associated receptors resulting in a Raf-MEK1/2-ERK cascade (Hagemann et al., 2001). Alternative, MEK-independent mechanisms involving a calcium-dependent activation of protein kinase C have also been demonstrated to cause ERK activation in some systems (Bapat et al., 2001). Depending on the specific stimuli and the cellular environment, the ERK cascade can regulate diverse cellular processes (Pearson et al., 2001). The two isoforms termed ERK1 and ERK2, often referred to as p42/p44 MAPK cascades are ubiquitously expressed (Hommes et al., 2003), although their relative abundance in tissues is variable. The importance of the ERK pathway in cell survival was demonstrated by gene knock-out experiments where disruption of one of the three Raf proteins (A-Raf, B-Raf or Raf-C), (Figure 1.5), known to activate ERK 1/2, was lethal in mice (Pearson et al., 2001; Kolch, 2000). However, in cells undergoing genotoxic stress such as exposure to UV or arsenite, this same pathway may be activated to regulate gene expression resulting in either cell cycle arrest or apoptosis (Ludwig et al., 1996; Tang et al., 2002). Although the predominant role of ERK 1/2-mediated signalling involves cell growth and proliferation, it is now increasingly clear that ERK activation is involved in several inflammatory processes. For example, it is suggested that ERK activation is crucial for T cell activation, as illustrated by the defective thymocyte maturation and reduced expression of the α and β chains of the T cell receptors in ERK 1 deficient mice (Hommes et al., 2003). Furthermore, ERK MAPK has been shown to play a key role in the regulation of the AP-1 family of transcription factors such as c-Jun, c-Fos, and ATF-2, which are important in the regulation of cell proliferation, differentiation and inflammation (Ding et al., 1999).
The activation of ERK has been implicated in the pathogenicity of a number of particle types. Crocidolite asbestos fibres have been shown to modulate and activate a number of intracellular signalling pathways including the ERK pathway in rat pleural mesothelial cells. This activation pathway has been implicated in asbestos fiber-induced apoptosis in this cell type (Jimenez et al., 1997; Mossman et al., 1997). Similarly, the enhanced phosphorylation of ERK and resultant activation of AP-1 has been implicated in the initiation and progression of silica induced-cancer (Ding et al., 1999).

1.7.2 c-Jun N-Terminal Kinases

The JNK branch of the MAPKs are otherwise known as stress-activated protein kinases (SAPKs) due to their increased activation in response to cellular stresses such cytokines, UV irradiation and DNA damaging agents (Martindale et al., 2002). The regulation of JNK pathway is highly complex, influenced by approximately 13 different MEKKs, enabling a diverse array of stimuli to influence this MAPK (Johnson et al., 2002). Activation of JNKs is involved in cytokine gene expression as well as stress-induced and developmentally programmed apoptosis, proliferation, actin reorganization and cell transformation (Ip et al., 1998; Chen et al., 2001b). In parallel with ERK1/2, JNK appears to be essential for AP-1 activation, phosphorylating and increasing the transcriptional activity of c-Jun and ATF-2 (Johnson et al., 2002). Indeed, although JNK deficient mice are viable, they display defects in apoptosis and immune responses (Weston et al., 2002). Defects in AP-1 activity, decreased proliferation and resistance to stress induced apoptosis were found in isolated embryonic fibroblasts from these mice (Tournier et al., 2000), illustrating the essential role for JNK in many aspects of normal cellular physiology. For example the JNK signalling has been implicated in a variety of pathological conditions including cancer, stroke, ischemic heart disease and inflammatory disorders (Davis, 2000; Hommes et al., 2003).
Although JNK signalling is mediated via a plethora of extra-cellular stimuli and plays a key role in inflammation and apoptosis, few studies have demonstrated its role in pro-inflammatory effects of air pollution particles. Timblin et al. (1998), found increased c-jun kinase activity and enhanced levels of phosphorylated c-Jun and AP-1 transcription in pulmonary epithelial cells following exposure to non-toxic concentrations of PM. In addition, increased cellular proliferation and/or unscheduled DNA synthesis was reported in these cells, implicating the JNK pathway in these pathologically related endpoints. Likewise, Samet et al. (1998), showed that metals present in ambient PM such as Arsenic (As), Vanadium (V), Zinc (Zn), caused rapid phosphorylation of ERK, JNK and p38 and subsequent IL-8 protein production in human bronchial epithelial cells.

1.7.3 p38 MAPK Signalling Pathway

The isolation and subsequent cloning of a mammalian 38 kDa protein, rapidly tyrosine-phosphorylated in response to LPS resulted in the identification a new member of the MAPK family p38 (Han et al., 1994). To date five p38 MAPK isoforms have been identified; p38α, p38β, p38β2, p38δ and p38γ; sharing approximately 60 % homology (Herlaar et al., 1999; Martindale et al., 2002). Both p38α, p38β are ubiquitously expressed (Ono et al., 2000), whereas p38γ is predominantly expressed in skeletal muscles and p38δ is found in the lungs, kidneys, testis, pancreas and small intestine. The expression of multiple isoforms in mammalian cells suggests that these isoforms may differ in their substrate specificity, thus allowing various p38 isoforms to activate different signalling events (Enslen et al., 1998). A plethora of extra-cellular stimuli such as UV, osmotic stressors, cytokines and oxidative stimuli activate p38 MAPK. In addition, a number of pathogens including LPS and herpes simplex virus 1 induce p38 phosphorylation via different Toll receptors (Ono et al., 2000). This broad range of stimuli are able to activate the MEK/p38 cascade due to the large group of membrane bound receptors and a variety of small GTP-binding proteins such as Cdc42 or Rac that act as
upstream in the signalling cascade (Herlaar et al., 1999; Hommes et al., 2003) (Figure 1.5).

The p38 pathway shares many similarities with the other MAPK, being associated with inflammation, cell growth, differentiation and apoptosis (Shi et al., 2002; Hommes et al., 2003). The main biological function of p38 signalling involves the synthesis of inflammatory mediators essential for leukocyte recruitment and activation, and as such, is thought to play a central role in the regulation of a wide range of immunological/inflammatory responses associated with numerous diseases. Consequently, specific p38 inhibitors have been developed as potential therapeutic agents for inflammatory diseases such as Crohn's disease. The anti-inflammatory agents pyridinylimidazoles, such as SB 203580, suppress the expression of pro-inflammatory cytokines through inhibition of both active and inactive forms of p38α and p38β in various animal models of inflammation (Badger et al., 1996). Additionally, SB 203580 can regulate IL-12 and IFN-γ responses in vitro (Kumar et al., 1999) and therefore represents an invaluable agent to investigate the physiological processes involving p38 MAPK.

With regard to particle-induced inflammation and toxicity, increased phosphorylation and activation of p38 has been identified following exposure to various different particle types including asbestos fibres in mesothelial cells (Swain et al., 2004), diesel exhaust particles in organ cultures (Dai et al., 2003) and ROFA in rat airway and alveolar epithelial cells and resident macrophages (Silbajoris et al., 2000). The involvement of p38 in the pro-inflammatory effects of welding fumes is discussed in Chapter 5 of this manuscript.

1.7.4 Redox Sensitive Transcription Factors

Phosphorylation of various members of the MAPK cascades results in the activation of downstream transcription factors. These are ubiquitously expressed DNA-binding or co-factor proteins that regulate the rate of transcription of numerous genes, many of which play a critical role in inflammation. There is increasing
evidence that oxidants generated directly by particulates or via activated inflammatory cells, initiate such intracellular signalling cascades. Two of the most well characterised redox sensitive transcription factors are nuclear factor-kappa B (NF-κB) and activator protein 1 (AP-1) (reviews by Blackwell et al., 1997; Karin et al., 1997; Haddad, 2002a).

1.7.5 NF-κB

The Rel/NF-κB family of transcription factors regulate the expression of numerous cellular and viral genes which play an essential role in immune and stress responses, inflammation and apoptosis (Baldwin, 1996; Ghosh et al., 1998). These proteins contain a highly conserved sequence homology in a 300 amino acid region termed the Rel homology domain (RHD) which is required for specific DNA binding, dimerization, nuclear localization and interaction with inhibitory proteins, IκB (DiDonato et al., 1996; Li et al., 1999a). To date, five mammalian NF-κB family members have been identified and cloned to include NF-κB1(p50/p105), NF-κB2 (p52/p100), Rel A (p65), Rel B and c-Rel, (Ashburner et al., 2001; Chen et al., 2001a; Blackwell et al., 1997). These subunits form various homodimers or heterodimers, which confer specific affinities for DNA and different trans-activation potentials required to activate specific genes. The classical activated form of NF-κB consists of a p50/p65 heterodimer (Chen et al., 2001a; Rahman et al., 1998a).

In resting cells, NF-κB is sequestered in the cytoplasm as an inactive module through its high-affinity association with a class of inhibitory proteins called IκBs (IκB-α, IκB-β, IκB-ε). These IκBs prevent nuclear translocation of NF-κB through masking of the conserved nuclear localisation signals (NLSs) by interaction with ankyrin-like repeats of NF-κB (Beg et al., 1992; Malek et al., 2001; Li et al., 1999a). NF-κB is activated by numerous extra and intra-cellular stimuli, including mitogens, oxidants, phorbal esters, bacteria and viruses (Sun et al., 1996; Rahman et al., 1998a). In addition, cytokines such as TNF-α and IL-1β active NF-κB via a distinct family of cell surface receptor TNFR1 and ILR1 (Karin et al., 2000). These
NF-κB is however able to auto-regulate its activation via a negative feedback loop involving de novo synthesis of IkB (Blackwell et al., 1997; Sun et al., 1996). Both IkB-α and IkB-ε genes contain a κB recognition sequence within their promoter regions, therefore, stimuli that activate NF-κB also induce the synthesis IkB-α and IkB-ε (Baldwin et al., 1996; Whiteside et al., 1997). IkB-α and IkB-ε can enter the nucleus and bind to the NLS of NF-κB causing rapid down-regulation of NF-κB-controlled gene transcription. The NF-κB/IkB complex is then sequestered back to the cytoplasm (Arenzana-Seisdedos et al., 1995). The importance of IkB inhibition is illustrated by the sustained NF-κB activation and severe widespread inflammation in IkB-α -/- mice (Klement et al., 1996). Compounds such as glucocorticoids and anti-proteases can inhibit NF-κB activation by preventing IkB degradation or by increased IkB-α synthesis. For example the antioxidant N-acetyll-L-cysteine (NAC) can prevent the inducible decay of IkB-α by blocking the upstream kinases (IKKα and IKKβ) (Oka et al., 2000). Indeed, ROS have been directly implicated as secondary messengers in the oxidation of conserved cysteine-SH group essential for optimal DNA binding or the ubiquitination and proteolysis processes required for NF-κB activation (Toledano et al., 1991). NF-κB activation
can also occur via mechanisms independent of Ik-B degradation. Imbert et al., (1996) demonstrated that a protein tyrosine phosphatase inhibitor, pervanadate, induced NF-κB activation via tyrosine phosphorylation of Ik-B which unmasked the NLS of NF-κB enabling cytoplasmic to nuclear translocation and subsequent activation.

The activity of NF-κB is regulated through its association with cellular co-activators such as (CREB)-binding protein (CBP) and its structural homolog p300 (Kwok et al., 1994). Interaction between the co-activator cAMP response element binding protein, CBP and p65, either via phosphorylation of p65 by protein kinase A (PKAc) or interaction of the C-terminal portion of p65 with CBP, appears to be necessary to optimise the transcriptional activity of NF-κB (Zhong et al., 1998; Gerritsen et al., 1997). However, CBP is present in finite amounts, and interacts with various other transcription factors such as c-Jun, c-Fos, p53 and glucocorticoid receptors, (Kwok et al., 1994; Chrivia et al., 1993; Arias et al., 1994). Competition between NF-κB and other transcription factors for CBP or reduced p65 phosphorylation have been suggested as playing an important role in regulating NF-κB transcriptional activity (Shumilla et al., 1999). Indeed, both in vitro and in vivo studies have demonstrated the direct competition between NF-κB and CREB for CBP (Shenkar et al., 2001).

Activation of the NF-κB signalling pathway has been demonstrated to play an important role in the inflammomogenicity of a variety of pathogenic particulates including asbestos (Driscoll et al., 1998; Luster et al., 1998; Brown et al., 1999), quartz (Schins et al., 2000; Duffin et al., 2001), ROFA (Quay et al., 1998; Samet et al., 2002) and PM (Jiménez et al., 2000) in both in vivo and in vitro models. The work presented in this thesis investigated the involvement of this signalling pathway in welding fume-induced inflammation.
The transcription factor AP-1 is known to play an essential role in cellular proliferation and differentiation (Karin et al., 1997) and is involved in the transcriptional regulation of numerous inflammatory mediators (Rahman et al., 1998a). AP-1 is a sequence-specific transcription factor composed of proto-oncogenes as homodimers and a heterodimer of Jun (v-Jun, c-Jun, JunB, JunD), Fos (v-Fos, c-Fos, FosB, Fra-1, Fra-2) or activating transcription factor, ATF-2, ATF3/LRF1, B-(ATF) and bZIP (basic region leucine zipper) proteins (Sen et al., 1996; Karin et al., 1997). Expression of different members of these families varies throughout the cell cycle (Gius et al., 1999). Dimerization of fos-jun or jun-jun via a “leucine-zipper” domain is a prerequisite for DNA binding and serves as a critical regulator in the expression of a wide variety of genes (Angel et al., 1991). Different AP-1 dimers preferentially bind promoter sites in specific genes. For example, Jun-Jun and Jun-Fos dimers bind to a 12-O-tetradecanoate-13-acetate (TPA) response element (TRE) (Karin et al., 1997), whereas Jun-ATF complexes interact with the 8 bp DNA sequence 5'-TGACGTCA-3', also known as cyclic AMP-responsive element (CRE) (Figure 1.6 A)(Hsu et al., 1991; Reddy et al., 2002). AP-1 proteins are able to regulate cell-type and stimuli specific gene expression by virtue of their selective dimerization and binding specificity (Reddy et al., 2002).

In response to extra-cellular stimuli, AP-1 activity is regulated via transcriptional and post-translational processes, which modulate protein abundance, stability and activity (Jackson et al., 2002; Xanthoudakis et al., 1996). Transcriptional regulation rapidly enhances levels of the early response genes, c-Fos and c-Jun, thus augmenting levels of AP-1 (Karin, 1996). Post-translational regulation of pre-existing and newly synthesized AP-1 components occurs via phosphorylation (Karin, 1996). In the case of c-Jun, phosphorylation at residues 227-252, located next to its basic region, inhibits DNA binding by c-Jun homodimers but not c-jun:c-fos heterodimers (Boyle et al., 1991). Phosphorylation of serine and threonine residues at the amino-terminal activation domain by protein kinases prevents ubiquitation and degradation, increasing protein stability (Musti et al.,
1997). In addition, phosphorylation of c-Jun by JNK on Ser-73 and Ser-63 within the trans-activation domain augments transcriptional activity (Smeal et al., 1994; Pulverer et al., 1991) (Figure 1.6 A).

Several cis elements within the promoter of the c-fos gene mediate its transcriptional activation in response to a plethora of extracellular stimuli (Figure 1.6 B). Induction of c-fos occurs via cAMP and Ca$^{2+}$-dependent signalling pathways involving protein kinase C (PKC) and calmodulin-dependent kinase up-regulation of c-fos via a CRE in response to neurotransmitters and polypeptide hormones (Sheng et al., 1991). Alternatively, in response to stimuli such as growth factors and cytokines, c-fos can be up-regulated through the serum response-element (SRE) which is generally bound by the serum-response factor (SRF) complexed to ternary complex factors (TCFs). Phosphorylation of Elk-1, one of several TCFs, via MAPK, enables TCF and SRF interaction to occur, thereby enhancing c-fos transcription. Similarly, stimuli activating the Janus protein kinases (JAK), mediate c-fos induction via a Sis-inducible enhancer/element (SIE). Unlike c-jun which positively auto-regulates its own transcription, c-fos transcription is suppressed by Jun-Fos dimers (Curran et al., 1993; Whitmarsh et al., 1996).

Activation of AP-1 has been implicated in the pathogenicity of numerous particles such as asbestos (Heintz et al., 1993; Swain et al., 2004), quartz (Shukla et al., 2001; 2003b) and PM$_{10}$ (Gilmour et al., 2001). Up-regulation of early response gene mRNA, such as c-Jun, concurrent with increased proliferation was observed in C10 cells following exposure to low concentrations of ultra-fine carbon black (ufCB) (Timblin et al., 1998). In contrast, enhanced expression of proto-oncogenes and apoptotic genes were noted following treatment with high particle concentrations indicating particles may differentially activate proto-oncogenes forming various AP-1 complexes which may determine whether cells undergo proliferation or apoptosis.
Figure 1.6. Schematic diagram illustrating the regulation of (A) c-jun and (B) c-fos transcription in response to extra-cellular stimuli. The major cis elements located relative to the transcriptional start site (+1) are shown. Adapted from Karin, 1995 and Reddy et al., 2002.
1.8 Particle Interaction with the Lungs

By virtue of their function, the lungs are continually exposed to many potentially harmful pollutants present in the ambient air, including inhaled oxidants such as oxides of nitrogen, ozone, cigarette smoke and particles. For particulates, their physical and chemical characteristics determine the site of deposition and subsequent clearance from the lungs (Churg, 1996).

The amount of particulate entering the respiratory tract is influenced by factors including breathing pattern, breathing via nose or mouth and the level of activity of the individual (MacNee et al., 1999). The properties of particles: shape, size and density determine the aerodynamic diameter of a particle and therefore where in the respiratory system that particle deposition occurs.

Particle deposition occurs primarily via three different mechanisms: inertial impaction, sedimentation and diffusion (Foster, 1999). Inertial impaction describes the mechanism, whereby a sudden change in the direction of the air stream carrying particles, results in the particles continuing on in the initial direction which may lead to collision and deposition (Tsuda et al., 1994). This is the predominant mechanism of deposition for particles > 3μm and occurs primarily at airway bifurcation (Raabe, 1999). Sedimentation arises due to gravitational forces with particles of a higher density than air settling within the respiratory tract (Darquenne et al., 2000). Brownian motion, whereby very small particles and gaseous molecules collide at random, results in particle deposition by diffusion (Hofmann et al., 2003). Unlike impaction and sedimentation, which increase in direct proportion to particle size, an inverse relationship exists between particle size and diffusion therefore diffusion is the principal mode of deposition for particles < 0.5 μm (Raabe, 1999). In addition, particle deposition may also occur via secondary processes such as interception, which principally involves particles of a fibrous shape (Zhang et al., 1996) and electrostatic interactions between particles and the airway walls (Scheuch et al., 1990).

The pattern of respiration greatly influences the mode, degree, site and extent of particle deposition. Increased tidal volume results in a greater proportion of
particles depositing and interacting with the respiratory epithelium. Likewise, respiratory flow-rate can influence particle deposition by velocity-dependent mechanisms such as inertial impaction and principally affects particles > 3μm diameter (Raabe, 1999).

1.8.1 Particle Clearance Mechanisms in the Lungs

Although the lungs are continually exposed to airborne particulates, a number of chemical, physical and biological clearance mechanisms are present within the respiratory system to effectively deal with deposited particles. The clearance mechanism deployed is dependent on the site of deposition and the chemical and morphological characteristics of the particle. Larger, relatively insoluble particles are normally deposited in the ciliated region of the conducting airways where they are efficiently removed via the mucociliary escalator (Foster, 1999). Soluble particles that readily dissolve in lung lining fluid may be cleared via the systemic circulation (Raabe, 1999). Smaller particles (< 3 μm diameter), which deposit in the centriacinar region are rapidly phagocytosed by the resident alveolar macrophages, with the majority of particles located intra-cellularly within one day. However, the clearance of particle-loaded macrophages from deep within the lungs may require several months for completion (Foster, 1999). Although alveolar macrophages provide one of the major clearance mechanisms within the lung, when high particle deposition occurs over a long time period in rats, the clearance capacity of these cells is exceeded, a condition known as “overload” ensues (Cullen et al., 2000). This overload phenomenon in rats is characterized by slowed alveolar clearance, resulting in prolonged interactions between the particles and epithelial cells, stimulating increase oxidant production, inflammation, epithelial proliferation and pulmonary injury (Oberdorster, 1995). Chronic instillation studies in rats producing excessive lung particle burdens leads to chronic inflammation, proliferation, pulmonary fibrosis and tumor formation (Morrow et al., 1996; Mauderly et al., 1994).
1.9 Hypothesized Mechanisms of Particle Induced Adverse Health Effects

Particulate air pollution in both occupational and ambient environments is known to induce a range of adverse pulmonary effects ranging from exacerbations of asthma to lung cancer. There is considerable evidence to suggest that oxidative stress-mediated mechanisms involving the generation of ROS may be fundamental in the initiation and development of these adverse effects. Virtually all particles inhaled in excess are able to initiate an inflammatory response. The pathogenicity of different particles varies depending upon their inherent physical and chemical characteristics. Current research investigating the molecular mechanisms involved in particle-induced inflammation have consistently highlighted particle size, endotoxins, organics and transition metals as key features driving pulmonary inflammation.

1.9.1 Ultra-fine Particles

Ultra-fine particles are defined as particles less than 100 nm diameter. The importance of small particle size in mediating toxicity was originally derived from a study by Ferin et al., (1992), who demonstrated greater interstitialization and an enhanced inflammatory response in rats following inhalation of ultra-fine titanium dioxide (ufTiO₂) (25 nm) a low toxicity, low solubility particle, compared with its larger counterpart (TiO₂) (200 nm). More recently, Brown et al., (2001), found a direct correlation between particle surface-area and inflammation in rats instilled with various sizes of polystyrene beads, suggesting that surface area is an important parameter in the pro-inflammatory activity of ultra-fine particles. Considerable evidence now supports this hypothesis. Stone et al., (1998) found that exposure of epithelial cells to ufCB induced oxidative stress and enhanced expression of the pro-inflammatory cytokine IL-8, in the absence of transition metals. Similarly, treatment of rat alveolar macrophages (Brown et al., 2002; 2004) and human monocyte derived macrophages with ufCB stimulated a significant production of both TNF-α mRNA and protein (Duffin et al., 2002). Furthermore, Stone et al., (2000) demonstrated that ufCB increased cytosolic Ca²⁺, possibly via opening of Ca²⁺ channels in the plasma membrane, which in turn caused activation of NF-κB resulting in inflammatory gene
expression. It is suggested that uf particles may exert their pro-inflammatory effects via ROS-mediated mechanisms, altering intracellular calcium concentrations, activating NF-κB and AP-1 and resulting in increased pro-inflammatory cytokine production (Brown et al., 2004).

1.9.2 Transition Metals

Transition metals have also been implicated as key components in the pathogenicity of numerous particle types (Antonini et al., 2004a; 2004b; Carter et al., 1997; Ghio et al., 1999; Schins, 2002). The catalytic activity of transition metals is well documented in biological systems, playing an essential role in maintaining normal cellular homeostasis, function and survival (Galaris et al., 2002)(section 1.6). However, there is considerable evidence to suggest that exposure to higher-than physiological levels of several metals catalyse oxidative deterioration of biological molecules (Stohs et al., 1995). The relative toxicity associated with many of these metals is thought to depend on their ability to generate free radicals. Transition metals such as iron (Fe), copper (Cu) and vanadium (V) generate free radicals via Fenton-type (Reaction 6) or Haber-Weiss (Reaction 7) reactions (Wardman et al., 1996; Halliwell et al., 1985).

\[ \text{[6]} \quad \text{Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{2+} \cdot \text{OH} + \text{OH}^- \]

\[ \text{[7]} \quad \text{Fe}^{3+} + \text{O}_2 \cdot \rightarrow \text{Fe}^{2+} + \text{O}_2 \]

\[ \text{[8]} \quad \text{Overall:} \quad \text{H}_2\text{O}_2 + \text{O}_2 \cdot \rightarrow \cdot \text{OH} + \text{OH}^- + \text{O}_2 \]

Metals can undergo oxidation or reduction via the addition or subtraction of electrons from their outer valence orbitals, thus enabling them to change their oxidative state. This inherent redox-cycling characteristic makes metal elements effective biological catalysts in many enzymatic reactions (Halliwell et al., 1985). In order to achieve stability, metals must achieve an octet configuration in their d-
orbital, either by gaining or losing electrons (Halliwell et al., 1985). Metal-dependent reduction of H₂O₂ as illustrated in reactions [6]-[8], results in the production of highly deleterious hydroxyl radicals, which in turn can cause DNA adducts or strand breakage, lipid peroxidation, protein modification and alter enzyme activity (section 1.6). In addition, these reactions also allow the non-enzymatic formation of partially reduced oxygen species such as H₂O₂, O₂⁻, which in turn participate in Fenton-chemistry. These oxidative effects on bio-molecules may also lead to increased cellular permeability, genetic mutations and altered gene transcription (Stohs et al., 1995). Indeed, it has been shown that certain metal such as Chromium (Cr), Cadmium (Cd) and Nickel (Ni) can directly inhibit DNA repair mechanisms thus enhancing mutations (Hartwig et al., 2002). These metals may replace Zn in zinc binding protein structures, resulting in conformational changes which may decrease the activity of DNA repair enzymes. Alternatively, these replacements may cause the production of free radicals at critical positions near the DNA (Galaris et al., 2002).

Reactive oxidants are also produced via indirect mechanisms such as the reduction of metals including Arsenic (As), Cadmium (Cd), Cobalt (Co) and Chromium (Cr) by cellular reductants such as ascorbate, glutathione and NAD(P)H-dependent reductases. In addition, certain metals such as Cd and Cr can form complexes with thiols, preferentially binding functional groups, which in turn can lead to the formation of thyl radicals (-S'). Although these radicals are relatively stable, directly interacting to form disulfide bonds, they can also interact with molecular oxygen resulting in the generation of ROS, increasing the redox-cycling mechanism (Galaris et al., 1989; 2002).

The specific toxicity of metals is thought to relate to their inherent oxidation status, solubility, chemical reactivity, transport and complex formation within the cell and is shown in Table 1.2.
Metal | Effect | Reference
--- | --- | ---
Iron (Fe) | Involved in Fenton chemistry resulting in ROS production and lipid peroxidation Interacts with xenobiotic agents; paraquat, TCDD and quinones contributing to their oxidative and toxic effects including tumor formation and carcinogenesis. Reduction of Fe(II)-Fe(III) caused DS + SS DNA damage. | Warman et al., (1996); Halliwell et al., (1989); Stohs et al., (1995); Puntarulo et al., (1989); Al Bayati et al., (1987); Toyokuni et al., (1993)
Copper (Cu) | Involved in Fenton chemistry resulting in ROS production and lipid peroxidation. Oxidizes thiol groups forming Thiyl radicals Copper poisoning → hepatic necrosis; Involved in oxidation of hydroquinones to benzoquinones → cytotoxicity and DNA damage. Cu(II) cause DS DNA damage. | Stohs et al., (1995); Halliwell et al., (1989); Li et al., (1993b); Ozawa et al., (1993)
Chromium (Cr) | Well known carcinogen (Cr⁶⁺ most toxic form) due to increased bioavailability. Reduction of Cr⁶⁺ → redox active forms (Cr⁵⁺ + Cr⁴⁺) → ROS production. Cr⁶⁺ causes 8-oxo-dG formation in Jurkat cells. Cr³⁺ involved in Fenton chemistry. Cr⁵⁺ end product of Cr⁶⁺ oxidation is strongly ligated by thiols → cross-links between DNA and protein. Cr³⁺ increase catalytic activity and decrease fidelity of DNA polymerases → contribute to mutagenic and carcinogenic capacity. | Stohs et al., (1995); Shi et al., (1999a, 1999); DeFlora et al., (1989); Zhitkovich et al., (1995, 1996); Snow et al., (1994)
Vanadium (V) | Involved in Fenton chemistry resulting in ROS production and lipid peroxidation V(V) reduction to V(IV). Cytotoxic to cells in culture. Toxic to humans. Potent protein tyrosine phosphatase inhibitor. | Shi et al., (1993a, b); Riley et al., (2003); Samet et al., (1999)

Table 1.2 Adverse Effects of Specific Metals. DS: Double-Strand; SS: Single-Strand; TCDD: 2,3,7,8-tetrachlorodibenzop-dioxin.
Transition metals have been implicated as crucial components in the pathogenicity of numerous particle types from both occupational and ambient environments. Indeed, many of the effects of asbestos, a highly carcinogenic mineral fibre, are thought to involve the availability of iron on the fibre surfaces which augments hydroxyl formation in the presence of $\text{H}_2\text{O}_2$ via Fenton-like reactions (Kamp et al., 1992; Gulumian et al., 1987; Mossman et al., 1990). The metal content, bioavailability and oxidant activity are thought to play a critical role in the toxicological and inflammatory effects of welding fumes (Antonini et al., 1997; 1998; 2004a; Taylor et al., 2003).

Residual oil fly ash (ROFA) is a metal-rich particulate by-product of the combustion of fossil fuel oil containing a low organic content. It is frequently used as a surrogate particle type to investigate the role of metals and the combustion-derived components of ambient PM (Ghio et al., 2002a). It is postulated that the inflammatory effects of ROFA are mediated by metal-catalysed oxidant generation or metal ion dysregulation of phosphoprotein tyrosine metabolism (Samet et al., 1999). Oxidative stress has been shown to play a fundamental role in the cytotoxic effects of ROFA, as illustrated by the protective effects of the antioxidant DMTU (Dye et al., 1997; 1999). Likewise, the increased mRNA and protein expression of cytokines including IL-6, IL-8 and TNF-α in human airway epithelial cells in response to ROFA (Carter et al., 1997; Quay et al., 1998) are attenuated in the present of the transition metal chelator, deferoxamine and antioxidant, DMTU, suggesting that a metal-induced oxidative mechanism is involved. Tyrosine phosphorylation (P-Tyr) and the MAPK phosphorylation (Silbajoris et al., 2000) and activation of the transcription factors NF-κB, ATF-2 and c-Jun in rats lungs following exposure to ROFA, substantiates the role of metal-induced ROS in its inflammatory effects (Su et al., 1995).

The contribution of particle-associated metals in the adverse effects of ambient PM was exemplified by studies in the Utah valley. Closure of the local steel mill, the principal source of particulate air pollution, over a period of one year, saw a significant fall in PM concentrations and a concomitant 50 % reduction in children’s
hospital admissions for respiratory diseases, mortality and morbidity (Ghio et al., 2001). In line with epidemiological data, toxicological studies found a higher metal content in PM pre and post mill closure than during the closure period (Dye et al., 2001). Furthermore investigations demonstrated that soluble transition metals, predominantly Zn, Cu and V, were responsible for the inflammatory and toxic effects of Utah PM (Molinelli et al., 2002; Pagan et al., 2003). Similar effects have been reported for Edinburgh PM, with Gilmour et al. (1996), showing the free radical activity of PM was related to the iron content. More recently, Jiménez et al. (2000), demonstrated that the activation of NF-κB and resultant pro-inflammatory effects of PM₁₀ were regulated via an iron-mediated mechanism.

The toxicological characteristics of various particle types and their associated health effects are discussed in the following sections.

1.10 PM₁₀

Airborne particulate matter (PM) is a complex pollutant, comprised of biological and chemical components from a wide range of natural and anthropogenic sources. Consequently the physical, chemical and biological characteristics of PM can vary greatly (Harrison et al., 1996). PM₁₀ is defined as particulate matter capable of passing through a size selective inlet of 10 μm aerodynamic diameter with a 50 % efficiency. PM₁₀ can be sub-divided into three classes, ultra-fine, fine and coarse according to its mean aerodynamic diameter. Ultra-fine (PM₀.₁) and fine (PM₀.₁-2.5) originate primarily from combustion sources including vehicles and fossil fuels. In contrast, coarse (PM₂.₅-₁₀) is predominantly generated by mechanical processes.

Epidemiological studies have shown a consistent association between increased concentrations of airborne particles (PM₁₀), and adverse-human health effects (Dockery et al., 1993; Pope et al., 1999b). These effects range from exacerbation of respiratory symptoms and increased emergency hospital admissions, to increased morbidity and mortality from respiratory and cardiovascular diseases (Seaton et al., 1995, Martin et al., 1997). Meta-analysis of epidemiological studies conducted throughout Europe and the USA have shown that a 10 μg m⁻³ increase in
the concentration of PM$_{10}$ is typically associated with an approximate 1 % increase in all cause daily mortality, with mortality increasing to 1.4 % and 3.4 % for cardiovascular and respiratory causes, respectively (Dockery et al., 1993; COMEAP, 1996; Pope et al., 1999a).

Much research has focused on the localised pulmonary effects of PM, with both human and animal models demonstrating an enhanced pulmonary inflammation characterised by an influx of leukocytes and increased expression of inflammatory mediators with accompanying oxidative stress (Churg et al., 2000; Peden, 2002; Holgate et al., 2003b; Ghio et al., 2004). These symptoms are more pronounced in susceptible individuals with pre-existing respiratory illnesses.

The PM-mediated inflammatory response also has systemic manifestations. Studies have shown increased plasma viscosity, fibrinogen and c-reactive proteins in both human and rodents following exposure to PM (Pekkanen et al., 2000; Huang et al., 2003; Ulrich et al., 2002). It is hypothesised that pulmonary inflammation could alter the coagulation system. This could be by release of IL-6 from alveolar macrophages, which stimulate the secretion of fibrinogen from hepatocytes or by local release of tissue factor from activated monocytes which initiate the conversion of factor X to factor Xa which is involved in fibrogenesis (Ottaway et al., 1984; Seaton et al., 1995; Helin, 1986). The resultant conditions could facilitate thrombogenesis and reduce blood flow in the coronary and cerebral microvasculature, increasing the likelihood of heart attacks and strokes (Donaldson et al., 2001a). Decreased PMN deformability may also play a role in the cardiovascular effects associated with PM$_{10}$. Oxidatively stressed PMNs are less able to deform, thus are slowed down in the pulmonary capillaries where they may produce injurious substances such as proteases and ROS, so contributing to both chronic and acute inflammation (MacNee & Donaldson, 1999). Furthermore, decreased PMN deformability caused by particle-derived or inflammatory derived oxidative stress may increase PMN sequestration in the pulmonary micro-vasculature (MacNee & Donaldson, 1999).

It has also been suggested that PM$_{10}$-induced lung inflammation may accelerate the progression of atherosclerosis by causing rupture of atherosclerotic
plaques (Suwa et al., 2002; Brooke et al., 2004). Using a rabbit model of atherosclerosis, the Watanabe heritable hyperlipidemic WHHL rabbit, Suwa et al., (2002), showed that repeated exposure of PM\textsubscript{10} caused a systemic inflammatory response, characterised by bone marrow stimulation causing an enhanced leukocytosis which was associated with the advanced progression of atherosclerotic process in the coronary artery and aorta. Analysis of the atherosclerotic process at both sites found the extent of PM\textsubscript{10} phagocytosis correlated with the phenotypes of coronary lesions with an increase in the volume of extra-cellular lipids in the aortic lesions of PM-exposed animals. This alteration in the composition of atherosclerotic plaques indicates lesions are more unstable and vulnerable to rupture resulting in thrombogenesis, (Fernandez-Ortiz et al., 1994; Falk et al., 1995), suggesting a potential relationship between PM and excess cardiovascular deaths. An additional hypothesis regarding the relationship between PM\textsubscript{10} exposure and cardiovascular disease involves the autonomic nervous system. The irritant effects caused by air pollutants may initiate a neural reflex, leading to changes in heart rate, increasing the risk of fatal changes in its rhythm (review by Brook et al., 2004).

1.11 Asbestos

Asbestos, a ubiquitous, naturally-occurring mineral silicate with a fibrous morphology, has been widely used in industry since the late 1800’s. Due to its high tensile strength and heat- and chemical-resistant properties, asbestos was frequently used in the manufacture of various insulation products, brake pads and linings (Mossman et al., 1990). However, substantial evidence linking asbestos to the development of malignant and fibrotic diseases of the lung and pleura, resulted in the classification of asbestos as a group A human carcinogen (IARC, 1977). Asbestos is classified into two mineral groups; the serpentines and the amphiboles based upon fibre morphology and chemistry. Chrysotile, the sole member of the serpentines, is characterised by curly white pliable fibres with high magnesium content. These break down into thinner fibrils in tissue and are relatively soluble and so are more...
rapidly cleared from the lungs and pleura (Bernstein et al., 2003). These properties contribute to its lower pathogenicity compared to amphibole. In contrast, the amphiboles, which include crocidolite (blue asbestos), amosite (brown asbestos) and anthophyllite are straight, do not fibrillate and are more chemically stable in the lung environment (Churg et al., 1994). In addition to its higher bio-persistence, the high iron content of crocidolite evokes enhanced production of deleterious ROS via Fenton chemistry (Mossman et al., 1990). Epidemiological evidence has consistently demonstrated a significant proportion of workers exposed to asbestos such as miners, millers and ship builders develop some form of asbestos related disease such as bronchogenic carcinoma, asbestosis or mesothelioma following a latency period of 15-30 years (Mossman et al., 1989). Moreover, combined exposure to asbestos and cigarette smoking has been shown to synergistically enhance the risk of lung cancer (Kamp et al., 1992). Pulmonary inflammation, lung injury, fibrosis and mesothelioma represent some of the adverse effects observed in human and animals exposed to asbestos (Donaldson, 1996; Mossman et al., 1998; Morimoto et al., 2001). Exposure of lung mesothelial and epithelial cells to asbestos can cause proliferation and apoptosis as well as activation of the MAPK pathway and transcription factors NF-κB and AP-1, demonstrating the complex mechanisms involved in the pathogenicity of asbestos (BeruBe et al., 1996; Ramos-Nino et al., 2002a; Shukla et al., 2003a; Swain et al., 2004).

1.12 Crystalline Silica (α-Quartz)

Silicon dioxides make up almost 28 % of the earth’s crust and can exist in either a crystalline or amorphous form, with α-quartz representing the prevalent crystalline polymorph in occupational exposures (Castranova et al., 2000). Inhalation of crystalline silica is associated with lung disease such as silicosis, lung cancer and several autoimmune diseases (Fenoglio et al., 2003). However, different forms of silica are known to differ in their biological reactivity both in vivo and in vitro and this phenomenon has been attributed to particle surface characteristics relating to the structural differences between crystalline polymorphs (Donaldson & Borm, 1998).
The generation of ROS and RNS, via the reduction of siloxyl radicals on the surface of freshly fractured silica, has been implicated in the initiation of inflammation, lung damage, oedema, lung scarring and fibrosis (Castranova et al., 2000). Up-regulation of inflammatory cytokines and chemokines, fibrogenic factors such as TGF-β and mitogenic factors have been associated with the initiation and progression of silica-induced pulmonary fibrosis and lung cancer (Driscoll et al., 1993). Mechanistic studies have demonstrated the involvement of the MAPK signalling pathway (Ding et al., 2001) and the transcription factors NF-κB and AP-1 (Kang et al., 2000; Shi et al., 1998; Chen et al., 1999). The surface reactivity has been demonstrated to play a fundamental role in the pathogenicity of quartz (Donaldson & Borm, 1998). This is illustrated by the reduction in surface reactivity in terms of hydroxyl generation, chemokine production, transcription factor activation and inflammation in rats following exposure to quartz coated in aluminium lactate compared to untreated quartz (Duffin et al., 2001).

Although the generation of ROS and resultant oxidative stress are thought to play a major role in the adverse effects of particles, distinct differences are apparent in the pulmonary diseases they cause, suggesting unknown mechanisms are involved in the underlying toxicity.
1.13 Welding Fumes

1.13.1 The History of Welding

The history of welding can be traced back to the Bronze Age. Small gold circular boxes made by pressure welding are the earliest known examples. During the Iron Age, the Egyptians and Eastern Mediterraneans made tools by welding pieces of iron, many of which date back to around 1000 B.C. In the middle ages, the art of Blacksmithing was developed and many items were welded together by hammering. It was not until the late nineteenth century that the current welding processes were developed. World War I created a huge demand for armament production and consequently welding became a highly important industrial process in the War campaign (Howard, 1998).

Automated welding was introduced in 1920 and was widely used in the automobile industry to repair worn motor shafts and crane wheels. The gas shielded metal arc welding process was successfully developed in 1948 with the introduction of flux core arc and plasma arc welding in the early 1960's (Howard et al., 1998). More recent developments in technology have resulted in friction and laser welding, however, manual metal arc, flux core arc and gas metal arc are still the most commonly used processes to date. Currently, it is estimated that approximately one million workers are employed as full-time welders world-wide, with over three million people using welding are part of their everyday work duties (Sundin, 1998).

1.13.2 Welding Processes

The process of welding is widely used in numerous light and heavy industries accounting for 1% of the work force in industrialised countries (Hewitt, 2001). It provides a powerful tool for high-quality joining of metallic and non-metallic compounds using either heat and/or pressure. Essentially, all metals and alloys can be welded, however, the complexity of the procedure varies depending on the metal/alloys and the type of bonding involved. Electrical arc welding has been in
commercial use since 1882 and is commonly used in approximately 80 different types of welding and allied processes, which include soldering, thermal cutting and brazing (Sferlazza et al., 1991). The more conventional processes encompass Manual Metal Arc Welding (MMAW), Flux Cored Arc Welding (FCAW) and Gas shielded Metal Arc Welding (GMAW) or Tungsten Inert Gas Welding. Each of these processes has advantages for different welding situations and are selected primarily with regard to metallurgical, efficiency and economic criteria (Hewitt, 2001).

The simplest and most widely used process for both construction and repair work is MMAW (Hewitt, 2001). Electricity passing from one electrical conductor to another through a gas filled gap creates an arc discharge of electrical energy at approximately 4000 °F, a temperature greater than the boiling point of most metals contained in the welding rods (Lockey et al., 1988). This process heats both the base metal pieces to be joined and the filler metal of the consumable electrode or wire fed into the weld (Sferlazza et al., 1991) (Figure 1.7). When heated, the consumable rod produces oxygen-free shield gas, thus protecting the weld from oxidation which would weaken the joint. Molten fluxes from the consumable electrodes aid the removal of welding impurities in a liquid stream. Fluxes, which may be present as an integral part of the consumable electrode, are comprised of a metal rod coated with mixture of chemicals such as fluoride and silica and as such are a major source of the hazardous welding fume (Antonini, 2003).
Figure 1.7. Schematic diagram of Manual Metal Arc Welding (MMAW). The electrical current between the core wire and base metal creates an electric arc stream in the intervening gap. The heat of the arc melts the base metal, core wire and electrode coating, with the weld forming between two pieces of base metal. The vaporised electrode coating produces a shield of gas, excluding oxygen thus preventing oxidation of the weld. Adapted from Sferlazza et al., (1991).

Flux Core Arc Welding (FCAW) utilizes a special hollow wire consumable electrode, containing a suitable flux which on combustion provides a protective gas to shield the molten metal. This shield may be augmented by an auxiliary continuous stream of carbon dioxide (CO₂) gas. Gas shield metal arc welding (GMAW) is a development related to FCAW but depends solely on the supply of an external shielding inert gas, such as CO₂, helium or argon.
1.13.3 Welding Fume Characteristics: Composition & Morphology

The composition and rate at which welding fumes are generated are characteristic of the welding process and influenced by the welding current, shielding gases, technique employed and the composition of the electrode, filler wire and fluxes (Lockey et al., 1988; Sferlazza et al., 1991; Yu et al., 2000). When heated by the electrical arc, evaporation of the metal electrode and fluxes occurs. On contact with air, these vaporised metals become oxidized forming complex metal oxides that condense into very small particulates, classified as welding fumes (Sferlazza et al., 1991). The chemical properties of welding fumes may be complex as different pure metals commonly found in welding materials evaporate at different rates at a particular temperature depending upon their inherent vapour pressure (Howden et al., 1988; Lockey et al., 1988). The majority of welding materials are alloys consisting of metals such as silicon (Si), chromium (Cr), manganese (Mn) and nickel (Ni), characteristic of different steels. Generally, fumes generated from stainless steels (SS) electrodes contain approximately 20 % chromium and 10 % nickel, whereas fumes from mild steel (MS) electrodes are primarily comprised of iron (> 80 %) (Antonini, 2003). The rates at which these alloying elements evaporate vary depending upon their concentration in the steel.

The size of welding fumes particulates is a critical factor in determining the hazard potential of the fumes. Morphologically, welding fumes are characterised by long chains of particulate aggregates, containing individual particles within the ultra-fine range (<0.1 μm) (Clapp et al., 1977; Lockey et al., 1988; Aksellson et al., 1976). Thus, upon inhalation, there is a high level of particle deposition in the lower respiratory tract beyond the mucociliary clearance mechanisms (Sferlazza et al., 1991; Yu et al., 2000).
1.13.4 Adverse Health Effects of Welding Fumes: Human Studies

Welding has been classified as one of the most potentially hazardous occupations due to the multitude of potentially harmful factors associated with the welding process (review by Antonini, 2003). These include exposure to physical hazards such as intense heat, electromagnetic radiation, noise and vibration. UV light produced by electrical arc welding results in virtually all welders experiencing an acute kerato conjunctivitis, or "arc-eye" during their working career (Sferlazza et al., 1991). Chemical hazards include noxious gases (CO, CO₂, NOₓ and O₃) and particulates, many of which are hazardous in their own right (Amdur et al., 1991). In addition, the composition of fumes is highly variable and differs according to the process employed, the work piece and the surrounding environment. A summary of the most common welding fume components and their associated health effects is shown in table 1.3.

The health of welders has been studied extensively. Epidemiological studies have consistently reported a greater incidence of respiratory illnesses amongst welders due to exposure and inhalation of high concentrations of fumes. A large proportion of welders experience some kind of transient or reversible pulmonary disorder throughout their working lifetime (Lockey et al., 1988; Sferlazza et al., 1991). These include airway irritation, acute and chronic bronchitis, metal fume fever, occupational asthma and infrequent cases of acute chemical or hypersensitivity pneumonitis (Sferlazza et al., 1991; Graeme et al., 1998; Antonini, 2003).
<table>
<thead>
<tr>
<th>Fume Component</th>
<th>Source and Health Effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromium Cr$^{3+}$, Cr$^{6+}$</td>
<td>Present in SS and steel alloys fumes. Fumes containing Cr$^{3+}$ mutagenic activity. Cr$^{6+}$ Human carcinogen (TLV-TWA 0.5 mg m$^{-3}$) Linked to possible increase in mortality from lung cancer of SS welders</td>
<td>Cohen et al. (1993); Stern et al., (1986); Becker et al., (1985); Sjogren et al. (1994)</td>
</tr>
<tr>
<td>Nickel Ni</td>
<td>Present in SS fumes. Human carcinogen. Correlation between exposure of soluble and insoluble nickel compounds and increase incidence of nasal and lung cancer. SS fumes containing nickel are potentially mutagenic, increased risk of lung cancer.</td>
<td>Costa et al. (1991); Lauwerys, (1989); NIOSH, (1977); Hedenstedt et al. (1977); Gerin et al. (1984); Langard et al. (1994)</td>
</tr>
<tr>
<td>Iron Fe</td>
<td>Main component from most welding processes. Iron oxide: nuisance dust. Accumulates in alveolar macrophages and lung interstitium. Long term exposure- pneumonocosis (siderosis)</td>
<td>Howden et al. (1988); Sferlazza et al. (1991); Doig et al. (1951)</td>
</tr>
<tr>
<td>Manganese Mn</td>
<td>Present in most fumes as a flux agent. Cytotoxic and neurotoxic. Hypothesised fumes may cause central nervous disease resembling Parkinsons’ disease.</td>
<td>Cooper et al. (1984); Chandra et al. (1981); Sjogren et al. (1996); Racette et al. (2001)</td>
</tr>
<tr>
<td>Silica Si</td>
<td>Coating of electrode and flux. Silica in fumes; low cytotoxicity, amorphous form</td>
<td>Pantucek, (1971)</td>
</tr>
<tr>
<td>Aluminium Al</td>
<td>Additive in many steels and nonferrous alloys. Welding of aluminium conducive to production of pneumotoxic gas ozone</td>
<td>Howden et al. (1988)</td>
</tr>
<tr>
<td>Cadmium Cd</td>
<td>Sometimes used in fluxes found in flux-cored electrode. Reported to cause acute chemical inhalation lung injury. Clinical manifestations include bilateral pulmonary infiltration representing inflammation, haemorrhage and/or oedema. Also implicated in development of metal fume fever</td>
<td>Anthony et al. (1978); Townsend, (1968); Patwardhan et al. (1976)</td>
</tr>
</tbody>
</table>

Table 1.3. Specific hazards associated with various fume components generated in the welding process. SS: Stainless Steel. TLV: Threshold Limit Value TWA Time Weighted Average
1.13.5 Metal Fume Fever

The most frequently described respiratory illness amongst welders is metal fume fever (MFF), an acute, transient, systemic, febrile illness which may occur during or after welding (Offermann et al., 1992; Lockey et al., 1988). It is caused primarily by inhalation of zinc oxide fumes although similar clinical symptoms are observed following inhalation of fumes containing magnesium, copper or cadmium. It occurs most frequently amongst welders joining or cutting galvanized zinc-coated steel or other zinc alloys (Gordon et al., 1993). The onset of metal fume fever occurs approximately 4 to 8 hr after inhalation with the characteristics of a flu-like illness (Van Pee et al., 1998). These symptoms include high fever and sweating, dry cough, a sweet or metallic taste in the mouth, thirst and general malaise associated with a pulmonary inflammatory response. This acute, self-limiting illness peaks 5-12 hr post exposure and resolves within 24-48 hr (Sferlazza et al., 1991; Antonini et al., 1996).

Although the aetiology of MFF has been well characterised, the pathogenesis of this illness is poorly understood, with allergic and immunological mechanisms most frequently postulated (Graeme et al., 1998). It is suggested that the release of pro-inflammatory cytokines such as TNF-α and IL-1 from alveolar macrophages may be involved in the pulmonary inflammation and systemic responses associated with MFF (Blanc et al., 1991; 1993; Kuschner et al., 1995). In support of this hypothesis, a significant increase in both macrophages and PMN were reported in the BALF of workers 22 hr after exposure to zinc oxide fumes (Blanc et al., 1991). Similarly, enhanced levels of TNF-α, IL-1, IL-6 and IL-8 were measured in the BALF of workers following welding of galvanised steel (Blanc et al., 1993). The transient increase in TNF-α found at 3 hr, led the authors to suggest that the release of TNF-α from activated macrophages played a key role in the initiation of MFF.
1.13.6 Chronic Bronchitis

An increased prevalence of symptoms characteristic of chronic bronchitis is frequently observed amongst full-time welders (Sferlazza et al., 1991). There is, however, conflicting evidence of a direct link between exposure to welding fumes and the increased prevalence of chronic bronchitis. An early study by Fogh et al., (1969) found no significant difference in the rate of chronic bronchitis in 156 Danish welders compared to 152 controls at the same plant. Similarly, an evaluation of English shipyard welders of ≥ 45 yr in whom the rate in welders and controls was 50 % smokers and 33 % ex-smokers, found no difference in the prevalence of chronic bronchitis (McMillan et al., 1984). In contrast, Barhad et al., (1975) found that chronic bronchitis occurred 1.5 more frequently amongst welders in a Romanian shipyard than in control subjects. More recently findings from an epidemiological cross-sectional study suggested that welding fumes and smoking may act synergistically to induce bronchitis (Mur et al., 1985). In support of this theory, Akbarkhanzadeh et al., (1980) examined the long term health effects of welding on respiratory symptoms and lung function and found chronic bronchitis only in welders who smoked or had a previous smoking history.

1.13.7 Respiratory Infection and Immunity

The severity, duration and frequency of acute upper and lower respiratory tract infections are greater amongst welders than the general population (Howden et al., 1988). This increased incidence of respiratory infection is thought to arise from the chemical irritation of the airway epithelium following exposure to welding fumes (Kennedy, 1994). Several studies have identified an elevated mortality rate from pneumonia amongst welders (Wergeland et al., 2001; Palmer et al., 2003). Early studies found increased numbers of small opacities on chest radiographs of asymptomatic welders, which correlated with iron oxide deposits in lung autopsies (Doig et al., 1936). This iron oxide pneumonosis in welders later became known as welders’ siderosis (Doig et al., 1964) and was associated with long-term exposure to
arc welding fumes in confined spaces. There is usually little or no fibrosis or impaired respiratory function associated with this benign condition (Lockey et al., 1988). A study by Coggon et al., (1994) which analysed three occupational mortality data sets for England and Wales, found a significant increase in mortality from pneumonia amongst welders and suggested that lobar pneumonia be considered as an occupational disease in welders. Recently, Norwegian physicians were issued with a warning from the Norwegian Labour Inspection Authority regarding the potentially lethal risk association of pneumonia with inhalation of welding fumes from thermal metal work (Wegeland et al., 2001).

It has also been suggested that the increased susceptibility to infection amongst welders may be due to immuno-suppression. Immunological screening performed on 74 healthy shipyard welders found evidence of cell-mediated immune deficiency in 21.6 % welders (Boshnakova et al., 1989) although there is little other evidence to support this theory.

1.13.8 Lung Cancer

In 1990, the International Agency for Research on Cancer (IARC) concluded that welding fumes were “possibly carcinogenic” to humans (IARC, 1990). The decision was based on the limited evidence in humans and insufficient evidence in animals. In the analysis of 22 epidemiological studies looking at the risk ratios for lung cancer amongst welders, 9 out 22 identified a significant increase in the risk ratios for cancer (Lockey et al., 1988). It was suggested that the slightly elevated risk may be due to the inhalation of fumes with a high content of hexavalent chromium, a known carcinogen, generated by SS welding. Several studies have specifically examined the rate of lung cancer amongst welders exposed to fumes containing nickel and chromium. Sjogren et al., (1987) compared 234 SS-welders exposed to high levels of chromium to 208 railway track welders exposed to low levels of chromium and all subjects had at least a 5 yr welding history. Five deaths due to pulmonary tumours occurred amongst welders exposed to high chromium concentrations compared to one death in the low-exposed welders. Although this was

57
not significantly greater than the corresponding mortality in the general population, it led the authors to suggest a possible association between exposure to SS welding fumes and an increased incidence of lung cancer. Likewise a study investigating possible relationships between lung cancer and nickel exposure found a three-fold increase in lung cancer amongst subjects exposed to nickel (Gerin et al., 1984). However, there is also considerable epidemiological evidence reporting no association between welding and lung cancer (reviewed by Antonini, 2003).

1.13.9 Toxicological Effects of Welding Fumes In Vivo

Animal models have been used frequently to evaluate the relative inflammogenicity and toxicity of different welding fumes. Although many of these studies deliver fumes via intra-tracheal instillation, which is less physiologic than inhalation, it ensures the actual dose is delivered accurately to the lungs and provides useful information if well-controlled. A number of studies adopting this technique have provided important information regarding the mechanisms involved in pulmonary inflammation following welding fume exposures. An early study by White et al., (1981) comparing the toxicity of mild steel (MS) and stainless steel (SS) welding fumes in rats, showed that a single intra-tracheal instillation of MMAW-SS fumes were more acutely toxic than the mild steel counterpart (MMAW-MS). In a similar study, animals were instilled with welding fumes generated using different processes and materials to try and establish which welding fumes cause inflammation and lung damage. SS welding fumes were found to be more pneumotoxic and were retained in lungs longer that MS fumes. In addition, enhanced levels of the pro-inflammatory cytokines TNF-α and IL-1β were measured in the BALF of these animals, suggesting the possible mechanism behind the pulmonary inflammation and injury induced by MMAW-SS and GMAW-SS fumes (Antonini et al., 1997; 1996). These studies illustrated that fumes generated from different processes and electrodes vary in their inherent toxicity and differ in their inflammogenicity. Furthermore, Antonini et al., (1998) showed that freshly-generated welding fumes induced a greater inflammatory response in rats than aged fumes. Surface-associated ROS may

Welding Fume: Small particulates of condensed metal oxides.
therefore play a role in welding fume-induced inflammation and lung disease amongst welders. In support of this theory, a recent study by Taylor et al., (2003) confirmed that welding fumes differ in toxicity and inflammogenicity. This study identified that metal content, solubility and free radical activity correlated with the pulmonary injury, demonstrated by enhanced levels of LDH and albumin in the BALF of rats. Moreover, both the soluble and insoluble fractions of MMAW-SS were required to induce maximal inflammation and injury, suggesting that the mechanism involved in welding fume-induced pulmonary toxicity is not exclusively regulated by soluble transition metals.

1.13.10 Toxicological Effects of Welding Fumes In Vitro

Numerous studies have utilized in vitro culture systems to examine the toxicity of welding fumes and their constituents, and to assess the potential fate of specific cell types which may play an important role in disease. Many of the findings using alveolar macrophage culture corroborate those reported in vivo. Hooftman et al., (1988), demonstrated a concentration-dependent reduction in bovine alveolar macrophage viability and phagocytosis following treatment with MMAW fumes generated from SS, compared to fumes from MS. Likewise, Antonini et al., (1997) found that MMAW-SS fumes induced a greater release of ROS, and were more cytotoxic to freshly isolated alveolar macrophages than SS fumes generated from GMAW welding. More recently, the soluble components of the same MMAW-SS fumes were identified as being the most cytotoxic, causing the greatest effect on macrophage function compared to GMAW-SS or MS fumes. The authors concluded that the differences in fume solubility and the presence of various transition metals may play an important role in the resultant pulmonary effects following inhalation of welding fumes.

Genotoxicity assays have frequently been used to investigate whether different welding fumes cause DNA mutations, a critical event in the development of cancer. Hansen et al., (1985), showed that fumes generated from MMAW of
stainless steel electrodes were cytotoxic and caused cellular transformation of baby hamster kidney (BHK) cells. This effect appeared to relate to the insoluble form of Cr$^{6+}$, which had a greater effect on BHK cell transformation than the soluble form. Although both the soluble and insoluble forms of Cr$^{6+}$ caused changes in sister chromatin exchange in cultured Chinese hamster lung (Don) cells, this could not be entirely explained by chromium, indicating other components present in MMAW-SS fumes have mutagenic activity (Baker et al., 1986).
1.14 Diesel Exhaust Particulates

1.14.1 The Morphological and Chemical Characteristics of Diesel Exhaust Particulates

Throughout the 1980s and early 1990s there was increased awareness of the contribution of fossil fuel combustion to greenhouse gas emissions-associated global warming. Subsequently, economic and political pressure to find more fuel-efficient alternatives to gasoline engines resulted in the development of new diesel exhaust technology (Nauss, 1995). Compared to petrol engines, diesel emissions are significantly lower in CO and hydrocarbons. However, due to the incomplete combustion of diesel fuel in compression-ignition engines, enhanced levels of NOx, aldehydes and particulate matter are produced. The amount of particulate matter produced by diesel engines is 100 greater than the gasoline equivalent (Hirafuji et al., 1995; QUARG, 1996) and subsequently diesel soot is a major component of PM$_{2.5}$ in urban environments (Nemmar et al., 2003).

Following combustion of diesel fuel, exhaust components form discrete spherical particles which combine to create chain-like aggregates. Electron microscopy studies identified that approximately 80 % of diesel exhaust particulates (DEP) have a mean aerodynamic diameter of $< 0.1 \, \mu m$, thus rendering them respirable and leading to deposition in the peripheral regions of the lungs (Scheepers et al., 1992). The particle fraction of diesel consists of a carbonaceous core with a large surface area to which heavy metal ions and an estimated 18,000 different high-molecular weight organic compounds are adsorbed (Salvi et al., 1999b).

1.14.2 Organics

The organic fractions of diesel emissions accounts for between 20-40 % of the particle mass encompassing compounds such aldehydes, heterocyclic compound and aliphatic hydrocarbons. However, polycyclic aromatic hydrocarbons (PAHs) and nitro-aromatic hydrocarbons are amongst the most abundant organics present in
diesel exhaust emissions. These constituents are formed by the incomplete combustion of diesel fuel by vehicle engines. The PAHs adsorbed onto DEP are predominantly comprised of three and four benzene-ringed compounds such as fluoranthene (19 %), phenanthrene (52 %) and pyrenes (10 %), including benz[a]pyrene \((C_{20}H_{12})\), many of which are highly toxic and carcinogenic (Kawasaki et al., 2001; Nel et al., 1998; Barfknecht et al., 1982; Westerholm et al., 1994). In addition, numerous studies have attributed the pro-inflammatory and oxidant capacity of DEP to the organic fractions (Bonvallot et al., 2001; Hiura et al., 2000; Kawasaki et al., 2001). For example, Boland and associates, (1999) demonstrated a marked reduction in DEP-induced cytokine expression in the presence of oxidant catalysts which reduced the PAH and nitro-PAH content of DEP by 50-60 %.

Due to the polarity and lipophilic nature of PAHs these compounds can easily diffuse through the cell membrane and bind to receptors within the cytoplasm such as the aryl hydrocarbon receptor (AhR), which is widely expressed in human tissue (Lorenzen et al., 1991). Cytoplasmic to nuclear translocation of this complex, results in its association with the AhR nuclear translocator, (Arnt), forming an active transcription factor involved in the activation of the CYP1A1 gene promoter. Cytochrome P450 1A1 (CYP1A1), a member of the cytochrome P450 reductase family is a substrate-inducible microsomal enzyme that oxygenates PAHs such as the carcinogen Benzo[a]pyrene (Whitlock, 1999). Many studies have shown that the toxicity of PAHs is related to their bio-activation by CYP1A1 to the adduct-forming epoxide. Bonvallot et al., (2001) reported that organic extracts of DEP induced the expression of CYP1A1 in human bronchial epithelial cells (16HBE14o). In accord, Sato et al., (2000), found that enhanced CYP1A1 gene expression is accompanied by increased mutation frequency in lung homogenates of Big Blue rats exposed to whole body diesel fumes, indicating a mechanism by which DEP cause mutagenic lesions in genomic DNA.
1.14.3 Quinones

Oxygenated derivatives of PAHs such as ketones, diones and quinones, are produced by incomplete combustion of diesel fuel and enzymatic conversion of PAH by cytochrome P450 1A1 in the lung (Cohen et al., 1999; Bolton et al., 2000; Takano et al., 2002; Ma et al., 2002). These have been identified as candidate chemicals that may contribute to the generation of ROS, due to their catalytic redox-cycling potential. Quinoid redox-cycling has been implicated in the toxicity of a number of combustion products including cigarette smoke particles, and involves the reduction of oxygen with generation of ROS and consumption of reducing compounds. This mechanism is depicted in Figure 1.8.

During redox-cycling, reduced quinoids reduce oxygen to produce superoxide and subsequently the hydroxyl radical. Hydroxyl radicals then oxidise biological molecules and trigger expression of pro-inflammatory genes (Pinkerton et al., 2000). Biological reducing equivalents such as NADPH and ascorbate provide the electrons required to reduce the oxidised quinoid substances back to their original states, thus sustaining redox-cycling (Chesis et al., 1984; Monks et al., 1992). The electron transport involved in this process may also be facilitated by enzymes including cytochrome P450 reductases and diaphorases, which reduce quinones to semi-quinones, producing ROS (Chesis et al., 1984). In addition, the ability of compounds to undergo redox-cycling may be affected by their complexion to metal ions. Dismutation or the reduction by hydroquinones or other electron donors by superoxide anions results in the formation of hydrogen peroxide. In the presence of transition metal ions, such as Fe$^{2+}$ and Cu$^+$, Fenton-like reactions may occur leading to the generation of cytotoxic hydroxyl radicals (Cadenas, 1989). Kumagai et al., (1997) demonstrated that the oxidative activity of DEP extracts were negated by the addition of NaBH$_4$, which reduces quinones to hydroxyl compounds, implying the participation of quinones in ROS production in vitro. More recently, quinoid redox cycling has been implicated as a mechanism for sustained free radical generation by PM$_{2.5}$ in the lung (Squadrito et al., 2001).
Figure 1.8 Quinoid Redox Cycling Mechanism. The figure illustrates the general mechanism involved in quinone cycling with reductants such as NADPH or ascorbate providing the electrons required to reduce quinones. This sustains the cycle by the continuous reduction of oxygen and formation of hydrogen peroxide, superoxide and hydroxyl radicals. Figure adapted from Squadrito et al., (2001).
1.14.4 Epidemiological Evidence for the Effects of Diesel Exhaust Particles in Occupational Environments

Epidemiological studies have consistently reported a weak association between exposures to diesel exhaust and lung cancer (HEI, 1995), as such, diesel exhaust was classified as a “probable human carcinogen” by the International Agency for Research on Cancer (1989). A summary of over 30 independent epidemiological studies suggest that long-term exposure to diesel exhaust in a variety of occupational setting is associated with a 1.2-1.5 fold increase in the relative risk of lung cancer compared to workers classified as unexposed (HEI, 1995). Although the possibility of confounding by cigarette smoking may bias the interpretation of these studies, Cohen et al., (1995), found that uncontrolled confounding by cigarette smoking could not explain the increased relative risk of lung cancer amongst diesel-exposed workers. Few epidemiological studies have considered other potential confounders including previous exposure to asbestos, other non-diesel particles and socioeconomic factors. As a result, there is insufficient evidence to conclude that these factors do not influence the overall interpretation of these studies (review by HEI, 1995; Bunn et al., 2002). As such although a number of studies have found a statistical association between the increased incidence of lung cancer and diesel exposure, there is insufficient evidence to demonstrate a positive causal association between diesel exposure and lung cancer (HEI, 1995; Muscat et al., 1995; Cox, 1997; Comstock, 1998).

1.14.5 Inflammatory Effects of Diesel Exhaust Particles

Diesel exhaust emissions have been shown to cause a number of adverse health effects on human subjects. Studies have shown that at high concentrations, DEPs act as non-specific irritants, causing symptoms such as eye and nasal irritation, headaches, nausea and dizziness in healthy subjects (Rudell et al., 1994; Rudell et al., 1996). At low concentrations, DEPs promote the release of cytokines, chemokines, immunoglobulins and oxidants in the upper and lower airways (Pandya
et al., 2002). Inhalation of DEPs has been shown to affect lung function in normal individuals causing increased airway resistance (Rudell et al., 1996). Marked systemic and pulmonary inflammatory responses characterised by significant increases in neutrophils, mast cells, CD4+ and CD8+ T lymphocytes have also been noted in normal subjects exposed whole body to DEP. This was accompanied by up-regulation of the endothelial adhesion molecules ICAM-1 and VCAM-1 in bronchial biopsies and significant influx of peripheral blood neutrophils and platelets (Salvi et al., 1999a). In line with these findings, Nightingale et al., (2000), demonstrated that acute exposure to DEP provoked an enhanced inflammatory response with an influx of activated neutrophils in induced sputum of normal healthy subjects. There was accompanying increases in the levels of exhaled CO implying that an oxidant mechanism may be involved in the inflammatory effects of DEP.

A similar acute inflammation was observed in mice instilled with DEP as shown by the marked influx of neutrophils into the alveolar spaces, alveolar-capillary lumen and increased levels in the BALF (Ichinose et al., 1995). It was suggested that damage to type I pneumonocytes and capillary endothelial cells may contribute to the pulmonary oedema and subsequent inflammatory response. A previous study by Sagai et al.,(1993), demonstrated that the pulmonary injury and toxicity of DEP in mice was largely prevented by pre-treatment with the free radical scavengers superoxide dismutase (PEG-SOD) and butylated hydroxytoluene (BHT), suggesting that the toxicity of DEP involves free radicals.

The enhanced ROS production induced by DEP and its associated PAHs, cause oxidative stress and trigger the release of numerous inflammatory mediators such as cytokines and adhesion molecules (Boland et al., 1999; Fahy et al., 1999). For example DEP has been shown to increase cytokine release in numerous cell types including BEAS 2B cells, primary cultures of nasal polyps, bronchial epithelial cells and nasal epithelial cells (Terada et al., 1997; Ohtoshi et al., 1998; Steerenberg et al., 1998). In addition, increased levels of adhesion molecules sICAM-1 accompanied by attenuated ciliary beat frequency have also been reported in human bronchial epithelial cells following DEP exposure (Bayram et al., 1998).
Studies investigating the molecular mechanisms involved in DEP-induced inflammatory gene expression have shown that signalling via MAPK pathways culminates in the activation of NF-κB (Takizawa et al., 1999; Hiura et al., 1999; Fahy et al., 2000; Xiao et al., 2003). Pre-treatment of human bronchial epithelial cells with the free radical scavenger NAC and pyrrolidine dithiocarbamate (PDCT), a specific NF-κB inhibitor, attenuated DEP-induced NF-κB DNA-binding and resultant IL-8 gene expression (Takizawa et al., 1999; 2000). Similarly, NAC was shown to inhibit DEP-induced p38 activation and resultant IL-8 and RANTES expression in bronchial epithelial cells (Hashimoto et al., 2000a). Overall, these inhibitor/intervention studies support a role for oxidative stress in the pro-inflammatory effects of DEP.

1.14.6 Adjuvant Effects of Diesel Exhaust Particles

In addition to its inflammatory effects, DEP has also been implicated in the development or exacerbation of asthma (Peterson et al., 1996). DEP has been shown to act as an adjuvant, enhancing allergen-specific IgE production, chemokine expression, eosinophil degranulation and alteration of cytokine production towards a TH2-like phenotypic response. An exacerbation of local mucosal inflammation resulting in the enhancement of local IL-4 production and histamine release has also been reported in both allergic and non-allergic subjects exposed to DEP. It is postulated that this enhanced IL-4 expression may play a key role in the TH2 adjuvant effects of DEP. In accord, a study by Diaz-Sanchez et al., (1996), demonstrated that exposure to DEP significantly increased cytokine gene expression. IL-4 protein and IgE levels were enhanced in the nasal lavage fluid of non-allergic subjects, suggesting a role for these cytokines in the increased incidence of allergic respiratory diseases associated with diesel exposure. Furthermore, the same group found that synergism between DEP and the natural allergen ragweed, resulted in the increased TH2-type cytokines (IL-4, IL-5, IL-6, IL-10 and IL-13) in nasal lavage fluid and decreased TH1-type cytokines. This suggests that DEP-mediated
adjuvanticity is a key mechanism involved in increasing allergen-induced respiratory allergic diseases (Diaz-Sanchez et al., 1997).

In support of these findings, in vivo studies have demonstrated that both the carbonaceous core and the adsorbed organic components of DEP possessed adjuvant activity for specific IgE production in mice. These adjuvant effects were seen following intra-tracheal and intra-nasal instillation of DEP or carbon black with ovalbumin and significant adjuvant effects on Japanese cedar pollen (JCPA)-specific IgE and IgG production (Lovik et al., 1997; Nilsen et al., 1997; Maejima et al., 1997). Intra-tracheal instillation also resulted in goblet cell hyperplasia and leukocyte infiltration (eosinophils, lymphocytes). This leukocyte influx was associated with the enhanced production of allergen specific IgG and IgE and expression of pro-allergic cytokines (IL-2, IL-4, IL-5) and GM-CSF (Takano et al., 1997). Further studies have shown increased local expression of pro-inflammatory cytokines such as IL-5 and GM-CSF following daily inhalation of DEP in mice (Takano et al., 1998a). In addition, the increased pulmonary resistance in animals inhaling acetylcholine combined with OVA and DEP support the hypothesis that DEP may act as an adjuvant for allergic immune responses and worsen the pathophysiology of asthma (Takano et al., 1998b).
1.15 Summary, Aims and Hypothesis

The lung has a unique function and physiology which renders it highly susceptible to damage from various agents including particulate matter. Numerous studies have examined the pathogenicity of different particle types from both occupational and ambient environments. These studies have provided much evidence to suggest that the physical and chemical characteristics of the particles are important determinants in their toxicity. Particle size/surface area and associated transition metals and organics are the main hypotheses explaining the adverse health effects of combustion derived components of ambient particulates. There is however, limited information regarding the relative roles for the various components of particulate matter. Recent studies have suggested the metal bioavailability and content are key factors influencing the inflammogenicity of particles such residual oil fly ash and ambient particulate matter (Costa et al., 1997; Dreher et al., 1997). Furthermore, mechanistic studies have consistently reported a role for free radical generation and oxidative stress responsive signaling pathways such as MAPK, NF-κB and AP-1, as potential mechanisms through which particle-associated adverse health effects arise.

The scope of the thesis centres on the role of transition metals in the pro-inflammatory effects of two different particles types; highly metal-rich welding fumes from an occupational environment and diesel exhaust particles which are predominant components of ambient PM and contain a relatively low metal content. As particles are known to interact with alveolar epithelium and stimulate an inflammatory response in epithelial cells, the majority of work in this thesis was performed using an alveolar type II epithelial cell line. Although the pathophysiology of welding fume exposure is well established, the molecular and cellular mechanisms involved in these effects remain unknown.

The aims of this thesis were to investigate the molecular toxicity of three compositionally different welding fumes to try and delineate the relative importance of the various components of welding fumes in the initiation of inflammation and the signalling mechanisms through which these events occur. In addition, the pro-
inflammatory effects of various diesel exhaust particulate components were investigated to determine whether similar mechanisms were involved. Overall, the relative importance of soluble transition metals in the pro-inflammatory events of highly metal-rich occupational particulates, welding fumes, was compared to that in a low metal-containing ambient particulate, diesel, to try and gain a greater understanding into the adverse health effects of these two particle types.

Hypothesis

The studies in this thesis are based upon the hypothesis that the soluble transition metal fraction of welding fumes were responsible for driving the pro-inflammatory events associated with welding fume inhalation.
Chapter 2

Materials & Methods
2.1 Culture of Human Type II Alveolar Epithelial Cells (A549)

The cell line A549, is a human cell line derived from a single patient with pulmonary adenocarcinoma. It was used as an in vitro model for human type II alveolar epithelial cells as it can be cultured for up to 50 passages whilst retaining properties of pulmonary type II pneumonocytes, such a cobblestone appearance and the potential to synthesize components for surfactant (Lieber et al., 1976).

The A549 cells were obtained from the European Collection for Animal Cell Culture (ECACC, Salisbury, UK; number 86012804). Cells were maintained in continuous culture in Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma-Aldrich, Poole, UK), supplemented with 10 % heat-inactivated foetal bovine serum (FBS) (Labtech International, East Sussex, UK), 2 mM glutamate, 100 IU.ml⁻¹ streptomycin and 100 µg ml⁻¹ penicillin (Invitrogen Life Technologies, Paisley, UK) at 37 °C in a humidified atmosphere containing 5 % CO₂. Once the cells had formed a confluent monolayer in 162 cm² tissue culture flasks (Costar, Corning, NY), they were washed with Ca²⁺/Mg²⁺-free phosphate buffered saline (PBS-CMF) and harvested with 5 ml Trypsin-EDTA (Invitrogen). After approximately 5 mins incubation, the cells were detached from the flask, at which point an equal volume of complete media was added to inactivate the trypsin. The cells were collected by centrifugation at 150 g for 5 mins and the cell pellet was re-suspended in fresh media and seeded at the concentration for the required experiment or diluted in complete media for continuous cell culture. Cells were used at passages 5-20 and were tested for mycoplasma using the VenorGeM® mycoplasma PCR detection kit (Sigma-Aldrich).

2.2.1 Welding Fume Particles

Samples of welding fume particles produced by two nickel based stainless steel welding consumables (NIMROD 182, and NIMROD c276) and one cobalt based stainless steel welding consumable (COBSTEL 6) were obtained from Health & Safety Laboratory, Sheffield, UK. These samples were generated under controlled conditions at The Welding Institute (TWI), Great Abington, Cambridge, UK, by
carrying out mechanical welding inside a fume box and collecting the fumes on paper filters. The resulting material was removed from the filters by brushing and stored in airtight glass containers until required. Welding fume suspensions were prepared at a stock concentration of 1 mg ml⁻¹ in DMEM containing 2 % FBS (2 % DMEM), sonicated for 10 mins and vortexed briefly to ensure complete dispersion. Welding fumes were diluted to the required concentrations in 2 % DMEM.

2.2.2 Diesel Exhaust Particles (DEP)

Diesel exhaust particles were obtained from the National Institute of Standards and Technology as Standard Reference Material® 2975 (Gaithersburg MD, USA). These particles were collected from an industrial diesel-powered forklift and certified analysis was performed detailing the concentrations of selected polycyclic aromatic hydrocarbons (Appendix 1). Diesel exhaust particle suspensions were prepared as for welding fumes and treatments were carried out in media containing 2 % FBS.

2.3 Particle Characterisation: Transmission Electron Microscopy

Welding fume particles and Diesel exhaust particles (DEP) were characterised by Transmission Electron Microscopy (TEM) using the method adapted from Brown et al.,(2001). Briefly, welding fumes or DEP were suspended at a stock concentration of 1 mg ml⁻¹ in deionised water and sonicated for 10 mins to ensure particles were dispersed evenly in solution. Carbon black (CB) (Huber 900; H Haefner, Cheptow, UK) and ultra-fine carbon black (ufCB) (Printex 90; Degussa, Frankfurt, Germany) were also prepared at a stock of 1 mg ml⁻¹ as reference particles. Five microlitres of each suspension were applied to the surface of 200-mesh size carbon-coated electron microscope (EM) grids (Agar Scientific, Stansted, Essex, UK). Grids were placed on filter paper (Whatman International Ltd, Maidstone, England), dried at room temperature and subsequently examined by
TEM. An image of each particle preparation was taken at three different magnifications (x 10,000; x 35,000 and x 125,000).

2.4 Treatment of A549 Cells with Particles

For all experiments, treatments were carried out in media supplemented with 2 % FBS, as initial studies investigating IL-8 response in A549 cells following treatments prepared in media containing various concentrations of FBS found 2 % FBS produced the optimal response. Subsequent to the initial dose-response studies, monolayers were treated with particle suspensions at concentrations ranging from 2-63 µg ml\(^{-1}\). When indicated, cells were exposed to either whole particles suspension, soluble fraction or washed particles. In the case of welding fumes, a concentration of 63 µg ml\(^{-1}\) was used throughout the study. Non-toxic concentrations of TNF-\(\alpha\) (10 ng ml\(^{-1}\); R&D systems, Abingdon UK) and CB (100 µg ml\(^{-1}\)) were used as a positive control and an inert particle respectively.

Soluble components were isolated from whole particle suspensions by centrifugation at 12,000 g (5 mins) to pellet any particulates. The resultant supernatant was transferred into a clean eppendorf tube and the procedure was repeated \(x\) 4 to ensure any particulate contaminant was removed prior to treatment.

To isolate the particulate fractions, whole particle suspensions were centrifuged at 12,000 g for 5 mins. The supernatant containing the soluble components was removed and the particle pellet retained. The particle pellet was repeatedly washed and centrifuged in PBS-CMF to ensure no soluble components were present. The resultant particles were re-suspended in media containing 2 % FBS and applied to monolayers for the time indicated. The same techniques were used to isolate the different fractions of diesel exhaust particles.

2.5 Cell Morphology

To examine the morphology of A549 cells following exposure to various treatments of welding fumes, the cells were seeded at a density of 0.15 x 10\(^6\) cells per
ml and grown on sterile (2.1 cm²) glass cover-slips (Chance Propper Ltd, UK) within 6-well plates (Corning, NY). The cells were grown under experimental conditions until approximately 80 % confluent when they were washed x 1 with PBS-CMF and the media changed to 2 % DMEM. At confluency, the cells were treated with welding fumes and incubated for a further 24 hrs. Following incubation, the medium was removed and the cover-slips were washed again (1 x PBS-CMF). The cells were fixed with methanol for 1 min, then stained with eosin for 30 seconds and finally counter-stained with methylene blue for a further 30 seconds then left to air dry (Romanowsky Dyes, Fisher, UK). Once dry, the cover-slips were mounted onto glass slides using DPX (BDH Laboratories, Pool, England). Images of the cells were captured at 2 different magnifications (x 400 and x 1000) using a Coolsnap camera connected to G4 Apple Mackintosh computer installed with Openlab software (Loughborough, UK).

2.6 MTT Assay for Cellular Metabolic Activity

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is a pale yellow tetrazolium salt that is converted to formazan (a dark purple insoluble product) by active mitochondrial dehydrogenases. As only viable cells are capable of cleaving the tetrazolium ring, this assay is routinely used to detect cell survival and proliferation (Mosmann, 1983). The conversion of the yellow substrate (MTT) to the dark blue formazan product can be measured spectrophotometrically once the formazan crystals have been dissolved in a solvent (H₂O₂). The absorbance measured is directly proportional to the number of viable cells and their metabolic activity.

A549 cells were seeded at a density of 0.1 x 10⁶ cells per well in 24-well plates (Costar, Corning, NY) and cultured overnight in a humidified atmosphere until approximately 80 % confluency in media containing 10 % FBS. Cells were washed with PBS-CMF and grown until confluent in 2 % DMEM. Monolayers were treated with particle-suspensions ranging in concentrations (1-250 μg ml⁻¹) for 6 – 48 hrs. Metabolic activity was assessed by adding 100 μl 3-(4,5-dimethylthiazol-2-yl)-2-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich-Aldrich) (0.5 mg ml⁻¹) 4 hrs prior to harvest. Following addition of the MTT, cells were incubated under normal
cell culture conditions for the remainder of the experiment. Following incubation, 500\(\mu\)l of solvent (0.1M HCl in Triton X-100-isopropanol {10% v/v}) was added to each well and mixed thoroughly to lyse the cells and solubilize the formazan products. The plates were then centrifuged at 250 g for 5 mins to sediment any particles that may interfere with the measured absorbance. Supernatants (200 \(\mu\)l) were transferred in triplicate to a flat-bottomed 96-well plate (Corning, NY) and the absorbance was determined spectrophotometrically at a wavelength of 570 nm (reference wavelength 630 nm) in a MPX microplate reader using Revelation (Version 2.0) software (DYNEX Technologies, West Sussex, UK).

2.7 Assessment of Cytotoxicity: LDH release

Particle toxicity was determined using the Lactate Dehydrogenase (LDH) cytotoxicity assay, (Roche Molecular Biochemicals, Lewes, UK) a colorimetric assay to determine plasma membrane leakage. The assay is a two stage assay with the initial step involving the reduction by LDH of \(\text{NAD}^+\) to \(\text{NADH} + \text{H}^+\) by oxidation of lactate to pyruvate, followed by the transfer of \(\text{H}/\text{H}^+\) causing the reduction of tetrazolium salt Iodotetrazolium chloride (INT) to formazan. An increase in LDH activity directly correlates with the amount of formazan present, which is proportional to the number of lysed cells. Formazan salt is detected spectrophotometrically at a wavelength of 490 nm.

Briefly, cells were seeded at a density of 9 x 10^4 cells per ml in 96-well flat bottomed culture plates in media containing 10 % FBS and grown until ~ 80 % confluent. The cells were then washed with PBS-CMF, cultured to confluency in media containing 2 % FBS and then exposed to particle concentrations ranging from 1-250 \(\mu\)g ml\(^{-1}\) for the various time points indicated. As a positive control (high control), cells were also treated with 0.1 % Triton X-100 (Sigma-Aldrich, Poole, UK) a detergent that will cause complete cell lysis. Following incubation, the plates were centrifuged at 250 g for 10 mins to pellet any cell debris. 100 \(\mu\)l of supernatant was removed and transferred into a clean 96 well plate. The supernatant was diluted 1:3 in 2 % DMEM and stored at 4 °C. LDH reagent was prepared by adding Reagent
1 (Diaphorase/NAD\(^+\)) to Reagent 2 (INT and sodium lactate) at a ratio of 1:45. 100 µl of LDH reagent was added to each well of the 96-well plate, which was then incubated in the dark for approximately 30 mins. The absorbance was read at 490 nm (reference wavelength 730 nm) on a MR5000 plate reader (Dynatech Laboratories, Inc. Chantilly, VA, USA). The percentage cytotoxicity was determined by:

\[
\text{Cytotoxicity (\%) } = \frac{\text{Experimental value} - \text{Low control}}{\text{High control} - \text{Low control}} \times 100
\]

Low control: Media alone
High control: 0.1% Triton X-100 treated cells

### 2.8 Determination of Protein: Bicinchoninic Acid (BCA) Protein Assay

The protein concentrations of samples were measured using the bicinchoninic acid method (BCA, Pierce, Rockford). This assay employs the principle of the biuret reaction.

\[
\text{Protein} + \text{Cu}^{2+} + \text{OH}^{-} \rightarrow \text{Cu}^{1+}
\]

\[
\text{Cu}^{1+} + 2 \text{BCA} \rightarrow \text{Cu}^{1+}/\text{BCA chromophore (562 nm)}
\]

(purple reaction product)

**Figure 2.1. Biuret Reaction**

The chelation of two molecules of BCA with one \(\text{Cu}^{1+}\) forms a purple reaction product, which exhibits a strong absorbance at 562 nm and is linear with increasing protein concentrations (Smith *et al.*, 1985). A Bovine Serum Albumin (BSA) (Sigma-Aldrich, Poole, UK) standard curve ranging from 0-1.2 mg ml\(^{-1}\) was prepared fresh for each experiment from a stock of 2 mg ml\(^{-1}\) BSA. Samples were diluted either 1:4, 1:5 or 1:10 in PBS-CMF, to ensure sample protein concentrations were within the BSA standard curve, and 5 µl of diluted samples and standards were
added to a 96-well plate (Costar, Corning, NY). BCA reagent was prepared by adding BCA Reagent A (Bicinchoninic acid) to BCA Reagent B (Copper (II) sulphate 4 % (w/v)) at a ratio of 50:1. 100 μl of the reagent was added to each well, which was then incubated at 37 °C for 30 mins. The absorbance was read at 570 nm using Revelation software (Version 2.0, Microsoft Windows). The concentration of protein was determined using linear regression.

2.9 φX174 RF1 Plasmid DNA Assay

The plasmid assay is a cell free assay frequently used to detect particle surface-associated free radical activity. The presence of free radicals can damage the bonds that hold the super-coiled plasmid DNA in place, causing the structure to unwind to form circular DNA. Further degradation can cause breakage in the circular DNA producing a linear molecule (Figure 2.2).

![Diagram of DNA structures](image)

**Figure 2.2** Free radical induced depletion of Supercoiled DNA

ROS : Reactive oxygen species

Free radical activity can be determined using agarose gel electrophoresis (Gilmour *et al.*, 1995). Smaller, compact super-coiled DNA molecules move more effectively through the agarose gel compared to unwound circular and linear molecules. Damage to the super-coiled DNA can therefore be detected by depletion in the intensity of the super-coiled band (Lehninger *et al.*, 1993).
To identify the potential of welding fumes or DEP to cause free radical damage, a closed-circular, superhelical, ϕX174 RF1 plasmid DNA was used (Song et al., 2003). This was deemed the optimum plasmid to use due to size and sensitivity to oxidative damage (Greenwell et al., 2003). Two hundred and ninety nanograms of the plasmid DNA (Invitrogen Life Technologies, Paisley, UK) were incubated with welding fumes or diesel exhaust particles ranging in concentrations from (100-500 μg ml⁻¹) prepared to final volume of 20 μl with deionised water (Donaldson et al., 1995). In some experiments welding fumes were spiked with H₂O₂ (100-500 μM). ϕX174 RF1 was also incubated with Residual Oil Fly Ash (ROFA) (100 μg ml⁻¹) as a positive particle control. A linear control was prepared by digesting the DNA with the restriction enzyme, PSTI (Providencia Stuattii) (0.5 μl), and its corresponding buffer H (2 μl). Untreated DNA was used as a positive super-coiled control. Samples were incubated at 37 °C for 8 hrs on a Thermomixer comfort (Eppendorf, Hamburg, Germany). Five microlitres of blue/orange 6x loading dye (Promega, Southampton, UK) was added to the controls and treated samples. The three plasmid forms; supercoiled, linearised or relaxed, were separated by electrophoresis for 1 hr at 100 V on a 0.8 % agarose gel (Sigma-Aldrich, Poole, UK) prepared in 1 x Tris Buffer EDTA (TBE) (Appendix II). The resulting agarose gel was soaked for 1 hr in 1xTBE containing 0.01% Ethidium bromide (Sigma-Aldrich, Poole, UK) on a Gyro-rocker (Stuart Scientific, Jencons PLS, UK). The proportion of the plasmid forms, which indicated free radical damage, was scanned and quantified by densitometry using a UVP High Performance Ultraviolet Transilluminator (UltraViolet Products, Limited, Cambridge, UK) and GRAB-IT software version 2.5. Free radical damage was expressed as percentage depletion of supercoiled DNA:

\[
\% \text{Depletion} = \left( \frac{\text{Relaxed} + \text{Linear}}{\text{Total DNA}} \right) \times 100
\]
2.10 Assessment of Total Glutathione Concentration (GSH + GSSG)

Total intracellular glutathione (GSH + GSSG) levels were determined by the DTNB/GSSG reductase recycling method originally described by Tietze, (1969) with slight modifications for use in a 96 well plate (Vandeputte et al., 1994). The assay works on the principle of the oxidation of GSH (reduced glutathione) by the sulfhydryl reagent, DTNB, to from a yellow derivative 2-nitro-5-thiobenzoic acid in the presence of β-NADPH, following the conversion of GSSG to GSH by the enzyme glutathione reductase (Figure 2.3). The rate of 2-nitro-5-thiobenzoic acid formation is determined by amount of total glutathione (GSH + GSSG) present in the sample and can be measured spectrophotometrically at an absorbance of 412 nm. The actual concentration of total intracellular GSH is determined using linear regression of a BSA protein standard curve to calculate the amount of GSH in nmols per microgram of protein.

![Figure 2.3. Principal of the DTNB/GSSG reductase recycling method of Tietze et al.,(1969) for analysis of total glutathione concentrations GR: Glutathione Reductase.](image)

A549 cells were seeded at a density of 0.2 x 10^6 cells ml⁻¹ in 6-well plates and grown under normal experimental conditions. Following treatment with either whole welding fumes or soluble fractions for the time intervals stated, cells were washed x 2 with 2 ml PBS-CMF and harvested with trypsin-EDTA (0.5 ml; Invitrogen Life Technologies) by incubation at 37 °C. Once cells were detached, the trypsin was neutralised by addition of 0.5 ml culture media and the cell suspensions were transferred into 1.5 ml eppendorf tubes and cell pellets were formed by centrifugation (13,000 g for 30 seconds). The cell pellets were lysed by the addition of 0.1 M extraction buffer (Appendix II) and homogenised on ice with a teflon pestle
The cell debris was collected by centrifugation (13,000 g) for 5 mins at 4 °C and the lysates were removed and transferred into pre-chilled eppendorf tubes.

Glutathione stock solution (Sigma-Aldrich, Poole, UK) prepared to a concentration of 1 mg ml⁻¹ in KPE (Appendix II) was used to create a GSH standard curve ranging from 0.125 μgml⁻¹- 8 μgml⁻¹ (0.406 - 26 nmoles ml⁻¹). 20 μl sample or standard was placed in a flat-bottomed 96-well plate (Costar, NY). 120 μl dithiobisnitrobenzioc acid (DTNB) (0.2 mg ml⁻¹) and glutathione reductase (GR; 1.67 Uml⁻¹) in phosphate buffer- EDTA were added to each well for 30 seconds to allow for the conversion of GSSG - GSH. 60 μl of β-NAPDH (0.66 μg ml⁻¹) was then added and the rate of DTNB reduction was measured spectrophotometrically every 30 seconds for 2 mins at a wavelength of 405 nm on an MRX microplate reader (DYNEX). The concentration of total glutathione present in the samples was determined using linear regression and was expressed as nmoles GSH/mg protein following the determination of protein using the BSA protein assay previously described.

2.11 Isolation of Total Cellular RNA

Following treatment, cells were washed with PBS-CMF and total cellular RNA was isolated using TRIzol Reagent® (Invitrogen), based on the method of Chomczynski et al.,(1987). TRIzol is a mono-phasic solution of phenol and guanidine isothiocyanate, which disrupts cells and dissolves cellular components, yet maintains the integrity of RNA. Briefly, 1 ml TRIzol was added per 10 cm² tissue culture dish which were then incubated at room temperature for 5-15 mins to allow complete lysis of the nucleoprotein complexes. The cell mixture was then transferred into sterile 2 ml eppendorf tubes. To isolate total RNA from the cells, 200 μl chloroform (Sigma-Aldrich) per ml TRIzol, was added and shaken vigorously and incubated at room temperature for 2-3 mins. The mixture was then centrifuged at 12,000 g for 15 mins at 4 °C. This separated the mixture into an upper, clear aqueous phase containing RNA, an interphase, containing DNA and a lower organic phase containing proteins. RNA was precipitated by transferring the clear aqueous phase into sterile 1.5 ml eppendorf tubes, adding 500 μl isopropanol (BDH, UK) and
incubating on ice for 10 mins. The mixture was then centrifuged at 12, 000 g for 10 mins at 4 °C to pellet the RNA. The RNA pellet was then washed by centrifugation at 7,500 g for 5 mins in ice cold 75 % ethanol (Fisher Scientific, Loughborough, UK) and allowed to air-dry. The RNA pellet was re-suspended in 50 μl RNase free water (diethylpyrocarbonate (DEPC) H2O) (Appendix II), heated at 55 °C for 10 mins to ensure the RNA was fully in solution and quantified spectrophotometrically using an Ultrospec 200 (UV/Visible spectrophotometer, Pharmacia Biotech). The concentration of RNA can be determined using Beer-Lambert’s Law (A= ε x C) by measuring the absorbance at 260 nm and using the molar extinction co-efficient of 0.025.

\[
s-s \text{ RNA (μg ml}^{-1}) = \frac{A_{260}}{0.025} \times \text{ dilution factor}
\]

s-s : single strand RNA

RNA was quantified at two wavelengths: A_{260} and A_{280} and the ratio calculated to determine the purity of the RNA. A ratio < 1.6 indicated that the RNA was not properly dissolved or there was contamination.

### 2.12 Preparation of complementary DNA (cDNA)

cDNA is a single-stranded DNA complementary to an RNA molecule. Therefore to make cDNA from the isolated RNA of treated cells, 2μg total RNA was prepared to a final volume of 25 μl in DEPC-H2O and then incubated at 37 °C for 60 mins with cDNA reaction buffer (Appendix II). The cDNA reaction buffer contained reverse-transcriptase derived from the moloney murine leukaemia virus (M-MLV) (Promega, Southampton, UK), oligo(dT) (Promega) primer which anneals to the poly(A) tail on the mRNA and enable transcription in the 5’ - 3’ direction and deoxynucleotide triphosphates (dNTPs) (Promega), which have energy-rich phosphate groups aiding nucleic acid synthesis. The reaction was stopped by heating at 94 °C for 10 mins and the resultant cDNA was stored at -20 °C until required.
2.13 Amplification of DNA by Polymerase Chain Reaction (PCR)

Reverse transcriptase polymerase chain reaction (RT-PCR) was used to measure the messenger RNA (mRNA) levels for the gene Interleukin-8 (IL-8) and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in cDNA prepared from A549 cell treated with welding fumes or diesel exhaust particles. PCR was performed using oligonucleotide primers (MWG-Biotech AG) and chosen according to Jimenez et al.,(2002).

Primer sequences:

**IL-8: sense**  5’- ATT GAG AGT GGA CCA CAC TGC GCC - 3’,
**Antisense**  5’- CAC TGA TTC TTG GAT ACC ACA GAG -3’.

**GAPDH: sense**  5’-CCA CCA TGG CAA ATT CCA TGG CA-3’,
**Antisense**  5’-TCT AGA CGG CAG GTC AGG TCC ACC-3’.

Briefly, 5μl or 3μl cDNA (IL-8 and GAPDH respectively) were added to 40 μl primer mix (Appendix II) to which 2 μl Taq polymerase (0.5u/μl) (Promega) was added. Samples were mixed gently by pipetting, and thermal cycling performed using a Peltier Thermal Cycler (PTC-200, MJ Research) according to table 2.1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Final Extension</th>
<th>No. Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8</td>
<td>94 °C, 1 min</td>
<td>60°C, 1 min</td>
<td>72°C, 1 min</td>
<td>72°C, 5 mins</td>
<td>31</td>
</tr>
<tr>
<td>GAPDH</td>
<td>72°C 90s</td>
<td>94°C 30 s</td>
<td>60°C 45 s</td>
<td>72°C 10 mins</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 2.1. Conditions for the RT-PCR of IL-8 and GAPDH
The amplification products stained with 5 μl, 6x blue/orange loading dye (Promega) were electrophoresed for 60-70 mins through a 1.5% agarose gel, in 1 x TBE buffer (Appendix II) containing 0.5 μg ml⁻¹ ethidium bromide. For each gel, a ladder consisting of known DNA fragment sizes (100 bp or 1 kb ladder) (Promega) was also resolved to indicate the size of the PCR fragment. The resultant bands were scanned and visualised using a UVP High Performance Ultraviolet Transiluminator and GRAB-IT software version 2.5. The intensity of the IL-8 (173 bp) and GADPH bands (600 bp) were determined by densitometry using GelBase/GelBlot software (UVP Laboratory Products, Cambridge, UK) and expressed as a ratio of the band intensity of IL-8: GAPDH or % Control IL-8:GAPDH.

2.14 Assessment of Intracellular ROS

The levels of intracellular ROS were determined by the change in fluorescence resulting from the oxidation of the fluorescent probe 2', 7'-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich). Dihydrochlorofluorescin Diacetate (DCF-DA) is a stable non-fluorescing compound that can be activated by hydrolysis to form 2', 7'-Dihydrochlorofluorescin (DCFH). DCF-DA was initially used to measure the production of hydrogen peroxide (H₂O₂) in aqueous solutions (Bass et al, 1983). H₂O₂ rapidly oxidises DCFH to form the highly fluorescent DCF (Bass et al, 1983). The extent of the DCF formation can then be detected and measured using flow-cytometry, fluorimetry or UV microscopy.

DCF-DA is a non-ionic, non-polar compound that can diffuse through cell membranes. Once inside, the diacetate (DA) moiety is enzymatically hydrolysed by intracellular esterase to yield the non-fluorescent DCFH. As a result of increased polarity, DCFH becomes trapped within the cell and in the presence of intracellular ROS is rapidly oxidised to highly fluorescent DCF (LeBel et al., 1992) (Figure 2.4).
Figure 2.4 Hypothetical Mechanism of DCFH-DA Assay
Sources: Bass et al., 1983 and Le Bel et al., 1992

A549 cells were seeded at a density of $0.2 \times 10^6$ cells ml$^{-1}$ in 6-well plates, and grown under normal experimental conditions. Cells were then exposed to either whole welding fume suspensions or soluble fractions only for the time indicated. Thirty mins prior to the end of the final exposure period, 40 μM the fluorescent probe Dichlorofluorescin-diacete (DCFA-DA) (Sigma-Aldrich) was added to the culture medium which was then incubated at 37 °C (Kobzik et al., 1990; Stringer et al., 1995). Following incubation, the cells were washed twice with 1 ml PBS-CMF and harvested by trypsinisation (0.25 % Trypsin-EDTA). Once detached, the trypsin was neutralised with culture media and the cells were transferred to 12 x 75 mm polypropylene FACS tubes (Becton Dickinson, Oxford, UK) and pelleted by centrifugation at 300 g for 5 mins. The cell pellets were re-suspended in PBS-CMF. The degree of fluorescence, corresponding to intracellular ROS, was measured immediately using FACS Calibular flow cytometry (excitation $\lambda$ 530 nm; emission 488 nm). The proportion of fluorescence cells was determined using Cell Quest software (Becton Dickinson) on Apple Mac (G3 work station) and expressed as percentage control geo-mean fluorescence. For all experiments, welding fume particles alone were incubated with the probe and the fluorescence measured to
ensure measurements were not the result of particle auto-fluorescence or potential interference with the assay.

2.15 Enzyme-Linked Immunosorbent Assay (ELISA): Interleukin-8 (IL-8)

Duo-set ELISA kits containing monoclonal and biotinylated anti-human IL-8 antibodies were initially obtained from the National Institute for Biological Standards and Control (NIBSC) (Pottersbar, Hertfordshire, UK). However, during the course of this thesis, the NIBSC ELISA kit was discontinued and therefore, for the latter experiments similar ELISA kits were supplied by R&D systems (Abingdon, UK). Flat-bottomed 96- well plates (E.I.A/R.I.A plate, Costar, Cambridge, UK) were coated with 100 µl IL-8 monoclonal capture antibody (2 µg ml⁻¹ in 1 x PBS-CMF for NIBSC ELISA; or 4 µg ml⁻¹ in 1 x PBS with Ca²⁺ + Mg²⁺ for R&D ELISA) and left at room temperature over night. The plates were then washed 3 times with wash buffer (1 x Tris Buffered Saline (TBS) pH 7.4, 0.05 % Tween 20 or 1 x PBS with Ca²⁺ + Mg²⁺ + 0.05% Tween 20) to remove any unbound antibody and incubated at room temperature on a plate rocker (Titramax 1000, Heidolph Instruments) for 1 ½ hrs with 100 µl/well blocking buffer (5 % sucrose, 1 % BSA in PBS with Ca²⁺ + Mg²⁺), to block non-specific binding sites.

Following incubation, the wells were washed again (x 3) with wash buffer and 100 µl sample or standard was added to each well. IL-8 standards were prepared from a stock of 2 ng ml⁻¹ in media (DMEM) over a range of 0-1000 pg ml⁻¹. Plates were then incubated on a plate rocker for a further 1 ½ hours at room temperature.

The plates were then washed again (x 3 with 100 µl/well wash buffer) to remove any unbound material and 100 µl of the biotinylated, polyclonal detection antibody (20 ng ml⁻¹ in reagent diluent (0.01% BSA in 1 x TBS, pH 7.4 0.05% Tween 20) was added to each well. The plates were incubated for a further 1 ½ hrs on the shaker to form an antibody-enzyme conjugate sandwich. Following incubation the plates were washed again (x 3 wash buffer) to remove any unbound conjugate. 100 µl streptavidin-horse radish peroxidase (HRP) (Dako, Denmark) (1:2000 dilution in reagent diluent) (Appendix II), was added to each well and further incubated for 20 mins on a plate shaker at room temperature.
Streptavidin is a specific substrate that binds to biotin producing a coloured product. Therefore the amount of IL-8 present in the samples was proportional to the colour produced. A stock of 10 mg ml⁻¹ 3,3',5,5'-tetramethylbenzidine (TMB) was prepared by dissolving TMB in dimethylsulfoxide (DMSO) (Sigma-Aldrich, Poole, UK) and wrapped in tin foil due to being light sensitive. The substrate buffer was prepared by addition of 100 µl TMB stock per 10 ml ELISA substrate buffer (Appendix II) with 0.05 % H₂O₂. The plates were aspirated and washed x 3 with wash buffer and 100 µl of substrate buffer was added to each well. The plates were covered in tin foil and allowed to incubate until a blue colour was apparent. The reaction was stopped by the addition of 50 µl sulphuric acid (H₂SO₄; 1 M) and the absorbance was read at a wavelength of 450 nm (reference λ 570 nm) on a Dynatech MR5000 plate reader. The values were determined by linear regression using a standard curve generated from recombinant IL-8 (NIBSC or R&D).

2.16 Preparation of Nuclear and Cytosolic Extracts

Nuclear and cytosolic extracts from cells exposed to either whole, soluble fraction or washed welding fumes were prepared according to the method described by Staal et al.,(1990) with minor modifications. Briefly, following treatment for the time points indicated, monolayers were washed x 2 with ice-cold PBS-CMF to remove any residual supernatant and detached from the tissue culture plates by gentle scraping. Cells from 3 replicate wells were pooled together and suspended in 1 ml PBS-CMF. The cell suspension was then transferred into sterile 1.5 ml eppendorf tube and collected by centrifugation at 12,000 g for 15 seconds. The cells were re-suspended in 0.4 ml buffer A (Appendix II) and incubated on ice for 15 mins. Following incubation cell lysates were formed by the addition of 25 µl buffer B (10 % Nonidet P-40) and vigorous mixing. The nuclei were collected by centrifugation at 12,000 g for 30 seconds and the cytosolic extracts were retained and stored -20°C for future analysis. Nuclei were then re-suspended in 50 µl buffer C (Appendix II) agitated on ice for 20 mins and centrifuged for 10 mins (8,000 g at 4 °C) to collect any cell debris. The nuclear extracts were retained and stored at -80 °C until required.
and the protein concentration was determined using BCA protein assay described previously (Section 2.8)(Pierce, UK).

2.17 Electrophoretic Mobility Shift Assay

10 µg nuclear protein from each treatment, HeLa cells or TNF-α (+ ve), no protein (-ve), were incubated at room temperature with 5 x binding buffer, (Appendix II), 1 mM DTT and 0.25 mg ml⁻¹ poly (dl: dC): poly (dl: dC) (Promega, Southampton, UK) for 20 mins, followed by addition of either [γ⁻³²P] labelled NF-κB or AP-1 double-stranded consensus oligonucleotide transcription factors (Promega). Samples were mixed gently and incubated for a further 30 mins to allow binding of the nuclear proteins to the radio labelled oligonucleotide.

The transcription factors sequences were obtained from Promega;

NF-κB (5'−AGT TGA GGG GAC TTT CCC AGG C-3')
AP-1 (5'−CGC TTG ATG AGT CAG CCG GAA-3')

To separate oligonucleotides bound by transcription factors, samples were electrophoresed on a 6 % non-denaturing polyacrylamide gel (Appendix II) in 1x TBE at 100-150 volts, and dried on Whatman filter paper under a vacuum using a HydroTech™ Vacuum pump and Gel Dryer (BIORAD, UK). Once dried, the gels were place overnight on a Phosphor screen (Molecular Dynamics, Buckinghamshire, UK). Quantitative analysis of the radioactive bands was performed with Storm 860 phospho-imager using Image Quant software (Molecular Dynamics). To ensure specificity of DNA binding a cold competitor and a non-competitor, i.e.100-fold excess (2.5 pmol) of un-labelled NF-κB or AP-1 were included in each experiment. The cold competitor would prevent labelled probe from binding thus no band would be apparent, whereas the non-competitor would have no effect on binding.
2.18 Determination of Lipid Peroxidation by Thiobarbituric Acid Reactive Substances (TBARS)

Malondialdehyde (MDA) is a secondary product of lipid peroxidation and its measurement is frequently used to determine the extent of oxidative membrane damage. Levels of MDA (and other lipid peroxides) can be determined using a colorimetric reaction with thiobarbituric acid (TBA) (Yagi, 1976; Ohkawa et al., 1979). Briefly, A549 cells were seeded at a density of 0.1 x 10^6 cells per ml and grown in 24-well plates under normal culture conditions. Following treatment with either whole, soluble or washed welding fume fractions or CB/ufCB for the time points indicated, monolayers were washed x 2 with PBS-CMF to remove any residual supernatant and cells were harvested using Trypsin-EDTA (Invitrogen). Cells from 3 replicate wells were pooled together and suspended in 1 ml 2 % DMEM. The cell suspension was then transferred into sterile 1.5 ml eppendorf tubes, collected by centrifugation at 12,000 g for 15 seconds and re-suspended in 100 µl PBS-CMF. Cell suspensions were transferred into 5 ml glass test-tubes and mixed with 100 µl 8.1 % Sodium Dodecyl Sulphate (SDS) (Sigma-Aldrich), 200 µl 0.8 % aqueous solution of TBA, 100 µl 20 % acetic acid (pH 3.5) and made up to a final volume of 1 ml with distilled H₂O. As the TBA reaction is highly dependant on pH (Ohkawa et al., 1978) the pH of acetic acid was adjusted using 10 M NaOH and checked prior to use. 1,1,3,3-tetramethoxypropane (TMP) standards ranging from 1-5 µM were prepared from a 10 µM stock. The reaction mixture was heated at 95 °C for 60 mins to allow maximal coloration and cooled with tap water. After cooling, 1 ml of N-butanol (Sigma-Aldrich) was added to each tube, which was then vortexed and centrifuged at 400 g for 10 mins. The organic layer, containing the MDA reaction products, was transferred into fluorimeter cuvettes (Sigma-Aldrich) and the fluorescence measured on a Fura-2 AM, Luminescence Spectrometer LS 50B (Perkin-Elmer Ltd. Beaconsfield, Bucks, UK.) using an excitation wavelength of 515 nm (emission λ 553 nm). The concentration of TBA reactive substances was determined using linear regression and expressed as µM TBA.
2.19 Western Blotting

2.19.1 Preparation of Cell Lysates

Whole cell lysates were prepared from cells following treatment with whole, soluble or washed welding fume fractions, TNF-α (positive) or carbon black (negative particle control). Following exposure for the time-points indicated, culture media was removed and cells were washed twice with ice-cold PBS-CMF. The cells were detached from the culture plates by gentle scraping using a cell lifter and lysed in 100 μl RIPA buffer (Appendix II). Three replicate wells were transferred to a 1.5 ml eppendorf, sonicated on ice for 10 mins and further incubated on ice for 30 mins on a rotating platform. Following incubation, lysates were passed through a 21 gauge needle 10 times to ensure no large clumps of DNA or cell debris were present and further centrifuged at 12,000 g (4 °C) for 20 mins to remove cell debris and nuclei. Whole cell lysates were transferred into pre-chilled 1.5 ml eppendorfs and stored at -80 °C until required. The protein concentration of lysates was determined using the BCA protein assay (Section 2.8).

2.19.2 Sodium Dodecyl Sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting

Protein samples were denatured by heating at 95 °C for 5 mins in SDS-reducing buffer to give a final volume of 20 μl (Appendix II). The denatured samples and 10 μl of pre-stained SDS-Page molecular weight standards (BioRad, Hercules, CA) were then subjected to electrophoresis on a 10 % SDS-poly-acrylamide gel, 4 % stacking gel (Appendix II) at 125 volts for approximately 60 mins in 1 x Running buffer (Appendix II). The gels were then removed and placed onto PVDF (Hybond P, Amersham Bioscicences, Bucks, UK), previously dampened in methanol for 10 seconds to activate the membrane. Two sheets of blotting paper (Whatman) and a fibre pad soaked in 1 x transfer buffer (Appendix II) were put on either side of the gel to create a sandwich. The sandwich was then placed into a gel cassette holder (Figure 2.5) and transferred into the electrophoresis apparatus, (BioRad, Hercules, CA) with the grey (negative side) closest to the gel to ensure the proteins were
transferred onto the positively charged membrane. The apparatus was filled with 1 x transfer buffer and an ice block and the proteins were transferred by electrophoresis at 80 mA per blot for 90 mins. The membranes were then stained briefly with Ponceau's solution (Sigma-Aldrich) to ensure protein transfer was successful then washed x 3 in 1 x TBS/0.05 % tween-20 for 5 mins (Appendix II). The conditions required for the specific proteins detected by western blotting are described in table 2.2. For detection of native p38, blots were stripped using 50 ml strip buffer (Appendix II) containing 400 μl β-mercaptoethanol per blot and heated at 65 °C for 45 mins. Following the stripping procedure, blots were washed thoroughly in 1x TBS/0.05 % tween-20 and blocked over night with 4 % Marvel in 1x TBS prior to re-probing with the native p38 antibody.

**Figure 2.5.** Schematic diagram illustrating the layering sequence of blotting paper, gel and gel cassette required for transfer of proteins in western blotting.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Blocking</th>
<th>1° Antibody</th>
<th>2° Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-p38</td>
<td>4% Marvel-TBS</td>
<td>Anti-phospho p38 (Biosource) (1:1000)</td>
<td>Anti-Rabbit IgG (SC) (1:5000)</td>
</tr>
<tr>
<td></td>
<td>O/N at 4 °C</td>
<td>5% BSA/0.05% Tween-20</td>
<td>5% Marvel/0.05% Tween-20</td>
</tr>
<tr>
<td>Native-</td>
<td>4% Marvel-TBS</td>
<td>Anti-native p38 (SC) (1:4000)</td>
<td>Anti-Rabbit IgG (SC) (1:5000)</td>
</tr>
<tr>
<td>p38</td>
<td>O/N at 4 °C</td>
<td>5% Marvel/0.05% Tween-20</td>
<td>5% Marvel/0.05% Tween-20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 hrs RT</td>
<td>1 hrs RT</td>
</tr>
</tbody>
</table>

O/N: Overnight
RT: Room Temperature

Antibody Supplier: (SC): Santa Cruz

Table 2.2. Antibodies and Conditions used for Western Blotting

### 2.20 In Vivo Model

#### 2.20.1 Intra-tracheal Instillation and Bronchoalveolar lavage

Healthy specific pathogen-free male Sprague Dawley rats (B&K Universal Ltd, Hull, UK) were used for all in vivo experiments. Rats were housed 5 per cage in a regulated animal facility where a 12 hr light/dark cycle was maintained. The rats were fed standard laboratory food and received water ad libitum. At 10-12 weeks old (350-400 g), rats were anaesthetized with an intra-peritoneal injection (1.1 ml) of a mixture of Hypnorm (0.315 mg of fentanyl citrate and 10 mg of Fluanisone per ml) and Hypnoval (95 mg of Midazolam Hydrochloride per ml) (McElroy et al., 2002). 500 µl of welding fume suspensions, NIMROD c276, (0.5mg ml⁻¹ in saline) of either whole, soluble, washed particulate fraction or chelex treated soluble fraction, were intra-tracheally instilled to give a final dose of 250 µg ml⁻¹ per animal. Control rats were instilled with 500 µl sterile saline only. 24 hrs post instillation the animals were killed by a single intra-peritoneal injection of 2 ml sodium pentobarbital (200 mg) (Vericore Ltd, Dundee, UK).

The lungs were exposed and the trachea cannulated prior to lavaging the bronchoalveolar space with 8 ml sterile saline (Baxter, Newbury, UK). The
procedure was repeated 3 times, with the primary lavage retained separately. All bronchoalveolar lavage (BAL) samples were centrifuged at 400 g for 5 mins at 4 °C and the pelleted cells from all lavages were combined and re-suspended in 1 ml sterile PBS-CMF. An aliquot of the primary lavage was retained at 4 °C for LDH analysis with the remaining sample stored at -80 °C until required for biochemical analysis (MIP-2 and total protein).

2.20.2 Preparation of Cytospins and Differential Cell Count

Total cell number in the BAL was determined using a haemocytometer chamber (Sigma-Aldrich) and cell viability was assessed by trypan blue dye exclusion. Cytospin preparations were made using 10,000 cells centrifuged at 15 g for 3 mins onto glass slides (Menzel-Glaser, Fisher Scientific). The cells were fixed with methanol and stained using Diff Quick dyes (Raymond Lamb, Eastbourne, UK). Differential cell counts of the cytospins were performed blind with a minimum of 300 cells counted per slide.

2.20.3 Enzyme-Linked Immunosorbent Assay (ELISA): Macrophage Inflammatory Protein-2 (MIP-2)

Cytoset ELISA kits containing monoclonal and biotinylated anti-rat MIP-2 antibodies were obtained from Biosource International (CA, USA), and employed peroxidase and tertamethylbenzidine (TMB) as the detection method. Briefly, flat-bottomed 96-well plates (E.I.A/R.I.A plate, Costar, Cambridge, UK) were coated with 100 µl MIP- monoclonal capture antibody (1.25 µg ml⁻¹ in coating buffer B Appendix II) and left at room temperature overnight. The plates were then washed x 3 with wash buffer (1 x TBS, pH 7.4, 0.05% Tween 20 or 1 x PBS with Ca²⁺ + Mg²⁺ + 0.05 % Tween 20) and 100 µl blocking buffer, (Appendix II), was added to each well to block non-specific binding sites. The plates were then incubated on a plate rocker at room temperature for 2 hrs. Following incubation, the wells were washed (x
3) with wash buffer and 100 µl sample or standard was added to each well. MIP-2 standards ranging from 0 - 1000 pg ml\(^{-1}\) were prepared from a stock of 2000 pg ml\(^{-1}\) in standard diluent (Appendix II). Plates were then incubated on a plate rocker for a further 1 ½ hrs. The plates were again washed (x 3) with 100 µl/well wash buffer to remove any unbound material and 100 µl of the biotinylated, polyclonal detection antibody (0.125 µg ml\(^{-1}\) in substrate diluent) was added to each well and incubated whilst rocking for 1 hr. Unbound conjugates were removed by washing (x 3 wash buffer) and 100 µl streptavidin- HRP conjugate (0.3 µg ml\(^{-1}\) in substrate diluent) was added to each well and incubated for 45 mins on a plate shaker at room temperature. A stock of 10 mg ml\(^{-1}\) 3,3’,5,5’-tetramethylbenzidine (TMB) was prepared by dissolving TMB in dimethylsulfoxide (DMSO) (Sigma-Aldrich) and wrapped in tin foil due to being light sensitive. The substrate buffer was prepared by the addition of 100 µl TMB stock per 10 ml ELISA substrate buffer (Appendix II) with 0.05 % H₂O₂. The plates were washed and 100 µl of substrate buffer was added to each well. The plates were then covered in tin foil and allowed to incubate until a blue colour was apparent. The reaction was stopped by the addition of 50 µl sulphuric acid (H₂SO₄; 1M) and the absorbance was read at a dual wavelength 450/650 nm on a Dynatech MR5000 plate reader. The values were determined by linear regression using a MIP-2 standard curve.

2.21 Statistical Analysis

Individual experiments were conducted in triplicate and the data shown in each figure represents the mean of at least 3 separate experiments ± the standard error of the mean (± S.E.M). Statistical significance was determined using One-Way Analysis of Variance (ANOVA) with the post-hoc test, Tukey’s pairwise comparison using Minitab (Version 10.5). This analysis allowed multiple comparisons yet minimised the inter-experimental error rate. Pearson-product moment correlation coefficients were calculated to investigate the linear-relationship between variables. The following nomenclature was used to denote significance for all the data
contained within this thesis. \*p<0.05; \**p<0.01 and \***p<0.001 compared to control values.
Chapter 3

The Pro-Inflammatory Effects of Welding Fumes *In Vitro*
3.1 Introduction

Welding fumes are a complex mixture of gases and small particulates formed by the vaporization and oxidation of metals (Lockey et al., 1988; Yu et al., 2000). The nature of respirable fumes can vary considerably and is dependent upon the composition of electrode, filler wire and fluxes, and the actual welding process (Lockey et al., 1988; Sferlazza et al., 1991; Yu et al., 2000). Due to the size of these particles (<0.5 μm) they are easily respirable, consequently many adverse respiratory health effects, such as bronchitis and chronic pneumonitis are associated with inhalation of welding fumes (Doig et al., 1951; Lockey et al., 1988). Metal fume fever (MFF), the most commonly described acute illness amongst welders, is characterised by fever, general malaise and acute pulmonary inflammation, peaking 5-12 hr post-exposure and resolving by 24-48 hrs (Sferlazza et al., 1991; Antonini et al., 1996). Although the pathogenesis of MFF is poorly understood, a cytokine or cytokine-like mechanism is frequently postulated (Blanc et al., 1993; Graeme et al., 1998). Numerous studies have reported elevated levels of the pro-inflammatory cytokines TNF-α, IL-1β and IL-8 following exposure to welding fumes supporting this hypothesis (Blanc et al., 1993; Antonini et al., 1996; Kuschner et al., 1998).

The importance of composition, bio-availability and concentration of metals in the induction of pulmonary inflammation and injury has been demonstrated following exposure to many environmental particulates including ROFA, PM_{10}, and stone-quarry particles (Gilmour et al., 1996; Pritchard et al., 1996; Dreher et al., 1997; Hetland et al., 2001). A common feature of particle-associated toxicity and inflammation is oxidative stress (Stohs et al., 1995; Martin et al., 1997; Tao et al., 2003). Mechanistic studies have reported that reactive particle-surfaces and particle-associated transition metals can generate reactive oxygen species (ROS), deplete antioxidants, such as glutathione and culminate in cellular damage and injury (Jiang et al., 2000; Ghio et al., 1999b; Gilmour et al., 1997).
The aims of the research in this chapter were to determine the toxicity of three compositionally different welding fumes on alveolar epithelial cells in relation to their pro-inflammatory effects by measuring the release of the chemokine IL-8. As the inflammatory effects of a number of other environmental particulates have been attributed to soluble transition metals, it was hypothesised that the soluble components of welding fumes may play a key role in causing inflammation. To test this hypothesis, welding fumes separated into either the soluble fraction which contained no particulates or the washed particulate fraction devoid of any soluble components, and the relative ability of both fractions to induce cytokine production was assessed. To try and delineate whether welding fume-induced inflammation was mediated via an oxidative stress mechanism, the levels of the antioxidant glutathione and the production of ROS were investigated.

An in vitro cell culture system was adopted for the majority of work in this manuscript as it provided a useful model for investigating the molecular mechanisms involved in welding fume-induced inflammation in type II pneumonocytes. The A549 cell-line used in this thesis has been widely accepted as a model lung alveolar epithelial cell, suitable to investigate potential in vivo responses. They display many of the characteristics of primary cells such as their ability to respond to particle and cytokine stimulation, regulation of glutathione homeostasis and expression of the chemokine receptor CXCR4 (Rahman et al., 1995b; Murdoch et al., 1999; Crestani et al., 1994; Kamp et al., 1998; Park et al., 1998).
3.2 Welding Fume Particle Morphology

Welding fumes are highly complex and inherently heterogeneous in nature. Transmission Electron Microscopy (TEM) was therefore used to characterise the morphology of the particulate components of the welding fumes used in this study. TEM images of the three welding fumes and reference particles carbon black (CB) and the ultra-fine counterpart (ufCB) are shown in Figure 3.1 A-F. These images show that a high proportion of welding fume particles are within the ultra-fine range; less than 0.1 μm (100 nm) in diameter (Fig 3.1 A-C) (Lockey et al., 1988; Antonini, 2003; Yu et al., 2000). Within this ultra-fine population, the majority of particles are roughly spherical in appearance, similar to that of ufCB (Figure 3.1 E.). Flux core arc welding was used to generate both NIMROD c276 and COBSTEL 6 and is known to create fumes of a uniform nature (Sferlazza et al., 1991). Some more angular particles are also present within this sub-population which may represent particles of crystalline silica, a known component of welding fumes produced from the coating of metal electrodes (Antonini, 2003). A number of larger particles and particle-aggregates, similar to those of CB (Figure 3.1.D), can be seen clearly in both nickel-based fumes, in particular NIMROD c276 (Fig. 3.1 B). In contrast, the majority of particles present in the cobalt-based fume, COSTEL 6 (Fig. 3.1 C), are more similar in both size and shape to that of ufCB (Fig. 3.1 F). Chain-like aggregates are also present in all three welding fumes as a result of the turbulent conditions created from heat generated in the welding process which enhances particle movement and collisions (Antonini, 2003).
Figure 3.1 Transmission Electron Micrographs showing Welding Fume Particles. All images represent particle concentrations of 1 mg ml\(^{-1}\). A. NIMROD 182. B. NIMROD c276. C. COBSTELE 6. D. Carbon Black (CB) (average diameter 260.2 ± 13.7 nm). E. Ultra-fine Carbon Black (ufCB) (average diameter 14.3 ± 0.6 nm). Magnification x 35,000.
3.3 The Effect of Welding Fumes on the Metabolic Activity of Alveolar Epithelial Cells

Welding fumes are known to contain toxic components such as chromium, nickel and crystalline silica (Costa, 1991; Dennis et al., 1996; Antonini, 2003). Therefore, to establish the effect of welding fumes on alveolar epithelial cell survival and proliferation, the metabolic activity of the three welding fumes, NIMROD 182, NIMROD c276 and COBSTEL 6 was determined by the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) to formazan, in the MTT assay (Mosmann, 1983). This assay detects the proportion of active mitochondrial dehydrogenases present in cell samples and therefore is indicative of the metabolic activity and viability of cells. Cells were exposed to welding fumes at concentrations ranging from 1-250 µg ml⁻¹ over various time-points. Figure 3.2 shows the dose-dependent decrease in the metabolic activity of A549 cells following 24 hr exposure to all three fumes. Cell viability was reduced by 50 % at concentrations greater than 63 µg ml⁻¹ by 24 hr with no significant difference observed between fumes. A similar, but less marked reduction in metabolic activity was seen at 8 hr with concentrations ≥ 125 µg ml⁻¹ required to cause a 50 % reduction in mitochondrial activity. Following 48 hr exposure the metabolic activity of alveolar epithelial cells was significantly reduced at concentrations ≥ 15 µg ml⁻¹ (Figure 3.3) however cells were slightly more metabolically active than at 24 hr suggesting possible cells recovery.

![Figure 3.2 Effect of Welding Fume Particles on the Metabolic Activity of Alveolar Epithelial Cells](image)

Figure 3.2 Effect of Welding Fume Particles on the Metabolic Activity of Alveolar Epithelial Cells

Metabolic activity was determined by the reduction of MTT following treatment with welding fumes (1-250 µg ml⁻¹) for 24 hr and is expressed as percent of the control value (untreated cells 0 µg ml⁻¹). The graph represents the mean of four experiments conducted in triplicate. The bars represent ± S.E.M. (n=4) *p<0.05, **p<0.01; ***p<0.001 compared to control.
Figure 3.3 Time course of Metabolic Activity of Welding Fume Particles. Metabolic activity was determined by the reduction of MTT following treatment with welding fumes (1-250 μg ml⁻¹) for 8, 24 or 48 hr and is expressed as percent of the control value (untreated cells 0 μg ml⁻¹). The graphs represent the mean of four experiments conducted in triplicate. The bars represent ± S.E.M. (n=4) *p<0.05, **p<0.01, ***p<0.001 compared to control A. NIMROD 182; B. NIMROD c276; C. COBSTEL 6
3.4 Cytotoxicity of Welding Fumes

Data from the previous section demonstrated the striking effect of welding fumes on the metabolic activity of A549 cells. This reduction in active mitochondrial dehydrogenases may however be the result of cells entering a state of mitotic arrest rather than death. To establish the cytotoxicity of NIMROD 182, NIMROD c276 and COBSTEM 6 the release of lactate dehydrogenase (LDH) into the supernatant was measured. LDH is a stable cytosolic enzyme present in all cells and is only released upon damage or rupture of the plasma membrane. The amount of enzyme activity detected in culture supernatant therefore correlates with the proportion of lysed cells. A549 cells were incubated with welding fumes ranging in concentration from 1-250 μg ml⁻¹ for the various time-points indicated. As shown in Figure 3.4, all three fumes caused a dose-dependent increase in toxicity, with concentrations greater than 63 μg ml⁻¹ causing significant cell death at 24 hr (p<0.01). NIMROD c276 appeared to be the most toxic fume, causing the greatest release of LDH at the highest concentration compared to control values (p<0.001), whereas, NIMROD 182 was the least toxic fume, (p<0.05). There was no significant difference between fume toxicity at the higher concentrations (≥ 63 μg ml⁻¹). The marked increase in LDH measured at concentrations greater than 63 μg ml⁻¹ suggests the reduction in metabolic activity shown previously (Section 3.3) was the result of cell death and not the case of cellular arrest.

A similar pattern was seen over all time points assessed, (2, 8 and 48 hr) with a time-dependent increase in toxicity observed for all fumes. Again, NIMROD c276 was most cytotoxic and NIMROD 182 least toxic (Table 3.1). Following 48 hr exposure, approximately 25 % cell death was observed for all fumes at concentrations greater than 31 μg ml⁻¹ (p<0.001).
Figure 3.4. Lactate Dehydrogenase (LDH) release from Alveolar Epithelial Cells following 24 hr Exposure to Welding Fumes. LDH release is expressed as percentage of total cellular LDH, measured in the cell lysates obtained following treatment with welding fumes (1-250 µg ml⁻¹) for 24 hr. The graph represents the mean of three experiments conducted in triplicate and the bars represent ± S.E.M. *p<0.05; **p<0.01; ***p<0.001 compared to 100% Cytotoxicity from cells treated with 0.1 % Triton X-100.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Conc.(µgml⁻¹)</th>
<th>Fume</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>15</th>
<th>31</th>
<th>63</th>
<th>125</th>
<th>250</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0</td>
<td>NIMROD 182</td>
<td>0.39±0.05</td>
<td>0.91±0.13</td>
<td>1.55±0.25</td>
<td>2.42±0.30</td>
<td>4.70±0.92</td>
<td>7.44±0.46</td>
<td>11.08±1.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>NIMROD c276</td>
<td>0.46±0.18</td>
<td>0.73±0.33</td>
<td>1.76±0.05</td>
<td>1.88±0.14</td>
<td>3.08±0.82</td>
<td>5.37±0.89</td>
<td>14.11±1.63</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1.72±0.32</td>
<td>COBSTELE 6</td>
<td>3.44±1.83</td>
<td>6.09±0.37</td>
<td>14.88±2.66</td>
<td>24.7±4.15</td>
<td>32.15±3.39</td>
<td>38.76±1.66</td>
<td>40.11±1.56</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>0.16±0.16</td>
<td></td>
<td>1.20±1.2</td>
<td>1.23±0.23</td>
<td>3.88±1.18</td>
<td>4.26±1.34</td>
<td>7.51±1.99</td>
<td>10.99±1.62</td>
<td>18.79±0.51</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.46±0.49</td>
<td></td>
<td>1.20±0.2</td>
<td>2.23±0.76</td>
<td>4.84±0.73</td>
<td>4.93±1.45</td>
<td>14.18±3.53</td>
<td>17.32±2.76</td>
<td>19.78±3.61</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1.44±0.45</td>
<td></td>
<td>5.88±1.57</td>
<td>18.83±1.79</td>
<td>25.02±4.85</td>
<td>28.33±2.86</td>
<td>37.74±6.73</td>
<td>41.67±3.95</td>
<td>60.74±5.86</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>0.27±0.17</td>
<td></td>
<td>0.71±0.64</td>
<td>2.02±0.16</td>
<td>2.99±2.54</td>
<td>5.00±0.96</td>
<td>7.21±1.16</td>
<td>16.22±2.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.10±0.05</td>
<td></td>
<td>1.37±1.31</td>
<td>3.02±0.94</td>
<td>2.99±1.68</td>
<td>9.33±1.68</td>
<td>13.21±3.29</td>
<td>18.09±0.82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1.37±0.64</td>
<td></td>
<td>3.46±0.59</td>
<td>10.21±3.42</td>
<td>22.02±5.21</td>
<td>27.31±1.33</td>
<td>32.33±2.15</td>
<td>47.21±1.16</td>
<td>59.55±1.82</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1. Time-Course of Lactate Dehydrogenase (LDH) release from Alveolar Epithelial Cells Exposed to Welding Fumes. LDH release is expressed as percentage of total cellular LDH, measured in the cell lysates obtained following treatment with welding fumes (1-250 µg ml⁻¹) for 2, 8 or 48 hr. No increase in LDH was observed following exposures to concentrations 0-1 µg ml⁻¹ (data not shown). Values represent the mean of three experiments conducted in triplicate ± S.E.M. *p<0.05; **p<0.01; ***p<0.001 compared to 100% Cytotoxicity from cells treated with 0.1 % Triton X-100.
To further investigate which components of welding fumes were causing the observed cell death, welding fumes were separated into the particulate fraction which had been washed to remove any soluble components (washed) or the soluble fraction depleted of particles (soluble). Cells were then exposed at the same concentrations and time-points as previously studied. A similar dose and time-dependent increase in toxicity was seen following exposure to both the washed particulate and soluble fractions for all three fumes (Figure 3.5). The degree of toxicity caused by either the soluble or the washed particulate fractions alone were significantly less than that shown by the whole welding fumes (Figure 3.4). The washed particulate fraction of each fume caused a greater release of LDH than the soluble counterpart at the highest concentration (250 µg ml⁻¹) (p<0.05), indicating the particulate fraction of welding fumes were more toxic than the soluble components. The combined toxicity following exposure to washed and soluble fractions was equivalent to that of the whole welding fumes confirming the previous results.

The toxic dose (TD) is a measurement of the degree of toxicity caused by a certain concentration of substance. The Toxic Dose (TD) or Lethal Dose (LD) of a substance is frequently referred to in toxicology; therefore the TD 10, 25 and 50 of welding fumes were calculated (Table 3.2 A and B).

<table>
<thead>
<tr>
<th>Sample</th>
<th>TD 10</th>
<th>TD 25</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIMROD 182</td>
<td>37.5</td>
<td>117.5</td>
</tr>
<tr>
<td>NIMROD c276</td>
<td>35</td>
<td>70</td>
</tr>
<tr>
<td>COBSTEL 6</td>
<td>42.5</td>
<td>108.75</td>
</tr>
</tbody>
</table>

**Table 3.2 A.** Toxic dose of welding fumes as determined by LDH.

<table>
<thead>
<tr>
<th>Sample</th>
<th>TD 25</th>
<th>TD 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIMROD 182</td>
<td>11.2</td>
<td>42</td>
</tr>
<tr>
<td>NIMROD c276</td>
<td>6.25</td>
<td>25</td>
</tr>
<tr>
<td>COBSTEL 6</td>
<td>4</td>
<td>11.875</td>
</tr>
</tbody>
</table>

**Table 3.2 B.** Toxic dose of welding fumes as determined by reduction of metabolic activity of alveolar epithelial cells. Values represent concentration of particles (µg ml⁻¹).
Figure 3.5. Cytotoxicity of Different Fractions of Welding Fumes on Alveolar Epithelial Cells. LDH release is expressed as % Total cellular LDH, measured in the cell lysates obtained following treatment with whole welding fumes, the soluble or the washed particulate fractions of welding fumes (1-250 µg ml⁻¹) for 24 hr. The graph represents the mean of three experiments conducted in triplicate and the bars represent ± S.E.M. *p<0.05; **p<0.01; ***p<0.001 compared to 100% cytotoxicity from cells treated with 0.1 % Triton X-100. $<0.05$ washed fraction compared to soluble fraction.
3.5 The Effect of Welding Fumes on Alveolar Epithelial Cell Morphology

The effect of welding fumes on the morphology of alveolar epithelial cells was examined by culturing A549 cells on cover slips and treating them with either the whole or soluble fractions of welding fumes or ufCB as a representative inflammogenic particle. Romanowsky dyes were used to stain the cells and allowed analysis by light microscopy. Following 24 hr exposure to whole fraction of all three welding fumes, there was no marked change in epithelial cell morphology, (Figures 3.6 A, C, F, I). Particles from the cobalt-based welding fume, COBSTEL 6, appear to be within the cytoplasm of A549 cells suggesting phagocytosis has occurred (Fig 3.6 J). However, due to the agglomerates formed by the two nickel-based welding fumes NIMROD 182 and NIMROD c276, it was difficult to determine whether particles were intracellular or just deposited on the cell surface (Fig.3.6 D, G). Compared to the polyhedral shaped control cells, (Fig. 3.6 A), those treated with the soluble fractions of welding fumes, particularly NIMROD c276, were more elongated, with a fibroblast-like appearance (Figures 3.6 E, H, K). In addition, although the cells were seeded at the same density (0.2 x 10^6 ml^{-1}), following treatment with the soluble fraction cells were less densely packed, having lost the typical cobblestone morphology of A549 cells, as displayed by the control cells (Fig. 3.6 A), indicating that a degree of cell death or inhibition of cell division had occurred. This confirms the data in section 3.4 which identified approximately 15 % cell death occurred at this concentration of welding fume (63 μg ml^{-1}). It is somewhat surprising that cells exposed to the whole welding fume fractions showed no apparent morphological changes as a significant degree of cell death was also shown in figure 3.5. A similar elongation effect was observed in cells exposed to ufCB, (Figure 3.6 B), confirming the validity of this particle as positive particle control.
Figure 3.6 Effect of Welding Fumes on Alveolar Epithelial Cell Morphology. A549 cells were cultured onto glass coverslips and exposed to either whole welding fumes or the soluble fractions (63 µg ml⁻¹) or ufCB (100 µg ml⁻¹) for 24 hr. The cells were then stained with Romanowsky dyes, visualised by light microscopy at either x400 or x1000 magnification and photographed.
3.6 The Effect of Welding Fumes on IL-8 Gene Expression in Alveolar Epithelial Cells

Numerous studies have demonstrated the inflammatory effect of welding fumes, both in vivo and in vitro (Blanc et al., 1993; Antonini et al., 1996; 1999; Taylor et al., 2003). The release of cytokines from activated macrophages and epithelial cells is a fundamental process in particle-induced lung inflammation resulting in the recruitment of leukocytes. The pro-inflammatory cytokine, interleukin-8 (IL-8) is one of the most potent neutrophil chemo-attractants and activators, (DeForge et al., 1993), produced by numerous cell-types including endothelial cells, alveolar macrophages and epithelial cells (Strieter et al., 1990; Roebuck, 1999; Schwarze et al., 2000). Previous studies have reported increased IL-8 mRNA and protein levels through mechanisms involving transition metals (Quay et al., 1998; Carter et al., 1997; Hetland et al., 2001). Furthermore, it is hypothesized that Metal Fume Fever (MFF), the most common respiratory condition seen in welders, is driven by cytokine or cytokine-like mechanisms, involving elevated circulating levels of TNF-α, IL-1β and IL-8 (Blanc et al., 1993; Graeme et al., 1998). Therefore to investigate the pro-inflammatory effects of welding fumes, reverse-transcriptase PCR (RT-PCR) was used to evaluate changes in steady-state IL-8 gene expression.

Alveolar epithelial cells were exposed to non-toxic concentrations of welding fumes (2-63 μg ml⁻¹) for various time points. Following 6 hr exposure to both NIMROD c276 and COBSTEL 6 (63 μg ml⁻¹) IL-8 mRNA levels were significantly increased compared to control values (p<0.05). A slight, but not significant, increase in IL-8 mRNA was observed following exposure to NIMROD 182 (Figure 3.7). IL-8 gene expression following exposure to all three welding fumes peaked at 6 hr, returning to control values by 24 hr (data not shown).
Figure 3.7. Effect of Welding Fume Particles on IL-8 gene Expression in Alveolar Epithelial Cells. A549 cells were treated with welding fume particles 2 - 63μg ml⁻¹ for 6 hr, RNA was isolated and IL-8 mRNA quantified by RT-PCR (Section 2.11-2.13). Representative IL-8 and GAPDH PCR gel showing the dose-response of A549 cells treated with welding fumes.
3.7 The Role of the Soluble Fraction of Welding Fumes on IL-8 Gene Expression

To determine which fraction of welding fumes was responsible for the increased gene expression, A549 cells were exposed to the whole, the soluble or the washed particulate fractions of welding fumes, (63 µg ml⁻¹), for 6 hr. A significant increase in IL-8 mRNA was found following exposure of the whole welding fumes for all three fumes, with no difference between fumes. More importantly, there was no significant difference in the level of gene expression between cells exposed to either the whole or soluble fraction of each fume (Figure 3.8). No increase in IL-8 mRNA was observed following exposure to the washed particulate fraction of any welding fume (Appendix IV). These results suggest that the soluble fraction of welding fumes is entirely responsible for the enhanced pro-inflammatory gene expression observed at 6 hr with welding fumes.

![Figure 3.8](image_url)

**Figure 3.8. Effect of Whole and Soluble Fractions of Welding Fumes on IL-8 gene Expression in Alveolar Epithelial Cells.** A549 cells treated with either the whole or soluble fraction of welding fumes (63µg ml⁻¹) for 6 hr. RNA was isolated and IL-8 mRNA quantified by RT-PCR (Section 2.11-2.13). A. Representative PCR gel for IL-8 and GAPDH for cells exposed to either whole (w) or soluble (s) fraction of welding fumes. B. The histogram represents the mean of four experiments conducted on pooled triplicate samples ± S.E.M. Values are expressed as the ratio of IL-8 mRNA/GAPDH % Control (untreated cells 0µg ml⁻¹). *p<0.05 compared to control.
3.8 The Effect of Welding Fumes on IL-8 Protein Production in Alveolar Epithelial Cells

IL-8 gene expression was significantly increased following exposure to both the whole welding fumes; that is the particulate matter plus the soluble components and also the soluble fraction alone (Section 3.7). To investigate whether this increased gene expression led to increased protein release the levels of IL-8 protein in supernatant after 6 or 24 hr exposure to various concentrations of whole welding fumes were measured. All three fumes caused a dose-dependent increase in IL-8 production (Figure 3.9 A), with both NIMROD c276 and COBSTEL 6 causing a significant increase above control levels at the highest concentrations (31 μg ml⁻¹ $p<0.01$; and 63 μg ml⁻¹ $p<0.001$). Following 6 hr exposure, NIMROD c276 induced the highest cytokine response in A549 cells (1309.82 ± 4.52 % IL-8 compared to control). At both time points, NIMROD 182 was the least potent fume causing a 4-fold increase above control levels at a 6 hr compared to the 13-fold and 8-fold increase induced by NIMROD c276 and COBSTEL 6 respectively. A similar, but less striking increase in IL-8 production was observed following 24 hr exposure to all three fumes (Figure 3.9 B) with NIMROD c276 again evoking the greatest pro-inflammatory response compared to both NIMROD 182 and COBSTEL 6 (NIMROD c276: 949.82 ± 162.01; NIMROD 182: 448.91 ± 106.89; COBSTEL 6: 717.21 ± 186.61 % IL-8 compared to control).

To further identify which fractions of welding fumes were responsible for the pro-inflammatory effects observed, alveolar epithelial cells were treated with either the soluble fraction alone or the particulate fraction which had been washed to remove any soluble components (washed fraction) and IL-8 release into supernatant was measured following 24 hr exposure. The soluble fraction of all three fumes caused a significant dose-dependent increase in IL-8 expression at concentrations greater than 31 μg ml⁻¹ ($p<0.001$) (Figure 3.9 C). Moreover, the measured levels of IL-8 were equivalent to those observed following exposure to the whole welding fume suspensions. No increase in IL-8 production was observed following 24 hr exposure to washed welding fume particles, (Figure 3.9 D) indicating that the pro-inflammatory effects of welding fumes were caused by the soluble fraction alone.
Figure 3.9. Effect of Welding Fume Particles on IL-8 Protein in Alveolar Epithelial Cells. A549 cells were treated with welding fumes (2 - 63 μg ml⁻¹) for A. 6 hr or B 24 hr. C. Cells were exposed to the soluble fraction of welding fumes or D. Washed welding fume particles for 24 hr and the supernatant was analysed for IL-8 protein. The values are expressed as percent control (untreated cells 0 μg ml⁻¹). The histograms represent the mean of four experiments performed in triplicate and the bars represent ± S.E.M. *p< 0.05, **p< 0.01, ***p< 0.001 compared to control. $p< 0.05, $$p< 0.01, $$$p< 0.001 NIMROD 182 v NIMROD c276, #p< 0.05, ##p< 0.01 NIMROD 182 v COBSTEL 6. A representative IL-8 standard curve is shown in Appendix V.
3.9 Time-Course of Release of Soluble Fractions of Welding Fumes

The release of IL-8 protein was greater following 6 hr exposure to welding fumes than at 24 hr and the soluble fraction alone appeared to be entirely responsible for this enhanced cytokine production. The time-scale over which the active soluble components of welding fumes were released was determined. Welding fumes were suspended in media and at various time-points, supernatant was separated from the particles. Alveolar epithelial cells were then exposed to these supernatants collected at the various time-points and IL-8 release was measured following 24 hr exposure. Supernatant obtained from all three fumes after 15-minutes caused a significant increase in IL-8 production (p<0.001) (Table 3.3). At all other time points over the 24 hr period, IL-8 levels were similar to those produced by supernatant collected after 15 minutes, demonstrating that all of the soluble activity was released within the first 15 minutes.

<table>
<thead>
<tr>
<th>Fume</th>
<th>Time mins</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>240</th>
<th>420</th>
<th>1440</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIMROD 182</td>
<td></td>
<td>50.24 ±5.2</td>
<td>258.44±5.2</td>
<td>221.67±5.4</td>
<td>191.8±10.3</td>
<td>216.62±15.9</td>
<td>227.65±15.9</td>
<td>210.64±12.5</td>
</tr>
<tr>
<td>NIMROD c276</td>
<td></td>
<td>50.24 ±5.2</td>
<td>291.34±7.4</td>
<td>265.56±6.5</td>
<td>285.56±7.1</td>
<td>310.84±4.9</td>
<td>294.75±7.1</td>
<td>337.95±5.6</td>
</tr>
<tr>
<td>COBSTEL 6</td>
<td></td>
<td>50.24 ±5.2</td>
<td>231.78±6.1</td>
<td>186.12±14</td>
<td>215.24±12.1</td>
<td>212.48±20.1</td>
<td>195.02±8.3</td>
<td>200.07±6.7</td>
</tr>
</tbody>
</table>

Table 3.3. Time Course of Release of Soluble Fractions of Welding Fumes. Welding fumes particles (63 µg ml⁻¹) were agitated over a 24 hr period and supernatant was collected after 15, 30, 60, 240, 420 and 1440 mins. Soluble fractions were isolated as described in section 2.3. A549 cells were then exposed to the soluble fractions for 24 hr and the supernatant was analysed for IL-8 protein. Values are expressed as picograms per ml ± S.E.M. * p<0.001 compared to control (untreated cells 0 µg ml⁻¹)
3.10 The Role of Soluble Transition Metals in Welding Fume-Induced IL-8 production

Due to the high metal content present in welding fumes, and the evidence demonstrating that the bio-availability and solubility of transition metals are critically important in the inflammatory and toxic responses to welding fumes (White et al., 1982; Antonini et al., 1999), the role of transition metals was addressed. As the composition of welding fumes is highly complex, containing a multitude of transition metals, fumes were pre-treated with the non-specific transition metal chelator, Chelex-100, (50 mg ml\(^{-1}\)) before addition to alveolar epithelial cells. Transition metals released or mobilised from the fumes would be chelated onto the beads and removed from solution. A complete attenuation in IL-8 protein release was seen following chelation of the whole and soluble fractions of all three welding fumes compared to untreated fumes (p<0.001) (Figure 3.10). These findings indicate soluble transition metals present in welding fumes play a pivotal role in the induction of the pro-inflammatory effects of welding fumes.

![Figure 3.10](image-url)

**Figure 3.10 Effect of Chelex Treatments on Cytokine Release by Welding Fume Particles and Soluble Fractions.** Whole welding fume particles and soluble fractions (63 µg ml\(^{-1}\)) were treated with chelex beads (50 mg ml\(^{-1}\)) for 4 hr. A549 cells were then treated with either the chelated fumes or untreated fumes for 24 hr and the supernatant was analysed for IL-8 protein. The values are expressed as percent control (untreated 0 µg ml\(^{-1}\)). The histograms represent the mean of three experiments performed in triplicate and the bars represent ± S.E.M *p<0.05; **p<0.01; ***p<0.001 compared to control. $$p<0.01, $$$p<0.001$$ untreated welding fumes
3.11 The Effect of Welding Fumes on Intracellular GSH levels in Alveolar Epithelial Cells

Oxidative stress is defined as an imbalance between oxidant and antioxidants (Cross et al., 1994) and is a common feature of particle-induced inflammation (Donaldson et al., 2002b). A characteristic of inflammogenic and toxic particles is their ability to produce free radicals which, when in excess, can cause a depletion of antioxidants, such as glutathione (GSH). To investigate the involvement of reactive oxygen species in the inflammatory and toxic responses previously shown, levels of the intracellular antioxidant glutathione were measured as an indicator of oxidative stress. Treatment of alveolar epithelial cells with all three welding fumes (63 μg ml⁻¹) caused a rapid, yet transient, depletion of GSH over a 24 hr period (Figure 3.11). A significant reduction in GSH was evident by 2 hr (p<0.05) which remained below control levels until 8 hr. However, when exposure was extended over a 24 hr period, a marked trend towards an increase in glutathione levels was seen suggesting a rebound effect.

Figure 3.11. Effect of Welding Fumes on Intracellular Glutathione Levels in Alveolar Epithelial Cells. Total intracellular GSH concentrations in A549 cells following treatment with whole welding fumes (63 μg ml⁻¹) for 0, 2, 4, 8 or 24 hr. The values are expressed as Total Glutathione % Control (untreated cells 0 μg ml⁻¹) at 0 hr. The graph represents the mean of five experiments conducted in triplicate and the bars represent ± S.E.M.*p<0.05 compared to control.
As soluble transition metals present in welding fumes were shown to play a fundamental role in IL-8 gene expression, A549 cells were treated with either the whole or soluble fractions of each fume to examine the mechanism by which soluble components exert these effects. Following 2 hr exposure to both the whole and the soluble fractions of welding fumes, levels of intracellular GSH were significantly decreased (p<0.05) (Figure 3.12). These data suggest that the soluble components of welding fumes exert their effects via an oxidative mediated mechanism.

![Graph](image)

*Figure 3.12. Effect of the Soluble Fractions of Welding Fumes on Intracellular GSH.*
Total intracellular GSH concentrations in A549 cells following treatment with whole welding fumes or soluble fractions (63 µg ml⁻¹) for 2 hr. The values are expressed as Total Glutathione % Control (untreated cells 0 µg ml⁻¹) at 0 hr. The graph represents the mean of five experiments conducted in triplicate and the bars represent ± S.E.M. *p<0.05; ***p<0.001 compared to control.
3.12 Acellular Production of Reactive Oxygen Species by Welding Fumes

Many transition metals present in welding fumes can undergo Fenton-chemistry resulting in the production of reactive oxidants. Previous studies have suggested the formation of ROS by bio-available metals may in part be responsible for oxidative damage induced by particles (Donaldson et al., 1996; 1997; Dick et al., 2003; Gilmour et al., 1996). Furthermore, surface-derived free radicals have also been implicated as a factor in the toxicity of a number of particle types such as asbestos, quartz and PM$_{10}$ (Li et al., 1996; Fenoglio et al., 2003; Donaldson et al., 1995; 1996; 1997). To investigate the oxidative capacity and reactivity of welding fumes the plasmid DNA scission assay was employed. This assay has been widely used to study particle and fibre-associated free radical damage to the model macromolecule, plasmid DNA (Donaldson et al., 1995). The assay works on the principle that free radicals associated with particle surfaces will cause changes in the quaternary structure of the DNA molecule by “nicking” the super-coiled DNA and causing it to unwind into a relaxed coil. Further free radical production will result in greater DNA damage and complete linearization followed by fragmentation.

Super-coiled DNA was incubated with the three different welding fumes at various concentrations ranging from 100–500 μg ml$^{-1}$. At all concentrations of welding fumes no DNA damage was observed as shown by the lack of linearization of the super-coiled DNA (Figure 3.13). Although this suggests that welding fumes do not produce free radicals, one of the main processes for transition metal mediated ROS production is via Fenton/Haber-Wiess reactions (Section 1.9.1 reactions [6]-[8]), which occur in reducing environments such as the epithelial lung lining fluid (Sun et al., 2001). As this assay was performed in an acellular, neutral environment, this may explain the lack of detectable DNA scission. To test this hypothesis, various concentrations of welding fumes were co-incubated with H$_2$O$_2$ and subsequent DNA damage was assessed. In the presence of H$_2$O$_2$, all three welding fumes caused a significant increase in DNA damage, indicating that Fenton-chemistry is involved in welding-fume associated free radical production (Figure 3.14). NIMROD 182 was shown to be the least reactive fume, causing 56.37 ± 2.52 % depletion of super-coiled DNA at a concentration of 100 μg ml$^{-1}$ in the presence of 100 μM H$_2$O$_2$. At
the same concentration NIMROD c276 caused significant depletion of the super-coiled DNA (81.56 ± 1.56 % depletion compared to DNA control). COBSTEL 6 was the most reactive welding fume causing complete fragmentation of the plasmid indicating the production of high concentrations of ROS. As H₂O₂ alone caused no depletion of the super-coiled DNA plasmid, this suggests that oxidants other than H₂O₂ are responsible for welding fume induced DNA damage. These data demonstrate that welding fume particles alone are highly reactive generating large quantities of free radicals in reducing environments.

![Figure 3.13. The Effect of Welding Fumes on Super-coiled DNA plasmid.](image_url)

Welding fumes (100-500 µg ml⁻¹) were incubated with DNA plasmid φX174 RF1 for 8 hr at 37 °C. DNA was used a control, a linear control was prepared using a restriction enzyme (PST1), ROFA and CB were used as positive and negative particle controls respectively. The figure shows a representative plasmid DNA gel. Treatments were repeated three times and the % depletion was quantified as: (Relaxed + Linear)/Total DNA.

![Figure 3.14. The Effect of H₂O₂ on Welding Fumes induced DNA Plasmid Scission.](image_url)

Welding fumes (100 µg ml⁻¹) were co-incubated with or without H₂O₂ (100 µM) with DNA plasmid φX174 RF1 for 8 hr at 37 °C. The same controls were used as above (Fig. 3.13) with the addition of H₂O₂ control (100 µM). Figure shows a representative gel. Treatments were repeated three times and the % depletion was quantified as: (Relaxed + Linear)/Total DNA.
3.13 The Induction of Intracellular Reactive Oxygen Species by Welding Fumes

As welding fumes were able to produce free radicals in an acellular environment, it was hypothesised that similar mechanisms may occur in the intracellular environment and this enhanced ROS production may be a mechanism by which welding fumes cause an inflammatory response. Therefore the levels of intracellular ROS in alveolar epithelial cells were measured using the fluorescent probe DCFH-DA and flow cytometry. DCFH-DA is frequently used to detect oxygen radical production and oxidative stress and although initially used to detect peroxide levels, its specificity is controversial. The diacetate moiety renders the molecule hydrophilic which enables the probe to diffuse through cell membranes, where it is enzymatically hydrolysed by intracellular esterases. In the presence of intracellular ROS, the DCFH is converted into the highly fluorescent dichlorofluorescein (DCF) (LeBel et al., 1992). Thus the intensity of fluorescence is directly proportional to the level of ROS present.

The fluorescence intensity was measured in A549 cells following treatment with either the whole or soluble fractions of the three welding fumes. To determine whether the rapid depletion of intracellular GSH shown previously could be attributed to increased levels of ROS, exposures were performed for 2 hr. Treatment of A549 cells with the whole and soluble fractions of both nickel-based welding fumes, NIMROD 182 and NIMROD c276, caused substantial increase in fluorescence indicating an elevation in intracellular ROS production as shown by the rightward shift in the histogram peaks illustrating the conversion of DCFH to DCF (Figure 3.15 A, B) and corresponding graphical representation (Figure 3.16). Furthermore, there was no significant difference in fluorescence intensity between the whole or soluble fractions. This suggests that the soluble fraction of nickel-based welding fumes induce oxidative stress in A549 cells by increasing intracellular ROS and depleting GSH. However, the cobalt-based fume, COBSTEL 6 appeared to have no effect on the oxidative status of epithelial cells as shown by the lack of fluorescence (whole; 96.76 ± 12.24; soluble; 95.18 ± 6.91; control; 100 %). This finding was somewhat surprising given that COBSTEL 6 caused complete fragmentation of the DNA plasmid, and also induced a similar depletion in GSH as both nickel-based fumes, suggesting a role for ROS.
Figure 3.15 Effect of Welding Fumes on Intracellular ROS production as measured by DCFH-DA. A549 cells were treated with either whole welding fumes or the soluble fraction for 2 hr in the presence of the fluorescent probe DCFH-DA. The figures show representative data from Flow Cytometer. Increased fluorescence is illustrated by increased DCF FITC indicates enhanced ROS production. The y-axis shows the number of cells counted at the corresponding fluorescence. Purple histogram: Control; Green histogram: Whole Welding Fumes; Pink histogram: Soluble Fraction. A. NIMROD 182; B. NIMROD c276; C. COBSTEL 6.
Figure 3.16. Intracellular ROS Production Induced by Welding Fumes in Alveolar Epithelial Cells. A549 cells were treated with either whole welding fumes or the soluble fractions (63 µg ml⁻¹) for 2 hr in the presence of the probe DCFH-DA and the levels of intracellular ROS were determined by flow cytometry. The values are expressed as % Control (untreated cells 0 µg ml⁻¹) Geo Mean Fluorescence. The histogram represents the mean of six experiments conducted in triplicate and the bars show ± S.E.M. *p<0.05; **p<0.01 compared to control; #p<0.05 NIMROD 182 (w) v COBSTEL 6 (w); §p<0.05 NIMROD 182 (s) v COBSTEL 6 (s); φφp<0.01 NIMROD c276 (w) v COBSTEL 6 (w); δδp<0.01 NIMROD c276 (s) v COBSTEL 6 (s).
Lipid Peroxidation following Exposure to Welding Fumes

Previous results showed an enhanced production of intracellular ROS and concomitant decrease in GSH in alveolar epithelial cells following exposure to welding fumes. This sequence of events can initiate or promote oxidative damage to cellular constituents such as proteins and lipids (Reuben et al., 2004). To further investigate the effects of welding fume-induced ROS production, the levels of malondialdehyde (MDA) a secondary product of lipid peroxidation were measured as an indicator of oxidative membrane damage. The levels of MDA were determined using a colorimetric reaction with thiobarbituric acid (TBA) (Yagi, 1976; Ohkawa et al., 1979). Alveolar epithelial cells were exposed to whole welding fumes, the soluble fraction alone or the washed particulate fraction for various time points and the levels of TBA reaction products (TBARS) were determined. A significant increase in TBARS was observed following 4 hr exposure to the whole welding fumes and washed particulate fractions of all three welding fumes (Figure 3.17). In accord with the depletion of GSH and enhanced ROS production at 2 hr, increased TBARS peaked at 4 hr illustrating the sequential effects of oxidative stress. Levels of TBARS returned to basal concentrations after 8 hr exposure demonstrating the transient effect of welding fume-induced cell injury (data not shown). NIMROD 182 and COBSTEL 6 caused the greatest degree of membrane damage (p<0.001 compared to control) and there was no difference between the whole welding fumes or the washed particulate fractions suggesting the particulate components were responsible for membrane damage. Although NIMROD c276 was previously identified as the most toxic (Fig. 3.4) and inflammagenic fume (Fig.3.9,3.16), with regards to lipid peroxidation it appeared to be the least potent welding fume (whole NIMROD: c276: 1.28 ± 0.13 v whole NIMROD: 182 2.61 ± 0.31 and whole COBSTEL 6: 2.01 ± 0.25 μM TBA). Furthermore, no increase in TBARS was found following exposure to the soluble fractions of all three fumes at any time point investigated (2, 4, 8, 24 hr), in contradiction to all of the previous data that clearly demonstrated the pro-inflammatory effects of welding fumes were driven by the soluble components.
Figure 3.17. Lipid Peroxidation in A549 cells following exposure to Welding Fumes determined by TBARS. A549 cells were exposed to whole welding fumes, the soluble fraction or the washed fractions for 4 hr and the levels of TBARS were measured. The graph represents the mean of four experiments conducted in triplicate and the bars show ± S.E.M. *p<0.05; ***p<0.001 compared to control #p<0.05; ###p<0.001 compared to soluble fractions.

As the particulate fraction was able to produce free radicals as illustrated by the depletion of super-coiled DNA plasmid (section 3.13), these anomalous results may be due to a direct reaction of the welding fume particulates with TBA. To test this hypothesis, the whole, soluble and washed particulate fractions of welding fumes were added directly to the TBARS reaction mixture (0.8 % TBA, 8.1 % SDS, 20% acetic acid and H₂O), heated at 95 °C for 1 hr and the levels of TBARS were measured. The whole and washed particulate fractions of all three welding fume fractions caused a striking increase in TBARS demonstrating the direct interaction of welding fume particulate with TBA. In accord, the soluble fraction, lacking any
particulates, failed to have any effect on the levels of TBARS. In support of these findings Hetland et al., (2001) demonstrated that various different stone-quarry particles significantly increased TBA reactive products in cell free conditions. These data provide a clear explanation for the increased levels of TBARS observed in A549 cells following exposure to the whole and washed particulate fractions (Figure 3.17). However the reasons why such apparently reactive particles fail to induce any inflammatory response in terms of cytokine production remains unclear.

Figure 3.18. Direct Effect of Welding Fumes on TBA. Whole welding fumes, the soluble fraction or the washed particulate fractions of welding fumes (63 μg ml⁻¹) were incubated with TBARS reaction mixture, heated at 95 °C for 1 hr and the levels of TBARS were measured. The graph represents the mean of two experiments conducted in triplicate ± S.E.M. (PBS: control).
3.15 The Effect of Anti-Oxidants on IL-8 Production by Alveolar Epithelial Cells

As shown previously, oxidative stress is likely involved in IL-8 production induced by welding fumes. Therefore welding fumes were treated with a variety of anti-oxidants to ascertain whether IL-8 production could be attenuated. The antioxidants employed were chosen due to their differing antioxidant capabilities. N-acetyl-L-cysteine (NAC) is a thiol antioxidant that acts directly as a free radical scavenger and indirectly by providing cysteine for enhanced intracellular GSH production (Morcillo et al., 1999); Vitamin E (α-tocopherol) is a fat-soluble compound which acts as a chain-breaking antioxidant by converting O₂⁻, 'OH and lipid peroxyl radicals to less reactive forms (Brigelius-Flohe et al., 1999); Mannitol acts as a free radical scavenger by forming stable complexes with H₂O₂, via hydrogen bonds and reacts with 'OH to form non-reactive mannitol dimers (Stohs et al., 1995; Halliwell et al., 1995).

Alveolar epithelial cells were incubated for 24 hr with either the whole or soluble fractions of each welding fume (63 μg ml⁻¹) in the presence of the above antioxidants and IL-8 protein production was determined. No attenuation of IL-8 protein production was observed with either the whole or soluble fractions of all three welding fumes in the presence of mannitol (Figure 3.19). Similarly, Vitamin E did not cause any reduction in the levels of IL-8 induced by welding fumes (Figure 3.20). In contrast, co-incubation of 5 mM NAC with both the whole and soluble fractions of all three welding fumes abrogated IL-8 production by approximately 50 % for all treatments (Figure 3.21).
Figure 3.19 Effect of Mannitol on Welding Fume induced IL-8 Production in Alveolar Epithelial Cells. A549 cells were treated with either whole welding fumes or the soluble fraction with or without 5 mM Mannitol for 24 hr and the supernatant was analysed for IL-8. The histograms represent the mean of three experiments performed in triplicate and the bars represent ± S.E.M. *p<0.05; **p<0.01; ***p<0.001 compared to control.

Figure 3.20 Effect of Vitamin E on Welding Fume induced IL-8 Production in Alveolar Epithelial Cells. A549 cells were treated with either whole welding fumes or the soluble fraction with or without 2 mM Vitamin E for 24 hr and the supernatant was analysed for IL-8. The histograms represent the mean of three experiments performed in triplicate and the bars represent ± S.E.M. ***p<0.001 compared to control.
Figure 3.21 Effect of N-Acetyl-L-cysteine on Welding Fume induced IL-8 Production in Alveolar Epithelial Cells. A549 cells were treated with either whole welding fumes or the soluble fraction with or without 5 mM NAC for 24 hr and the supernatant was analysed for IL-8. The histograms represent the mean of three experiments performed in triplicate and the bars represent ± S.E.M. *p<0.05, **p<0.01 compared to control; $p<0.05 compared to untreated fraction.

### Summary

<table>
<thead>
<tr>
<th>Fume</th>
<th>Fraction</th>
<th>LDH (24 hr)</th>
<th>IL-8 mRNA</th>
<th>IL-8 Protein</th>
<th>GSH</th>
<th>DCFH-DA</th>
<th>TBARS</th>
<th>*IL-10 Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIMROD</td>
<td>Whole</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>182</td>
<td>Soluble</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>NE</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Washed</td>
<td>+</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>+++</td>
<td>NE</td>
</tr>
<tr>
<td>NIMROD</td>
<td>Whole</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C276</td>
<td>Soluble</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>NE</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Washed</td>
<td>+</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>+</td>
<td>NE</td>
</tr>
<tr>
<td>COBSTEL 6</td>
<td>Whole</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>NE</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Soluble</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>NE</td>
<td>NE</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Washed</td>
<td>+</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>+++</td>
<td>NE</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 3.3 Summary of data illustrating differences in biological reactivity between whole, soluble and washed welding fume fractions.* IL-10 data is shown in appendix VI. + p<0.05; ++ p<0.01, +++ p<0.001 compared to control values. NE: No effect.
3.15 Discussion

It is well documented that exposure to a variety of airborne particulates in both occupational and environmental settings can cause adverse health effects ranging from the exacerbation of pre-existing lung inflammation to fibrosis (Levy et al., 1984; Rendell et al., 1994; Hauser et al., 1995; Dockery et al., 1993; Schwartz et al., 1992; Seaton et al., 1995). The biochemical mechanisms responsible for particle-induced adverse health effects are partially elucidated and for many particles the generation of reactive oxygen species (ROS) plays an important role (Carter et al., 1997; Donaldson et al., 1997; Quay et al., 1998; Kennedy et al., 1998).

Epidemiological studies have consistently reported a higher incidence of respiratory illnesses, such as bronchitis and metal fume fever (MFF) amongst welders exposed to high concentrations of welding fumes (Doig et al., 1951; Sferlazza et al., 1991; Antonini et al., 1996). A common characteristic of many of the respiratory conditions associated with welding fume exposure is the increased expression of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-8 (Lockey et al., 1988; Kuschner et al., 1995; Blanc et al., 1993; Antonini et al., 1996; 1997). Furthermore, the acute inflammatory response and systemic fever observed in welders suffering from MFF is thought to be driven via a cytokine or cytokine-like mechanism (Blanc et al., 1991; 1993). This hypothesis is supported by both in vivo and in vitro studies reporting enhanced inflammatory responses characterised by neutrophilia, increased protein and elevated cytokine levels in bronchoalveolar lavage fluid (BALF) following exposure to welding fumes (Blanc et al., 1991; 1993; Antonini et al., 1996; 1997; Yu et al., 2000).

The chemical composition of welding fumes is highly complex and can vary considerably between fumes, consequently the toxicity and inflammogenicity may also differ (Kuschner et al., 1995; Antonini et al., 1996; 1997). For example, due to their inherent toxicity, some metal constituents of welding fumes such as chromium, nickel and manganese, create a potentially more harmful fume (Antonini, 2003). A recent study by Taylor et al., (2003) showed that the acute lung injury and
inflammation observed in rats following instillation with a variety of welding fumes was dependent on the composition and solubility of the fume. Similarly, previous studies by Antonini et al., (1999) demonstrated that the increased cytotoxicity and reduced activity of rat alveolar macrophages was due to the soluble fraction of Manual Metal Arc (MMA)-fumes. However, other studies have suggested that both soluble and insoluble fractions of ambient air particulates contribute to pulmonary inflammation (Ghio et al., 1999b). Therefore, the aim of this chapter was to determine the toxicity and inflammogenicity of three compositionally different welding fumes; two nickel-based fumes: NIMROD 182 and NIMROD c276 and a cobalt-based fume: COBSTEL 6; and identify the relative importance of the various components, i.e. the particulate or the soluble fractions, in welding fume-induced inflammation.

In this study, all three welding fumes had a marked effect on alveolar epithelial cell morphology, proliferation and viability. Exposure of A549 cells to a range of concentrations of welding fumes caused a time- and dose- dependent decrease in mitochondrial enzyme activity, which was accompanied by a significant increase in LDH release, a marker of cell death. Furthermore, following 24 hr exposure to the various fractions of welding fumes, the cell morphology was altered resulting in a change from polyhedral to fibroblastic-like appearance (Fig.3.6), indicating changes in the cellular cytoskeleton. This change in epithelial morphology may be the result of oxidation of cysteine residues in tubulin proteins that form the cytoskeletal microtubules, causing conformational changes in these proteins and consequently altering the cells shape. This phenomenon has was reported in 3T3 cells following exposure to nickel and cadmium compounds (Li et al., 1993a), and therefore a similar mechanism may be involved in the morphological changes caused by welding fumes. Although the welding fumes used in this study varied in composition, there was no obvious difference in their relative toxicity at the highest concentrations tested (Table 3.1).

The similarity in fume toxicity, particularly the nickel-based fumes, is interesting given that these fumes were generated by the different welding process,
Gas Metal Arc (GMA) welding and Manual Metal Arc (MMA) welding. The welding process employed to generate fumes can have a marked effect on the resultant fume toxicity (Antonini et al., 1998; Taylor et al., 2003) as illustrated by the greater abundance of hexavalent chromium and other toxic compounds in MMA welding fumes compared to GMA fumes (Kallimaki et al., 1986). This effect was highlighted by studies which showed that stainless steel fumes generated from MMA welding were more toxic to macrophages than fumes from a variety of other welding processes (Stern et al., 1983; 1986). This variability in fume toxicity may be explained by the fact that welding fumes used in this study were not freshly generated, thus any potential differences in short-lived particle-surface associated species would not be observed. This aging effect was illustrated by Antonini et al., (1998), who found freshly generated welding fumes induced a greater lung inflammation in rats than “aged” fumes.

It is widely accepted that transition metals such as iron, copper and chromium undergo redox-cycling. Although not classically redox-active, nickel and cadmium also generate ROS (Stohs et al., 1995; Halliwell et al., 1995). Both mechanisms can cause the depletion of antioxidants such as glutathione (GSH), resulting in oxidative stress (Stohs et al., 1995; Halliwell et al., 1985; 1995; Toyokuni, 1996). Welding fumes contain many of these reactive metals in a greater or lesser proportion, which depend on the composition of the fluxes and filler wires used (Sferlazza et al., 1991; Antonini, 2003). Recent studies have attributed the inflammatory and toxicological effects of a variety of other particulates to the soluble transition metal content (Adamson et al., 1999; Carter et al., 1997; Kennedy et al., 1998; Kodavanti et al., 1997). Indeed, particles containing easily solubilized metals appear to cause a more rapid onset and severity of acute pulmonary injury (Dreher et al., 1997).

There is also evidence to suggest the insoluble fractions of particulates play an important role in particle-induced inflammation. In a study by Imrich et al., (2000) the insoluble material present in concentrated ambient particles (CAPs) was almost entirely responsible for the activation and cytokine secretion in alveolar
macrophages. Likewise Lewis et al., (2003), identified a greater degree of tissue injury and ROS production in rats instilled with the insoluble fraction of ROFA compared to the soluble fraction.

To try and identify the toxic components of the welding fumes used in this study, alveolar epithelial cells were treated with the particulate fraction which was isolated and washed to remove any soluble components and was thus referred to as the washed fraction or the soluble fraction alone. Although both fractions of welding fumes caused an increase in cell death, the washed particulate components were more potent than the soluble counterparts at equivalent concentrations. The level of cell death observed for both soluble and washed particulate fractions however were approximately half that shown after treatment with whole welding fumes (Fig.3.5). This indicates that both the particulate and soluble fractions of welding fumes were involved in the toxicity of welding fumes. This finding is supported by those of Taylor et al, (2003), who showed an additive effect of both the insoluble and soluble fractions of MMA-SS welding fume-induced toxicity.

Although all fractions of the three welding fumes were cytotoxic at the higher doses (> 63 µg ml⁻¹), the pulmonary deposited mass following inhalation exposures is likely to be very low with particle deposition primarily occurring throughout the lower respiratory tract (Yu et al., 2000). Furthermore, as clearance mechanisms, such as the mucociliary escalator and alveolar macrophages are present within the lung, the inflammation observed in welders is probably the result of mechanisms involving cell stimulation rather than toxicity. Therefore, to investigate the role of cell stimulation in the inflammatory effects of welding fumes, non-lethal concentrations were utilised for the remainder of the study.

Numerous studies have shown an increased expression of pro-inflammatory cytokines such as IL-6, IL-8 and TNF-α in a variety of cell types following exposure to airborne particulates such as ROFA, PM₁₀ and quartz (Becker et al., 1996; Driscoll et al., 1997; Dreher et al., 1997; Donaldson et al., 1998; Hetland et al., 2004). In this study, a dose- and time-dependent increase in IL-8 was observed in alveolar epithelial cells exposed to the three different welding fumes. NIMROD c276
and COBSTEL 6 induced a significant increase in IL-8 expression at gene and protein levels with a more pronounced effect at 6 hr than 24 hr. NIMROD 182, was the least potent fume in terms of IL-8 production, causing only a 4-fold increase above control levels in comparison to 13-fold and 8-fold increases observed for NIMROD c276 and COBSTEL 6 respectively. This result supports previous toxicity data which highlighted NIMROD 182 to be the least toxic fume. As welding fumes are know to vary considerably in composition, the difference in potency noted may be due to the lower total metal concentration or metal bio-availability present in NIMROD 182 compared to both NIMROD c276 and COBSTEL 6. Previous studies have shown that different fluxes and shielding gases used during the welding process may affect the oxidation state and solubility of the fumes (Eagar et al., 1997). Consequently the pulmonary responses caused by welding fumes vary according to the materials and processes used (Antonini, et al., 1996; 1997).

The pulmonary toxicity and inflammogenicity of many different particles can be associated with the types and concentrations of soluble transition-metals (Carter et al., 1997, Kennedy et al., 1998; Frampton et al., 1999; Adamson et al., 2000). There is also considerable evidence however supporting the role of ultra-fine (uf) particles (<0.1μm diameter) in mediating such responses (Donaldson et al., 2001b; 2002a). Previous work has demonstrated that ultra-fine particles induce a greater inflammatory response and free radical production than their larger counterparts and this enhanced reactivity could be attributed to their greater surface area (Ferin et al., 1992; Li et al., 1996; Brown et al., 2001). Several studies have reported that fumes generated in the welding process are <0.5 μm (Aksellson et al., 1976; Howden et al., 1988). As all three welding fumes were composed predominantly of ultra-fine particles (Section 3.2 and Appendix III) any soluble metals present on particle surfaces may enhance free radical activity and thus induce a greater inflammatory response. This interaction between uf particles and transition metals was recently illustrated by Wilson et al., (2002) who suggested a potentiative increase in the levels of PMN in the BALF of rats following instillation of both ufCB and FeCl_3 compared to either component alone. Likewise, instillation of the metallic ultra-fines of cobalt and nickel produced an enhanced inflammatory response in rat
lungs via a mechanism involving free radical production (Zhang et al., 1998a; 1998b; Dick et al., 2003).

Both the soluble and particulate components were required to induce the maximal toxicity of all three fumes. Therefore to further investigate the specific components of welding fumes responsible for inducing the pro-inflammatory response, alveolar epithelial cells were exposed to either the soluble fraction or the washed particulate fractions of each fume. Following 24 hr treatment with the washed particulate fraction, IL-8 levels remained at basal levels. In contrast, by 6 hr, the soluble components of all three fumes significantly increased IL-8 mRNA expression and subsequent protein release. More importantly, these results mimicked those observed following exposure to the whole welding fumes. These findings support the original hypothesis that the soluble components of all three welding fumes studied were entirely responsible for the increased pro-inflammatory response observed in epithelial cells, and in the present study, particle size or surface reactivity did not contribute to the pro-inflammatory response as has been proposed for a range of low toxicity particles (Duffin et al., 2001).

As the previous results demonstrated that the soluble fraction of welding fumes played a fundamental role in initiating a pro-inflammatory response, the involvement of transition metals in this cytokine release was examined by the use of a non-specific metal chelator, Chelex-100. Although a number of other investigators have used a combination of desferrooxamine and ferrozine to chelate transition metals, these compounds are specific iron chelators, chelating Fe\(^{3+}\) and Fe\(^{2+}\) ions respectively. As the welding fumes examined in this study contained a variety of metals other than iron, Chelex-100 was used as it works on the principle that any cations present in solution are chelated onto the bead surfaces, and thus any released or mobilized transition metals are removed from solution along with the beads (Gao et al., 2002). Although there is some evidence to suggest a small amount of non-metallic chemical constituents or organic material such as DNA can be removed by chelation, this only occurs under alkaline conditions (Hemmasi et al., 1975; Giraffa et al., 2000; Molinelli et al., 2002). As all treatments were slightly acidic, chelation treatment should only remove transition metals. In accord with Molinelli et al.,
(2002) who showed that chelex-treated extracts of Provo PM$_{10}$ attenuated cellular responses in BEAS-2B human alveolar epithelial, pre-treatment of welding fumes with chelex-100 completely abrogated IL-8 protein release compared to untreated fumes confirming that transition metals have a pivotal role in the induction of inflammatory gene expression by welding fumes. These data corroborate findings by Pritchard et al., (1996), Kadiiski et al., (1997) and Dye et al., (1999), which demonstrated an association between the soluble metal content and enhanced pulmonary responses for other particle types. Furthermore, as components of all three fumes were solubilized within a few minutes in vitro, one could predict that the same would occur in vivo in the epithelial lung lining fluid, leading to the acute effects seen in MFF. In support of this theory, a recent human study by Huang et al., (2003) linked the specific soluble components of fine ambient particles such as Cu, Zn, Fe and sulphate to pulmonary neutrophil influx and increased levels of systemic blood fibrinogen. More importantly, these results provide vital information in understanding the effects of particles on the human pulmonary and systemic systems.

Glutathione (GSH) is one of the major intracellular antioxidants in the lung, playing a vital role in maintaining the intracellular redox status (Hayes et al., 1999; Rahman et al., 2000a). GSH has been implicated in numerous cellular processes, such as signal transduction, mitochondrial regulation, remodelling of extra-cellular matrix, apoptosis, proliferation and pro-inflammatory responses within the lung (Rahman et al., 2000a). It has been suggested that many of the toxic effects of airborne particles may be the result of alterations in glutathione homeostasis as illustrated by the depletion of GSH observed in lung tissue following intra-tracheal instillation of PM$_{10}$ and ufCB (Li et al., 1997; Hetland et al., 2001). In the present study, all three welding fumes caused a rapid depletion of intracellular GSH following 2 hr exposure with a trend towards a rebound increase observed at 24 hr. Further examination demonstrated that the depletion in GSH could be attributed to the soluble fraction of welding fumes. In support of these findings a number of other particles such diesel exhaust particles, ufCB and quartz have been shown to cause a reduction in intracellular GSH in bronchial epithelial cells and macrophages in vitro (Matsuo et al., 2003; Zielinski et al., 1999; Stone et al., 1998; Fenoglio et al., 2003). In addition, previous studies using ROFA, a transition metal-rich emission PM
sample, showed that metal-mediated mechanisms were responsible for the depletion of GSH in rat tracheal epithelial cells and guinea pig tracheal epithelial cells (Dye et al., 1999; Jiang et al., 2000).

The profile of welding fume-induced GSH depletion was characterized by a rapid transient depletion in GSH followed by a rebound enhancement. A similar kinetic response was previously demonstrated by Rahman et al., (1998b) who reported a transient depletion followed by up-regulation in GSH in A549 cells exposed to the oxidants H2O2 and menadione. A depletion of GSH is a characteristic feature of many inflammatory pulmonary disorders such as acute respiratory distress syndrome (ARDS) (Bunnell et al., 1993), cystic fibrosis (Roum et al., 1999), and idiopathic pulmonary fibrosis (IPF) (Cantin et al., 1989). As MFF is a transient condition, characterised by an acute inflammatory response, this initial reduction in GSH may be the result of an antioxidant/oxidant imbalance due to metal-mediated ROS production. The elevated levels of GSH observed 24 hr later could act directly as free radical scavengers thus could help to explain the rapid resolution of the acute inflammation associated with MFF. Further evidence to support this theory is shown by the IL-10 protein response (Table 3.3, Appendix VI). The significant increase in the anti-inflammatory cytokine, IL-10 was observed following 24 hr treatment with both the whole and soluble fractions of welding fumes represents a potential mechanism behind the acute nature of MFF.

Oxygen radicals and their metabolites have been shown to play a pivotal role in pulmonary toxicity caused by inhalation of different particles and fumes (Vallyathan et al., 1997). In vitro studies using various cell lines have demonstrated the involvement of oxidants such as reactive oxygen and nitrogen species in particle associated inflammation (Dye et al., 1999; Pritchard et al., 1996). It is suggested that the mechanism by which transition metals elicit their pro-inflammatory effects may be via the generation of ROS through the catalysis of Fenton-like reaction and subsequent oxidative stress (Stohs et al., 1995). Alternatively, metals could act via mechanisms independent of their oxidant catalytic activity (Dye et al., 1999), such as direct interaction of particles with endogenous antioxidant molecules (Fenoglio et
et al., 2003), interactions with metal response elements (Dalton et al., 1996) or metal-associated inhibition of tyrosine phosphatase activity (Kresja et al., 1997).

The depletion of GSH shown previously could be attributed to enhanced ROS production, via mechanisms involving transition metals. Although welding fumes alone had no effect on the super-coiled plasmid DNA, which indicated a lack of free radical activity, this may be due to the metals present in welding fumes being in a fully oxidised state and thus are unable to support electron transfer and redox reactions (Pritchard et al., 1996). Indeed, in the presence of the reductant H$_2$O$_2$, welding fumes caused a significant depletion in super-coiled plasmid DNA, indicative of hydroxyl radical generation (Gilmour et al., 1997). In accord with these findings, a recent study by Song et al. (2003) suggested that the degree of DNA damage induced by a variety of particles was dependent on the proportion of mobilised metals. The oxidant activity of welding fumes was confirmed in vitro using the fluorescent probe dichloro-dihydrofluorescein diacetate (DCFH-DA). A significant increase in intracellular ROS was observed in alveolar epithelial cells treated with the soluble fractions of both nickel-based welding fumes, NIMROD 182 and NIMROD c276 as shown by the enhanced fluorescence compared to control cells. A greater accumulation of ROS was observed following exposure to NIMROD c276 than NIMROD 182 which may explain the higher levels of pro-inflammatory cytokine expression induced by this fume shown previously. There are a number of possible mechanisms by which the nickel-based welding fumes may induce intracellular ROS production. Firstly, as the type II epithelial cells used in this study (A549 cells) are known to produce H$_2$O$_2$ (Kinnula et al., 1991) this would enable soluble transition metals released from welding to partake in Fenton-like reaction resulting in the enhanced ROS production. Secondly, this rise in intracellular ROS production could be attributed to the depletion in GSH, which would reduce the capacity of the cells to scavenge oxygen radicals (Haddard, 2000). Alternatively both scenarios could occur concurrently and thus potentate the cellular oxidative stress.

Although the welding fume COBSTEL 6 contains numerous transition metals known to undergo redox-cycling, no rise in intracellular ROS was measured
using this fluorescent probe. This disparity is somewhat surprising given the previous results illustrating the complete fragmentation of super-coiled plasmid DNA and the significant reduction in GSH similar to that caused by both nickel-based fumes, implicate the involvement of ROS. A number of possible factors may account for this apparent anomaly. Although previous studies have used the DCFH-DA fluorescent probe to investigate metal-induced ROS production, the majority of these studies concentrated on specific metal compounds such as Ni, Cr and Co (Huang et al., 1993; Martin et al., 1998; Salnikow et al., 2000). The lack of ROS detected in the present study may therefore be due to the highly complex chemical properties of welding fumes, and the interactions between different metals may synergise, attenuate or even block free radical formation detectable by this method. Likewise, the bioavailability of metals to undergo Fenton-like chemistry determines their ability to produce free radicals. The lack of ROS measured following exposure to the cobalt-based welding fume may be the result of metals present within this fume being in an inactive state, thus unable to undergo Fenton reactions. The lack of ROS production observed following exposure to the cobalt-based fume is consistent with the findings of Salnikow et al., (2000) who using the same DCFH-DA probe saw no detectable ROS in A549 cells treated with CoCl₂ at concentration of 100 μM, significantly greater than the concentration present in COBSTEL 6.

The data presented in this chapter illustrating the enhancement of intracellular ROS production and concomitant GSH depletion indicate that welding fume-induced inflammation may be regulated via a metal-mediated oxidative stress mechanism. To further characterise the oxidants involved in this process, a variety of antioxidants were tested. Of all the antioxidants employed, only N-acetyl-L-cysteine (NAC) was able to reduce welding fume-induced IL-8 protein production. As a cysteine derivative NACs ability to attenuate IL-8 production may be due to its capacity to directly quench oxygen radicals and replenish intracellular GSH stores thus suppressing downstream cytokine-dependent signalling pathways (Haddad, 2002b). In support of these findings, Dick et al., (2003) reported a significant reduction in ufNi induced TNF-α production in alveolar macrophages in the presence of NAC and GHSme. Likewise, ROFA-induced injury to rat tracheal
epithelial cells was attenuated by co-exposure with the free radical scavenger DMTU (Dye et al., 1996). These results further strengthen the role of transition metal derived free radial production in welding-fume induced inflammation.

It is interesting to note that over the range of concentrations examined, mannitol, a specific hydroxyl radical (·OH) scavenger appeared to have no effect on welding fume-induced IL-8 production. Numerous metals present in welding fumes are involved in Fenton-chemistry (Stohs et al., 1995) resulting the production of hydroxyl radicals (Gilmour et al., 1997). Indeed, the significant depletion of the super-coiled plasmid DNA strongly suggests welding fumes cause the generation of hydroxyl radicals. Furthermore, as ROS including ·OH have been directly linked to increased pro-inflammatory gene expression such as IL-8, via the redox-sensitive transcription factors NF-κB and AP-1 (Holtmann et al., 1999; Meyer et al., 1994), one could predict mannitol to have some inhibitory effect on welding fume induced IL-8 production.

The results presented in this chapter have demonstrated that the pro-inflammatory effects of welding fumes are entirely attributable to soluble transition metals. Furthermore, the transient depletion of intracellular glutathione concomitant with the increased levels of ROS and attenuated IL-8 production following co-treatment with the antioxidant NAC, strongly suggests the involvement of an oxidative mediated mechanism in driving the pro-inflammatory effects of welding fumes in vitro.
Chapter 4

Metal Composition of Welding Fumes
4.1 Introduction

Welding fumes are highly complex particulates containing numerous different metal compounds. The bioavailability, content and composition of these compounds can differ greatly between fumes as a result of the differing composition of the filler wire, flux and electrode used and the welding process employed (Lockey et al., 1988; Sferlazza et al., 1991). Consequently, the inherent toxicity of fumes also varies, with those generated from stainless steel manual metal arc welding causing a greater pulmonary inflammation and injury than mild steel counterparts and those generated via flux-core arc welding (Antonini et al., 1997). It is suggested that the differing toxicity is a result of the elemental composition and solubility of these fumes with significant levels of chromium and nickel present in stainless steel fumes, in contrast to mild steel fumes which are predominantly comprised of iron (Antonini et al., 2004b).

Transition metals such as iron, copper, zinc and vanadium have been implicated as key components driving the inflammatory events of various different particle types including ambient PM and ROFA (Kenndey et al., 1998; Rice et al., 2001; Samet et al., 2003). As the results of the previous chapter demonstrated that the pro-inflammatory effects of welding fumes were entirely attributable to the soluble transition metal fraction, the aim of this chapter was to establish the soluble metal content of the three welding fumes and determine whether their differing degree of toxicity and inflammogenicity was related to different soluble transition metals. In addition, to elucidate the role of specific metals or the potential for synergistic or additive interactions, various combinations of metals were employed to try and replicate the pro-inflammatory effects of welding fumes.
4.2 Metal Analysis of Welding Fumes

The metal content present in various welding fume fractions; whole welding fume, the soluble extract, the washed particulate fraction and the soluble extract following pre-treatment with the transition metal chelator chelex-100, was analysed by inductively coupled plasma-mass spectrometry (ICP-MS) by Dr Hibbs, at the Department of Chemistry, University of Edinburgh and Dr Gibson, Department of Civil Engineering, University of Strathclyde. Metals selected for analysis were chosen for their potential to participate in redox-cycling and/or their ability to generated reactive oxidants (Stohs et al., 1995). The sample concentrations were quantified against 11-point calibration graphs constructed using a multi-element ICP standard.

The total concentration of all metals analysed in the various fractions of welding fumes are shown in Figure 4.1. The figure clearly shows that the concentrations of metals in the whole welding fumes were similar to that of the soluble fractions, with COBSTEL 6 containing the highest total metal content of all the three welding fumes. A considerably lower total metal concentration was measured in the washed particulate fraction which is synonymous with the particle core. The chelex-treated samples also contained a very low concentration of metals demonstrating that the chelation technique effectively removed any transition metals from solution. The relative total metal concentration present in the various welding fume fractions closely mimics the observations of the pro-inflammatory and oxidative activity of these fumes. NIMROD c276 and COBSTEL 6 contained a greater total metal content than NIMROD 182 and this difference was reflected in the degree of pro-inflammatory cytokine release evoked in alveolar epithelial cells. Furthermore, chelation of soluble welding fumes removed transition metals and the associated inflammogenicity, thus providing strong evidence that the soluble transition metal content of welding fumes mediate the pro-inflammatory effects.

Although the proportion of different metals varied between fumes, chromium (Cr) dominated the soluble metal content of all three fumes (> 80% of all metals measured). COBSTEL 6 also contained a significant amount of cobalt (14 %)
in the soluble extract of the total metals measured. Nickel was previously thought to be the predominant metal type in both NIMROD 182 and NIMROD c276 due to the high Ni content present in the NIMROD electrode. Data from the ICP-MS analysis however did not support this theory as Ni only represented approximately 3 % of the total metal content of the soluble fraction. The remaining soluble metal content of all three fumes was comprised predominantly of manganese (Mn), Ni and iron (Fe) with measurable amounts of tin (Ti) and vanadium (V) in NIMROD c276.

Figure 4.1 Metal Content of Whole, Soluble, Washed and Chelex-treated Soluble Welding Fume Fractions. Transition metal content of welding fumes (63 µg ml⁻¹) was analysed by ICP-MS and quantified using an 11-point calibration graph from multi-element ICP standards. The values are expressed as µg ml⁻¹ total metal. The soluble proportion of these measured metals represents 3-5 % of the total particle weight.
4.3 Correlation between Metal Concentration of Welding Fumes and IL-8 Production in Alveolar Epithelial Cells

Correlations were performed to determine the relationship between the soluble metal content of the three different welding fumes and IL-8 production from alveolar epithelial cells following 24 hr exposure. A significant positive correlation was found between the total soluble metal concentration and levels of IL-8 protein production for all three welding fumes (correlation co-efficient NIMROD 182 \( r = 0.869 \) \( p < 0.01 \), NIMROD c276 \( r = 0.993 \) \( p < 0.001 \) and COBSTEL 6 \( r = 0.982 \) \( p < 0.001 \) confirming the relationship between soluble transition metals and the pro-inflammatory effects of welding fumes. As chromium (Cr) was the predominant metal present in the soluble fraction of all three welding fumes (> 80 %) the relationship between Cr concentration and IL-8 was also investigated. Figure 4.2 illustrates the strong positive correlation between IL-8 release and concentration of Cr present in the soluble metal fractions with the greater levels of IL-8 observed for NIMROD c276 and COBSTEL 6 compared to NIMROD 182 being reflected by the higher Cr concentrations.

![Figure 4.2 Correlation between Soluble Chromium concentrations and IL-8 Production. Correlations were performed between the Cr concentration present in welding fume concentrations from 2-63 µg ml\(^{-1}\) and the associated IL-8 protein production (n= 4).](image-url)
4.3.1 Ability of Chromium to Mimic the Pro-Inflammatory Effects of Welding Fumes

Data from the previous sections identified a strong correlation between Cr present in the soluble fraction of all three welding fumes and IL-8 release. Therefore, to determine whether the pro-inflammatory effects of welding fumes could be attributed to the soluble Cr content, alveolar epithelial cells were treated with various soluble chromium salts at concentrations comparable to those present in the soluble welding fume extracts and the resultant IL-8 production was determined (Table 4.1).

<table>
<thead>
<tr>
<th>Fume</th>
<th>CrCl₃.6H₂O</th>
<th>CrO₃</th>
<th>K₂Cr₂O₇</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIMROD 182</td>
<td>4.1</td>
<td>1.6</td>
<td>2.3</td>
</tr>
<tr>
<td>NIMROD c276</td>
<td>9.8</td>
<td>3.7</td>
<td>5.5</td>
</tr>
<tr>
<td>COBSTEL 6</td>
<td>13.3</td>
<td>5.0</td>
<td>7.4</td>
</tr>
</tbody>
</table>

Table 4.1. Concentration of chromium salts (μg ml⁻¹) required to equal the concentration of soluble chromium present in welding fumes NIMROD 182, NIMROD c276, COBSTEL 6.

As previously demonstrated, the soluble fraction of all three welding fumes significantly increased IL-8 protein production in alveolar epithelial cells following 24 hr exposure (p<0.01) (Figure 4.3). When treated with CrCl₃.6H₂O at a similar concentration as that measure in each welding fume, a marked increase in IL-8 was noted, although the levels of IL-8 released did not equal that of the welding fume alone (NIMROD c276: 342.7 ± 16.7 v CrCl₃.6H₂O: 263.1 ± 22.8 IL-8 % Control). Similarly, the enhanced IL-8 production following exposure to CrO₃ did not mirror that of any of the three welding fumes. Furthermore, as K₂Cr₂O₇ failed to induce any inflammatory response in alveolar epithelial cells, this indicated that other components in addition to Cr were also involved in the pro-inflammatory effects of welding fumes. A somewhat surprising result was the lack of IL-8 induced by all three Cr compounds in the case of COBSTEL 6. To equal the concentration of Cr in COBSTEL 6, A549 cells were exposed to concentrations of Cr compounds 3-fold and 1.3-fold greater than NIMROD 182 and NIMROD c276, however, the levels of
IL-8 measured in the supernatant were comparable to those noted at the lower Cr concentrations.

Figure 4.3. Effect of Various Chromium Compounds on IL-8 protein production in Alveolar Epithelial Cells. A549 cells were treated with different chromium compounds at concentrations comparable with those present in the soluble fractions of welding fumes for 24 hr and IL-8 protein was measured. The bars represent the mean of five separate experiments conducted in triplicate ± S.E.M *p<0.05; **p<0.01 compared to control (untreated cells 0 μg ml⁻¹).
4.3.2 Ability of Various Metal Combinations to Mimic the Pro-Inflammatory Effects of Welding Fumes

The welding fumes used in this study contain numerous different reactive metals (Figure 4.1), thus there was the potential for synergistic or additive interactions to occur between metals. As the inflammatory effects of welding fumes could not be attributed to Cr alone, various combinations of metals were tested for their ability to mimic the pro-inflammatory effects of the soluble fractions of welding fumes. After Cr, nickel (Ni) was the second most abundant metal present in both NIMROD 182 and NIMROD c276, representing approximately 3% of the total soluble metal content. Therefore, combinations of different Cr and Ni compounds at comparable concentrations to those found in welding fumes were tested. The concentration of NiCl₂ present in the soluble fractions of NIMROD 182 and NIMROD c276 were 0.06 and 0.11 µg ml⁻¹ respectively. The effects of various Cr-Ni combinations on IL-8 production are shown in Figure 4.3.2. Ni alone and in combination with the Cr compounds CrCl₃.6H₂O and CrO₃ evoked an increase in IL-8 protein production in alveolar epithelial cells following 24 hr exposure. However, the levels of IL-8 measured were not significant and did not equate to that produced by either NIMROD 182 or NIMROD c276. These data indicate that more complex interactions between the various welding fume components are involved in the underlying inflammogenicity of these fumes.
Figure 4.3.2. Effect of Various Combinations of Ni and Cr on IL-8 production in Alveolar Epithelial Cells. A549 cells were treated with different combinations of Ni and Cr compounds at concentrations comparable with those present in the soluble fractions of welding fumes for 24 hr and IL-8 protein was measured. The bars represent the mean of five separate experiments conducted in triplicate ± S.E.M. *p<0.05, **p<0.01, ***p<0.001 compared to control (untreated cells 0 μg ml⁻¹).
In addition to Cr, cobalt (Co) was also present in high concentrations (14%) in the COBSTEL 6 fume. Similar combination experiments were performed for COBSTEL 6 replacing the Ni compounds with various Co compounds at the appropriate concentrations. Concentrations of Co equal to that present in the soluble fraction of COBSTEL 6 were 2.1 μg ml\(^{-1}\) for Co(NO\(_3\))\(_2\) and 0.9 μg ml\(^{-1}\) for CoCl\(_2\). Both Co compounds alone significantly increased IL-8 production in A549 cells (\(p<0.05\)), yet these levels were lower than COBSTEL 6 (% IL-8: 187.9 ± 16.1 CoCl\(_2\), 184.8 ± 15.3 Co(NO\(_3\))\(_2\) compared to 235.6 ± 5.7 COBSTEL 6) (Figure 4.3.3). All combinations of Co and Cr evoked a marked increase in IL-8, with the combination of CoCl\(_2\) + CrO\(_3\) virtually mimicking the effect of COBSTEL 6. These data suggest that the pro-inflammatory effects of the COBSTEL 6 fume may involve an additive interaction between soluble Co and Cr species.

![Figure 4.3.3. Effect of Various Combinations of Co and Cr on IL-8 production in Alveolar Epithelial Cells.](image)

A549 cells were treated with different combinations of Co and Cr compounds at concentrations comparable with those present in the soluble fraction of COBSTEL 6 for 24 hr and IL-8 protein was measured. The bars represent the mean of five separate experiments conducted in triplicate ± SEM \(*p<0.05\); **\(p<0.01\) compared to control (untreated cells 0 μg ml\(^{-1}\)).
4.4 Discussion

The previous chapter of this thesis demonstrated that the pro-inflammatory effects of welding fumes could be entirely attributed to the soluble metal fraction. Therefore, the aims of this chapter were to determine the metal composition of the three welding fumes and to try and identify which metal compound(s) in particular were responsible for these effects.

The welding fumes used in this study were generated from compositionally different electrodes (NIMROD and COBSTEM), fluxes and welding processes, variables which are known to greatly influence the composition and oxidation state of the resultant welding fumes (Eagar et al., 1997). These differences were highlighted by the variable metal content and concentration in the three fumes (Figure 4.1). In terms of total metal concentration, COBSTEM 6 was found to contain the highest total metal concentration (3.15µg ml⁻¹ whole welding fume), with NIMROD 182 and NIMROD c276 containing approximately 2.15 and 2.29 µg ml⁻¹ respectively. The magnitude of the differences in total metal concentration (1.3-1.5-fold) could strongly influence the toxicity and inflammogenicity of these fumes as many of the metals present in welding fumes redox-cycle to produce oxidants (Stohs et al., 1995; Halliwell et al., 1995). However, previous studies have suggested that other factors such as the bio-availability of particle-associated metals may be more important than actual mass in determining the overall pathogenicity (Costa et al., 1997; Dreher et al., 1997; Adamson et al., 1999; Smith et al., 2000).

When comparing the total metal concentration of the various welding fume fractions; the whole, the soluble and the washed particulates, it was clearly evident that the majority of these metals were present in welding fumes in a soluble form. This was illustrated by the strong similarity in metal concentrations between the whole and soluble fractions for all three fumes and the notable decrease in metal concentration in the washed particulate fractions. Furthermore, following pre-treatment of the soluble welding fume extracts with the non-specific metal chelator Chelex-100, a significant reduction in the total metal concentration was observed for
all three fumes. This pattern of high metal concentration in the soluble fractions of welding fumes correlates strongly with the IL-8 production in alveolar epithelial cells (r = 0.869 NIMROD 182; r = 0.993 NIMROD c276; r = 0.982 COBSTEL 6), implying that soluble transition metals are likely to be responsible for the pro-inflammatory effects of welding fumes.

As implied by the names, NIMROD 182 and NIMROD c276 are both nickel (Ni) based welding fumes, whereas, COBSTEL 6 is predominantly comprised of cobalt (Co). It was therefore expected that nickel and cobalt would be the principal metals in these respective welding fumes. Metal analysis by ICP-MS however, revealed chromium (Cr) to be the predominant soluble metal present in all three fumes, constituting > 80 % of the total metal content in the soluble fume extract. Although this result was somewhat unexpected, Cr is known to be present in various oxidation states in stainless steel welding fumes and metal alloys frequently used in welding (Stern et al., 1986; 1988). Significant quantities of both trivalent (Cr III) and hexavalent (Cr VI) have been measured in welding fumes (Antonini, 2003). Cr is however, predominantly found in its strongly oxidised hexavalent state in either a soluble or insoluble form depending on the specific welding technique employed (Cohen et al., 1998). Analysis of various welding fumes has demonstrated that the concentration of Cr (VI) present in the fume is a function of the shielding gas used and can therefore vary considerably between different welding techniques (Hewitt et al., 1986). As Cr was the predominant metal in the soluble extracts of all three welding fumes, correlation analyses were used to investigate the relationship between Cr and IL-8. An almost linear relationship was found between Cr concentration and IL-8 protein production for all three welding fumes (correlation co-efficients 0.952 - 0.998) strongly suggesting the pro-inflammatory effects of welding fumes are mediated by soluble Cr species. As Cr naturally occurs as stable III or VI forms, both of which have been identified as components of welding fumes, different Cr compounds were selected to try and mimic the pro-inflammatory effects of welding fumes. The concentration of available Cr present in the soluble hexavalent compounds K₂Cr₂O₇ and CrO₃ and the trivalent CrCl₃ compound were calculated and used at a concentration equivalent to that present in the soluble
fractions of each welding fume. Although both K₂Cr₂O₇ and CrO₃ enhanced IL-8 production in alveolar epithelial cells, the levels of IL-8 were not comparable to that of the soluble welding fume fractions. These results contradict the previous correlation data, which strongly implicated Cr as the main component driving the pro-inflammatory effects of welding fumes. However, there are a number of possible reasons for this apparent discrepancy. Firstly, the biological effects associated with Cr are diverse and are highly dependent upon bio-availability, metal speciation and solubility, as well as on intracellular reductants and chelators (O'Brien et al., 2003). With regard to the ions formed in solution, both Cr(VI) compounds yield anions; K₂Cr₂O₇ yields Cr₂O₇²⁻ and CrO₃ can produce Cr₂O₇²⁻ and/or CrO₄²⁻. Due to their isostructure with respect to phosphate and sulphate they readily enter cells via non-specific anion transport channels (Arslan et al., 1987; Wetterhahn, 1981). Once intracellular, they can be chemically reduced by compounds such as cysteine, glutathione, NAD(P)H and ascorbate (DeFlora et al., 1989; Suzuki et al., 1990). The metabolic reduction of Cr(VI) induces oxidative stress and generates a whole spectrum of reactive intermediates. These include Cr(V), Cr(IV), thiyl radicals and hydroxyl radicals in addition to Cr(III) complexes which can bind directly to DNA and proteins (Shi et al., 1990; Martin et al., 1998; Shrivastava et al., 2002; Bagchi et al., 2001). In contrast, trivalent Cr such as CrCl₃ yields cations in solution such as Cr³⁺ probably as hydrated ions like [Cr(H₂O)₆]³⁺ which are unable to enter cells via membrane transporters. Thus Cr(III) is generally considered non-toxic (O'Brien et al., 2003). The failure of CrCl₃ to mimic the effects of welding fumes may be the result of its inability to enter the cells, enhance ROS production and activate redox-sensitive transcription factors such as NF-κB and AP-1 involved in IL-8 expression.

In the case of the hexavalent Cr compounds (K₂Cr₂O₇ and CrO₃) a number of mechanisms may explain the failure to induce a similar inflammatory response. Due to its high reactivity with Cr(VI) and high intracellular concentration, it has been suggested that glutathione (GSH) plays an important role in Cr(VI) metabolism (Meister et al., 1983; Wiegand et al., 1984). A549 cells are known to have a high basal GSH concentration and the concentrations of Cr(VI) utilised in this study were extremely low. The transient depletion of GSH induced by Cr(VI) may cause a
compensatory up-regulation of GSH synthesis, thus effectively protecting cells from Cr(VI)-induced oxidative damage and inflammation. This “rebound” effect was observed following exposure to welding fumes (section 3.12) and also after exposure to other oxidants, such as cigarette smoke (Rahman et al., 1995a). Alternatively, Cr can form Cr-GSH complexes via ligand interactions to thiol groups, which under certain conditions can lead to the formation of thiyl radicals (Galaris et al., 2002). Although these radicals can interact with molecular oxygen resulting in the generation of ROS, they can also interact with each other forming non-radical disulfide bonds (Kalyanaraman, 1995). This prevents the activation of oxidant-mediated signalling events involved in pro-inflammatory cytokine production. Furthermore, it has been suggested that very low concentrations of Cr(VI), such as those used in this study, selectively inhibit the transcriptional activity of NF-κB. This is caused by decreasing p65/CREB interactions, thereby preventing the NF-κB dependent gene expression such as IL-8 (Shumilla et al., 1999). The most likely explanation, however, is the fact that welding fumes are highly complex particulates. They contain numerous metals in various different oxidative forms, therefore synergistic or additive interactions are likely to occur between various metals. By investigating the effects of Cr in isolation, any potential interactions are removed, which may be required to evoke the optimal pro-inflammatory response.

Previous studies have suggested complex interactions between various soluble metals such as Fe, Cr, Cu, Mn and Ni may be important factors determining the pulmonary responses observed following welding fume exposure (Antonini et al., 1999). Similarly, the inflammmogenicity of other environmental particulates such as ROFA have been attributed to the complex interactions between various metals such as Fe, Ni and V (Dreher et al., 1997; Ghio et al., 2002a). It was therefore hypothesised that the pro-inflammatory effects of welding fumes were the result of synergistic or additive interactions between various metals. Ni constituted approximately 3 % of the total soluble metal concentration in both NIMROD 182 and NIMROD c276 and previous studies have shown an enhanced IL-8 expression in airway epithelial cells following exposure to Ni (Allermann et al., 2002; Salnikow et al., 2004). A549 cells were therefore treated with NiCl2 or various combinations of
soluble Cr-Ni at equivalent concentrations to those present in NIMROD 182 and NIMROD c276. Although all combinations of Cr-Ni enhanced IL-8 production in A549 cells, the magnitude of IL-8 release was less than either of the soluble welding fume extracts themselves or that of the individual metals. A number of other investigators have reported similar negative interactions between metals. Kodavanti et al., (1997) showed that cytokine production induced by a mixture of Fe, V and Ni was lower than that caused by Ni alone. Likewise Dreher et al., (1997) found that the pulmonary injury and inflammation in rats instilled with a combination of Ni and V was less severe than that of Ni. Metal-metal interactions are known to be highly complex, influenced by numerous factors such as concentration, ionic status, solubility which greatly affect the intracellular bioavailability of metal ions, in addition to pH and the presence of reductants and chelators (Hartwig, 1998). These factors, as well as the potential interactions with other reactive metals present in welding fumes such as Fe, V and Mn (Carter et al., 1997; Kodavanti et al., 1998) may in part explain the inability of the artificial mixtures of metals to reproduce the pro-inflammatory effects of soluble NIMROD based welding fume extracts.

Similar strategies were adopted to identify potential metal interactions involved in the pro-inflammatory effects of soluble COBSTEL 6 welding fumes. In addition to Cr, the only other metal present in any significant concentration was Co, comprising 14 % of the total soluble metals. Alveolar epithelial cells were therefore exposed to soluble divalent Co compounds (Co(NO₃)₂ and CoCl₂) alone, or combinations of the Co and Cr compounds. As for Ni, both Co compounds alone significantly increased IL-8 production (p<0.05 compared to control), yet not to the same extent of COBSTEL 6. Although all Co-Cr combinations evoked an inflammatory response, the levels of IL-8 measured were comparable to that of Co(II) alone and less than that of COBSTEL 6. However, the combination of CoCl₂ and CrO₃ virtually mirrored the effect of the soluble COBSTEL 6 extract (227.8 ± 8.2 v 235.5 ± 5.7 IL-8 % Control). This suggests that the inflammmogenicity of COBSTEL 6 may be mediated via the additive metal interaction of the Co(II) and Cr(VI) forms. As both Co(II) and Cr(VI) are able to generate ROS (Stohs et al., 1995), the enhanced levels of IL-8 may be the result of increased transcriptional
activation of NF-κB and AP-1 via oxidant-mediated mechanisms (Roebuck, 1999; Chen et al., 2002).

Overall, these data illustrate the complexity of welding fume-induced inflammation and highlight the variability in metal composition, bio-availability and activity of fumes generated from compositionally different electrodes. All of these parameters have an effect on the resultant inflammogenic and toxic properties of welding fumes.
Chapter 5

Signalling Mechanisms Involved in the Pro-Inflammatory Effects of Welding Fumes
5.1 Introduction

The mitogen activated protein kinases (MAPKs) are important regulatory proteins that act by phosphorylating a number of proteins and transcription factors. This provides a signalling mechanism through which extra-cellular signals are transduced into intracellular responses such as gene transcription (Davis, 1994; Robinson et al., 1997; Hommes et al., 2003). The p38 MAPK cascade responds to a variety of cellular stresses such as heat shock, UV irradiation and inflammatory stimuli. A number of inflammatory mediators such as the cytokines IL-1β, IL-6 and TNF-α have also been shown to be potent activators of p38 MAPK in several cell types (Beyaert et al., 1996; Su et al., 1996; Garrington et al., 1999; Wang et al., 1998). In addition, p38 MAPK has been implicated in regulating the transcription (Lee et al., 1994) and synthesis (Rutault et al., 2001) of pro-inflammatory cytokines.

An important role of these signalling cascades is regulating the transactivation of transcription factors, such as NF-κB and AP-1 that are involved in the expression of genes critical in mediating cellular events including proliferation, apoptosis, cell survival, and production of inflammatory cytokines (Su et al., 1996; Garrington et al., 1999). Furthermore, many inducible genes that encode inflammatory cytokines and chemokines such as IL-8 and TNF-α contain NF-κB and AP-1 binding sites within their promoter or enhancer regions (Blackwell et al., 1997; Siebenlist et al., 1994; Lakshminarayanan et al., 1998; D’Angio et al., 2004).

Although a number of investigators have suggested that cytokine or cytokine-like mediated mechanisms are involved in welding fume-induced inflammation (Gordon, 1991; Blanc et al., 1993; Kushner et al., 1998), the key events involved in the initiation of pulmonary inflammation by welding fumes remains unclear. Previous studies investigating the toxicity of welding fumes have identified an important role for the generation of reactive oxygen species in welding fume-induced inflammation (Antonini et al., 1998). Numerous studies in the field of particle inflammation have shown that various MAPK signalling cascades are critically involved in the activation of transcription factors such as NF-κB and AP-1,
and resultant inflammatory gene expression (Mossman et al., 1997; Samet et al., 1997; Chen et al., 2000; Swain et al., 2004). It was therefore hypothesised that the regulation and activation of the MAPKs and downstream transcription factors may play a role in welding fume-induced inflammation. Given the central role identified for soluble transition metals in the inflammatory response, the effects of the soluble and particulate fractions of welding fumes on p38 MAPK and NF-κB and AP-1 were assessed.
5.2 Activation of Nuclear Factor-kappa B by Welding Fumes in Alveolar Epithelial Cells

Components of welding fumes rapidly solubilize (within 15 minutes) and induce a significant increase in IL-8 gene and protein expression in alveolar epithelial cells after 6 hr exposure. In addition, maximal glutathione depletion concomitant with an increase in intracellular ROS was noted following 2 hr treatment with both the soluble and whole welding fume fractions suggesting that welding fumes induce a rapid, acute pro-inflammatory response. It was hypothesised that due to the high metal content present in the welding fumes, NF-κB would be rapidly activated either directly by ROS produced via Fenton-chemistry or as a result of cellular oxidative stress. Therefore, to investigate the effect of welding fumes on NF-κB-DNA binding, electrophoretic mobility shift assays (EMSA) were performed.

Nuclear extracts were prepared from A549 cells exposed to NIMROD c276 (63 μg ml⁻¹) or a positive control (TNF-α 10 ng ml⁻¹) at various time points ranging from 2 to 6 hr and the degree of NF-κB-DNA binding was investigated. Figure 5.1 illustrates a typical EMSA gel following exposure to welding fumes. The specificity of NF-κB-DNA binding was confirmed using a consensus unlabelled NF-κB oligonucleotide as the cold-competitor and AP-1 as a non-competitor. The extent of DNA binding was shown by the intensity of NF-κB protein bands as determined by densitometry (Figure 5.1). A 2.1-fold increase in NF-κB-DNA binding was observed following 4 hr exposure to welding fumes. A slight increase in DNA binding was still evident at 6 hr (1.4-fold increase compared to control) demonstrating the transient effect of welding fumes on DNA binding to NF-κB.
Figure 5.1. Time Course of NF-κB-DNA Binding in Alveolar Epithelial Cells. A549 cells were treated with the welding fume, NIMROD c276 (63 μg ml⁻¹) or TNF-α (10 ng ml⁻¹) for 2, 4 or 6 hr. Nuclear proteins were isolated (as described in section 2.16) and incubated with radio-labelled NF-κB oligonucleotide and resolved on a polyacrylamide gel. Nuclear extracts from TNF-α exposed cells were incubated in the presence of either the cold competitor or non-competitor. The figure shows a representative NF-κB gel. C: control; TNF: Tumour necrosis factor-α; W: welding fume. nc: non-competitor; cc: cold-competitor. Fold increase represents the mean of three separate experiments.
5.3 The Role of Welding Fume Fractions in Nuclear Factor-kappa B-DNA Binding in Alveolar Epithelial Cells

To assess the importance of the soluble and particulate fractions of welding fumes in the activation of NF-κB, A549 cells were exposed to the whole, soluble or washed particulate fractions of welding fumes and NF-κB-DNA binding was investigated following exposure for 4 hr. A striking increase in NF-κB-DNA binding was observed in response to both the whole and soluble fractions of all three welding fumes compared to control levels (Figure 5.2 A). More importantly, the extent of binding to NF-κB was comparable between the whole and soluble fractions for both NIMROD c276 and COBSTEL 6. Interestingly, the soluble fraction of NIMROD 182 caused a significantly greater increase in DNA binding than the whole counterpart (420.2 ± 43.5 compared to 293.5 ± 31.7 relative units p<0.05) (Figure 5.2 B). No NF-κB response was observed in A549 cells exposed to the washed particulates from any of the three welding fumes (Figure 5.2 A & B).

Figure 5.2 A. Effect of Whole, Soluble or Washed Particulate Fractions of Welding Fumes on NF-κB-DNA Binding in Alveolar Epithelial Cells. A549 cells were treated with either whole, soluble or washed particulate fractions of welding fumes (63 μg ml⁻¹) for 4 hr. Nuclear extracts were prepared and 10 μg protein was then incubated with radio-labelled NF-κB consensus oligonucleotide. The figure shows a representative EMSA gel with the arrow indicating the position of NF-κB. C: control; TNF: Tumour Necrosis Factor-α; W: whole; S: soluble; Wa: washed particulates; nc: non-competitor; cc: cold competitor.
Figure 5.2 B. Effect of Various Welding Fume Fractions on NF-κB-DNA Binding in Alveolar Epithelial Cells. A549 cells were treated with either whole, soluble or washed particulate fractions of welding fumes (63 μg ml⁻¹) for 4 hr. Nuclear extracts were prepared and 10 μg protein was then incubated with radio-labelled NF-κB consensus oligonucleotide. The histogram represents the band density of individual bands from three independent experiments. The values are expressed as % Control (untreated cells 0 μg ml⁻¹). ***p<0.001 compared to control; #p<0.05 soluble compared to whole; $$$p<0.001 whole or soluble compared to washed particulates.
To further characterise the active nuclear translocated NF-κB dimer following exposure to welding fumes, super-shift assays were performed. Prior to treatment with the NF-κB radio-labelled probe, nuclear extracts from welding fume-treated cells were incubated with specific antibodies against p50 and p65, the components of the classical NF-κB heterodimer. As shown in Figure 5.3, retarded bands (super-shifted) were observed in the polyacrylamide gel in the presence of antibodies against both p50 and p65 indicating the formation of protein complexes and confirming the involvement of both subunits in NF-κB-DNA binding.

![Figure 5.3. NF-κB Super-Shift Assay in Alveolar Epithelial Cells Exposed to Welding Fumes](image)

Figure 5.3. NF-κB Super-Shift Assay in Alveolar Epithelial Cells Exposed to Welding Fumes. Nuclear proteins isolated from A549 cells treated with the welding fume NIMROD c276 (63 μg ml⁻¹ for 4 hr) were incubated overnight with either p50 or p65 antibodies or rabbit serum prior to the addition of the radio-labelled NF-κB oligonucleotide. The DNA-protein complexes were resolved on a polyacrylamide gel. The arrows indicate the position of the NF-κB complex and super-shifts (s/s). W: whole welding fumes; nc: non-competitor; cc: cold-competitor; W + p50: welding fume + p50 antibody; W + p65: welding fume + p65 antibody; serum: rabbit serum control
5.4 The Effect of Welding Fumes on Activator Protein-1 in Alveolar Epithelial Cells

To investigate the effect of welding fumes on AP-1-DNA binding, nuclear proteins were isolated from alveolar epithelial cells exposed to various fractions of welding fumes and incubated with a radio-labelled AP-1 oligonucleotide. Although not as striking as seen with NF-κB, a significant increase in AP-1-DNA binding was shown in cells following exposure to the whole and soluble fractions of all three welding fumes (Figure 5.4 A). Moreover, there was no difference in the degree of DNA binding between the whole and soluble fractions for all three fumes (e.g COBSTEL 6 whole: 228.4 ± 41.8 v soluble: 228.3 ± 47.7 % Control) (Figure 5.4 B). NIMROD 182 appeared to be the least potent fume with NIMROD c276 causing the greatest increase in DNA binding (soluble NIMROD 182: 192.8 ± 22.5 v soluble NIMROD c276: 306.4 ± 43.3 % Control). As with NF-κB, the washed particulate fraction of welding fumes caused no marked affect on AP-1 DNA binding (Figure 5.4 A & B).

![Figure 5.4 A. Effect of Various Welding Fume Fractions on AP-1-DNA Binding in Alveolar Epithelial Cells. A549 cells were treated with either whole, soluble or washed particulate fractions of welding fumes (63 μg ml⁻¹) for 4 hr. Nuclear extracts were prepared and 10 μg protein was then incubated with radio-labelled AP-1 oligonucleotide. The figure shows a representative EMSA gel of AP-1-DNA binding in alveolar epithelial cells treated with welding fumes. The arrows indicate the position of AP-1 and non-specific binding. C: control; TNF: Tumor Necrosis Factor-α; W: whole; S: soluble; Wa: washed particulate; nc: non-competitor; cc: cold competitor.](image-url)
Figure 5.4 B. Effect of Various Welding Fumes Fractions on AP-1-DNA Binding in Alveolar Epithelial Cells. A549 cells were treated with either whole, soluble or washed particulate fractions of welding fumes (63 μg ml⁻¹) for 4 hr. Nuclear extracts were prepared and 10 μg protein was then incubated with radio-labelled AP-1 consensus oligonucleotide. The histogram represents the band density of individual bands from three independent experiments. The values are expressed as % Control (untreated cells 0 μg ml⁻¹). *p<0.05; **p<0.01; ***p<0.001 compared to control; $p<0.01; $$p<0.001 whole or soluble compared to washed particulates.
5.5 The Role of Reactive Oxygen Species in NF-κB and AP-1-DNA Binding in Alveolar Epithelial Cells Exposed to Welding Fumes

Welding fumes enhance the levels of intracellular reactive oxygen species in alveolar epithelial cells. To assess whether ROS were involved in welding fume-induced NF-κB-DNA binding, A549 cells were co-exposed to either whole welding fumes or soluble fractions and N-acetyl-L-cysteine, (NAC), a precursor for intracellular thiols containing antioxidant properties. Complete inhibition of DNA binding to the NF-κB oligonucleotide was observed in the presence of NAC compared to untreated welding fume fractions as illustrated by the absence of a band in these lanes (Figure 5.5 A). Consistent with the previous results, (section 5.3), there was no marked difference in the extent of DNA-protein complex formation between the whole and soluble fractions of all three fumes (Figure 5.5 B) (NIMROD 182 whole: 464.1 ± 29.8 v soluble: 644.8 ± 72.6; NIMROD c276 whole: 540.5 ± 11.2 v soluble: 497.3 ± 44.7; COBSTEL 6 whole: 390.8 ± 6.8 v 460.4 ± 25.9 % Control). These data indicate that activation of NF-κB by the soluble fractions of welding fumes is mediated via an oxidative stress mechanism.

Figure 5.5 A. Effect of NAC on NF-κB-DNA Binding in Alveolar Epithelial Cells. A549 cells were treated with either whole or soluble fractions of welding fumes (63 μg ml⁻¹) with or without NAC (5 mM) for 4 hr. Nuclear extracts were prepared and 10 μg protein was then incubated with radio-labelled NF-κB oligonucleotide. The figure shows a representative EMSA gel showing NF-κB-DNA binding in alveolar epithelial cells treated with welding fumes with or without 5 mM NAC. Arrows indicate the position of NF-κB and non-specific binding C: control; NAC: N-acetyl-L-cysteine; W: whole; W+: whole + NAC; S: soluble; S+: soluble + NAC; nc: non-competitor cc: cold competitor.
Figure 5.5 B. Effect of NAC on NF-κB-DNA Binding in Alveolar Epithelial Cells. A549 cells were treated with either whole or soluble fractions of welding fumes (63 μg ml⁻¹) or whole or soluble fractions of welding fumes plus 5 mM NAC for 4 hr. Nuclear extracts were prepared and 10 μg protein was then incubated with radio-labelled NF-κB oligonucleotide. The histogram represents the mean of three experiments and values are expressed as % Control (untreated cells 0μg ml⁻¹). ***p<0.001 compared to control, $$$p<0.001 welding fume + NAC compared to untreated fume.
To investigate whether a similar oxidant-related mechanism was involved in AP-1-DNA binding, nuclear proteins were isolated from A549 cells treated with the whole or soluble fractions of the welding fumes, with or without 5 mM NAC for 4 hr, then incubated with an AP-1 radio-labelled oligonucleotide. Following treatment with NAC, binding to the AP-1 sequence was markedly reduced for all three fumes compared to the untreated welding fumes (Figure 5.6 A) (Whole NIMROD 182: 150.1 ± 9.1 v Whole NIMROD 182 + NAC: 94.8 ± 5.4 % Control). There was no significant difference in the ability of NAC to reduce AP-1-DNA binding between the whole and soluble fractions of all three welding fumes (1.3-fold and 1.5-fold reduction in AP-1 following co-incubation of the whole and soluble fractions of COBSTEL 6 with NAC). Notably, the ability of NAC to prevent AP-1-DNA binding was markedly less than that shown for NF-κB, as demonstrated by 1.3-fold reduction in soluble NIMROD c276 DNA-protein complex formation in the presence of NAC, compared to the 3.9 fold reduction in NF-κB-DNA binding (Figure 5.5 A & B).

Figure 5.6 A. Effect of NAC on AP-1-DNA Binding in Alveolar Epithelial Cells. A549 cells were treated with either whole or soluble fractions of welding fumes (63 μg ml⁻¹) or whole or soluble fractions of welding fumes plus 5 mM NAC for 4 hr. Nuclear extracts were prepared and 10 μg protein was then incubated with radio-labelled AP-1 oligonucleotide. The figure shows a representative EMSA gel showing AP-1-DNA Binding in A549 cells treated with welding fumes with or without 5 mM NAC. Arrows indicate the position of AP-1 and non-specific binding. C: control; NAC: N-acetyl-L-cysteine; W: whole; W +: whole + NAC; S: soluble; S +: soluble + NAC; nc: non-competitor cc: cold competitor.
Figure 5.6 B. Effect of NAC on AP-1-DNA Binding in Alveolar Epithelial Cells. A549 cells were treated with either whole or soluble fractions of welding fumes (63 μg ml\(^{-1}\)) or whole or soluble fractions of welding fumes plus 5 mM NAC for 4 hr. Nuclear extracts were prepared and 10 μg protein was then incubated with radio-labelled AP-1 oligonucleotide. The histogram represents the mean density of DNA-protein bands measured by densitometry from three separate experiments. The values are expressed as % Control (0 μg ml\(^{-1}\)). *p<0.05; **p<0.01 compared to control; $p<0.5; $$p<0.01$ welding fume + NAC compared to untreated welding fume.
5.6 The Effect of Welding Fumes on the Activation of p38 MAPK

As welding fumes had been shown to induce intracellular oxidative stress the involvement of the oxidant-sensitive p38 MAPK pathway was investigated. To this end, the effect of welding fumes on the phosphorylation of p38 was determined by Western blotting. Whole cell lysates prepared from A549 cells treated for various times (0.5, 1, 2 or 6 hr) with the whole, soluble or washed particulate fractions of the welding fumes; NIMROD 182, NIMROD c276 and COBSTEL 6 (63 μg ml⁻¹), were analysed by western blotting and SDS-PAGE electrophoresis, using a specific anti-phosphorylated p38 antibody. At all time-points examined both the whole and soluble fractions of all three welding fumes induced a significant increase in phosphorylated p38 with the maximal levels of phosphorylation occurring following 2 hr exposure (Figure 5.7 & 5.8). Both the whole and soluble fractions of NIMROD 182 and COBSTEL 6 caused a greater effect on p38 phosphorylation at 30 minutes than NIMROD c276 (NIMROD 182: whole 0.72 ± 0.08; soluble 0.78 ± 0.06; NIMROD c276: whole 0.59 ± 0.06, soluble 0.64 ± 0.01 v COBSTEL 6 whole 0.43 ± 0.09, soluble 0.45 ± 0.06 ratio pp38:p38). By 2 hr however, NIMROD c276 was the most active fume (Figure 5.7). As with previous data, the particulate fractions of welding fumes had no significant effect on p38 phosphorylation (Figure 5.7 & 5.8). To normalise for protein loading, blots were stripped and re-probed with a native p38 antibody and the data presented as a ratio of phosphorylated p38 to native p38 (pp38:p38). Levels of native p38 remained entirely consistent regardless of time or treatment, demonstrating equal protein loading and that the enhanced phosphorylation of p38 was proportional to overall p38 (Figure 5.8). To examine the specificity of this response to welding fumes, carbon black was used as a particle control. Although a slight increase in phosphorylated p38 was observed at the earlier time points (0.5 and 1 hr);(Figure 5.8) no appreciable change was noted with levels on average 19- fold lower than for welding fumes at 2 hr time point.
Figure 5.7. Effect of Welding Fumes on p38 MAPK Phosphorylation

A549 cells were treated with either the whole, soluble or washed particulate fractions of welding fumes (63 μg ml⁻¹) for 2 hr and whole cell lysates were prepared as described in section 2.19.1. Protein samples were separated by SDS-PAGE and analyzed by immunoblotting with an antibodies specific for phosphorylated (pp38) or native p38 (p38). The figure shows representative western blots for A. NIMROD 182; B. NIMROD c276; C. COBSTEL 6. Arrows indicate phosphorylated p38 (pp38) or native p38 (p38).
Figure 5.8. Effect of Welding Fumes on p38 MAPK Phosphorylation

A549 cells were treated with either the whole, soluble or washed particulate fractions of welding fumes for 0.5, 1, 2 or 6 hr and whole cell lysates were prepared (section 2.19.1). Protein samples were separated by SDS-PAGE and analyzed by immunoblotting with an antibodies specific for phosphorylated or native p38. The histograms show the mean ratio of pp38:p38 from three separate experiments. Values are mean ± S.E.M. *p<0.05; **p<0.01, ***p<0.001 compared to control; $p<0.05; $$p<0.01; $$$p<0.001 compared to washed particulate; #p<0.05; ##p<0.01; ####p<0.001 compared to CB.
5.7 The Effect of a p38 Inhibitor on Welding Fume induced NF-κB & AP-1 DNA binding

p38 MAPK signalling has been shown to be redox-sensitive and to cross-talk with both the NF-κB and AP-1 pathways (Schulze-Osthoff et al., 1997; Maselli et al., 2002; Rahman et al., 2000b). To investigate whether p38 signalling influenced NF-κB and AP-1-DNA binding, A549 cells were treated with either the whole or soluble fractions of welding fumes with or without the specific p38 inhibitor SB 203580 then incubated with an NF-κB or AP-1 radio-labelled probe.

A striking reduction in the level of welding fume-induced NF-κB-DNA binding was shown in cells following co-treatment with 10 μM SB 203580 compared to cells exposed to welding fumes alone (p<0.001) (Figure 5.9 A & B). As with previous data, there was no difference in DNA-protein complex formation between the whole and soluble fractions of all three welding fumes. This further supports a key role for the soluble components in welding fume-induced inflammation. The specificity of inhibition is demonstrated by the absence of a band in lane 2, (SB), which shows an SB 203580 control.

Figure 5.9 A. The Effect of SB 203580 on NF-κB-DNA Binding in A549 cells following 4 hr exposure to Welding Fumes. A549 cells were pre-treated with 10 μM SB 203580 for 30 mins then exposed to welding fumes with or without SB 203580 for 4 hr and nuclear extracts prepared. Figure A shows a representative EMSA gel for NF-κB following treatment with SB 203580. The arrow indicates the position of NF-κB. C: control; SB: SB 203580; W: whole; W+: whole + SB 203580; S: soluble; S+: soluble + SB 203580; nc: non-competitor cc: cold competitor.
Figure 5.9 B. Effect of SB 203580 on NF-κB-DNA Binding in A549 cells following 4 hr exposure to Welding Fumes. A549 cells were pre-treated with 10 μM SB 203580 for 30 mins then exposed to welding fumes (63 μg ml⁻¹) with or without SB 203580 for 4 hr and nuclear extracts prepared and incubated with radio-labelled NF-κB oligonucleotide. The histogram represents the mean density of DNA protein bands from three separate experiments and the values are expressed as % Control (untreated cells 0μg ml⁻¹). ***p<0.001 compared to control; $$$p<0.001 welding fumes + SB 203580 compared to welding fumes alone.
As with NF-κB, the levels of welding fume-induced AP-1-DNA binding were significantly reduced in the presence of the p38 inhibitor SB 203580 (Figure 5.10). Although still significant, the effect of SB 203580 on AP-1-DNA binding following exposure to COBSTEL 6 was less than for NIMROD 182 and NIMROD c276 (whole fractions 1.5-fold reduction in COBSTEL 6 + SB 203580 v 2.1 and 2.2-fold reduction for NIMROD 182 and NIMROD c276 + SB 203580 respectively).

Figure 5.10. Effect of SB 203580 on AP-1-DNA Binding in Alveolar Epithelial Cells. A549 cells were pre-treated with 10 μM SB 203580 for 30 mins then exposed to whole or soluble fractions of welding fumes (63 μg ml⁻¹) with or without SB for 4 hr and nuclear extracts prepared and incubated with a radio-labelled AP-1 oligonucleotide. A Representative EMSA gel showing AP-1-DNA Binding activity in alveolar epithelial cells treated with welding fumes with or without SB 23580. The arrow shows the position of AP-1. C: control; SB: SB 203580; W: whole; W+: whole + SB 203580; S: soluble; S+: soluble + SB 203580; nc: non-competitor; cc: cold competitor. B. The histogram represents the mean density of DNA-protein bands from three separate experiments. Values are expressed as % Control (untreated cells 0μg ml⁻¹).*p<0.05; **p<0.01; ***p<0.001 compared to control values. $p<0.05; $$p<0.01; $$$p<0.001 welding fumes + SB 203580 compared to untreated welding fumes.

175
5.8 The Role of p38 in Welding Fume induced IL-8 Expression

The role of p38 signalling in welding fume-induced IL-8 production was examined by exposing A549 cells to either the whole or soluble fractions of welding fumes (63 μg ml\(^{-1}\)) for 24 hr with or without 10 μM of the specific p38 MAP kinase inhibitor SB 203580. This concentration was chosen as previous studies investigating the role of p38 in the induction of various cellular responses have shown an inhibition of cytokine expression at this concentration (Matsumoto et al., 1998; Furuichi et al., 2002).

In the presence of SB 203580, welding fume-induced IL-8 production was completely abolished for both the whole and soluble fractions of all three welding fumes, thus confirming a pivotal role for phosphorylation of p38 in the signalling mechanisms involved in welding fume-induced inflammation (Figure 5.11).

![Figure 5.11. Effect of the p38 Inhibitor SB 203580 on Welding Fume-induced IL-8 production. A549 cells were pre-treated with 10 μM SB 203580 for 30 mins then exposed to the whole or soluble fractions of welding fumes (63 μg ml\(^{-1}\)) with or without SB 203580 for 24 hr and the supernatant was analysed for the presence of IL-8 protein. The histogram represents the mean of three separate experiments conducted in triplicate and the bars shown ± S.E.M. ***p<0.001 compared to control (untreated cells 0 μg ml\(^{-1}\)); $$$p<0.001 welding fumes + SB 230580 compared to welding fumes alone.](image-url)
5.9 Discussion

Epidemiological studies have consistently shown that the majority of welders' experience some type of respiratory illness throughout their working career (Howden et al., 1988; Lockey et al., 1988). A common feature of many of these illnesses is inflammation, characterised by leukocyte recruitment and enhanced expression of inflammatory mediators such as chemokines, pro-inflammatory cytokines and adhesion molecules (Driscoll et al., 1990; Strieter et al., 1993; Larsen et al., 2000). Indeed, the most frequently observed condition amongst welders is metal fume fever, (MFF) an acute, transient flu-like illness. Although the aetiology of MFF is well characterised, the molecular mechanisms underlying the pathogenicity of welding fumes are as yet unknown. The aim of this chapter was to try and delineate the molecular signalling mechanisms involved in welding fume-induced inflammation.

In the present study both the whole and soluble fractions of welding fumes NIMROD 182, NIMROD c276 and COBSTEL 6 enhanced NF-κB-DNA binding in a type II alveolar epithelial cell line (A549). In contrast, the washed particulate fractions of all three fumes failed to cause any response in NF-κB-DNA binding above control levels. Furthermore, there was no difference in the extent of DNA binding between cells exposed to the whole or soluble fractions of all three fumes, demonstrating a crucial role for soluble transition metals in welding fume-induced inflammation. In support of these findings Quay et al., (1999) showed that ROFA, a highly metal-rich combustion particle, induced IL-6 expression in human airway epithelial cells (BEAS-2B) via activation of NF-κB. Likewise, a metal-mediated mechanism involving activation and enhanced NF-κB-DNA binding has been implicated in the inflammatory effects of PM from both Utah and Edinburgh (Kennedy et al., 1998; Jiménez et al., 2000). Moreover, in the latter study, these effects were replicated using the soluble extract confirming an important role for soluble transition metals in particle-induced inflammation.
One aspect of NF-κB activation where welding fumes appear to differ from other environmental particulates is the time scale at which maximal transcription factor-DNA binding occurred. Previous work from this laboratory showed that PM$_{10}$ caused a 3.5-fold increase in NF-κB-DNA binding compared to control in A549 cells following 2 hr exposure (Jimenez et al., 2000). Likewise, Quay et al., (1998) demonstrated that ROFA significantly increased the activation and DNA binding of NF-κB in human airway epithelial cells (BEAS-2B) after 1 hr exposure. In contrast to both PM$_{10}$ and ROFA, DNA binding to NF-κB was not apparent at the early time point of 2 hr following exposure to welding fumes. This slight delay in transcription factor-DNA binding may be due to differences in the bio-availability and speciation of metals present in welding fumes compared to ROFA and PM$_{10}$ or interactions between the various metals present in welding fumes. In addition, differences in the culture conditions i.e. 0 % serum compared to 2 % serum, may account for the slight delay in transcription factor activation observed in this study.

The involvement of AP-1 in the pro-inflammatory effects of welding fumes was demonstrated by the significant increase in DNA binding to AP-1 following exposure to both the whole and soluble fractions of all three welding fumes. Moreover, as the washed particulate fraction had no effect on AP-1 this confirmed that the increased DNA binding could be entirely attributed to the soluble fraction. AP-1 activation has been implicated in the pathogenicity of a number of particle types. One of the first studies highlighting the involvement of AP-1 in particle toxicity was by Heintz et al., (1993) which demonstrated a persistent dose-dependent increase in c-fos and c-jun mRNA in rat pleural mesothelial cells in response to crocidolite asbestos. Since then, crystalline silica, PM$_{2.5}$, ufCB and diesel particles have been shown to selectively up-regulate the expression of c-jun, junB, c-fos and fra-1 mRNA in numerous cell types including pulmonary epithelial cells, rat mesothelial cells and C10 cells (Ramos-Nino et al., 2002b; Shukla et al., 2001; Timblin et al., 1998; Brown et al., 2004; Zhang et al., 2004). The role of transition metals in the activation of AP-1 is supported by the study of Samet et al., (2002) which showed increased expression of ATF-2 and c-Jun and enhanced AP-1-DNA binding in rat lungs following instillation with the highly metal rich particulate
ROFA. Similarly, Huang et al., (2002) identified the iron content of coal dust as the component responsible for the increased AP-1 and NFAT activation in mouse epidermal cells JB6.

Welding fumes are highly metal rich particulates and thus are able to generate intracellular ROS via Fenton/Haber-Weiss chemistry and autoxidation, (Shi et al., 1992; Stohs et al., 1995; Halliwell et al., 1995). ROS can activate the redox-sensitive transcription factors NF-κB and AP-1 either directly or via second messengers as a consequence of altering the cellular redox-status, particularly intracellular thiol status, resulting in the expression of inflammatory genes such as IL-8 and TNF-α (Khan et al., 1995; Sen et al., 1996; Rahman et al., 1998a). The involvement of ROS as signalling molecules in welding fume-induced NF-κB and AP-1-DNA binding was assessed by co-treating A549 cells with the thiol antioxidant N-acetyl-L-cysteine (NAC) and welding fumes. In the presence of NAC DNA binding of both NF-κB and AP-1 was significantly reduced compared to untreated welding fume fractions indicating that welding fume-derived ROS production is critically involved in the activation of NF-κB and AP-1. Transition metals have been shown to play an important role in particle-induced toxicity both in vivo and in vitro (Carter et al., 1997; Quay et al., 1999; Adamson et al., 1999; Jiménez et al., 2000; Roberts et al., 2004). Metal-induced cellular and molecular events are however complex and highly concentration dependent. For example, high concentrations of certain metals including Cr(VI) and V(V) can disrupt NF-κB-DNA binding in vitro by modifying protein thiols, altering the specific interactions between transcription factors and co-factors required for gene expression (Shumilla et al., 1999; Chen et al., 2002). In contrast, low metal concentrations can enhance NF-κB activation via oxidative stress-mediated mechanisms, as demonstrated by the ability of antioxidants such as catalase, SOD and D-mannitol to block this response (Chen et al., 2000; Kaltreider et al., 1999; Kim et al., 2003b). Similar oxidative mechanisms have been implicated in metal-induced AP-1 activation as illustrated by the inhibition of CoCl₂ and Cr(VI)-induced AP-1-DNA binding in the presence of antioxidants (Zou et al., 2001; Chen et al., 2000).
The reduction in welding fume-induced NF-κB and AP-1-DNA binding by NAC may occur via several mechanisms. As a cysteine derivative, NAC enhances levels of intracellular GSH by increasing the substrate concentration for the synthesis of γ-GCS, the rate-limiting enzyme for glutathione synthesis (Rahman et al., 1999a; 1999b; 2000a). Indeed, there is evidence demonstrating that NAC supplementation enhances GSH levels in A549 cells by 4 hr (Antonicelli et al., 2002). In turn, the increased levels of GSH alter the GSH/GSSG ratio and return the intracellular redox balance to the reduced state, preventing the activation of oxidative stress-signalling pathways. NAC can also directly scavenge ROS, thus removing the stimuli causing increased transcription factor-DNA binding (Zafarullah et al., 2003). Although the exact mechanisms by which NAC causes its effects on welding fume-induced DNA binding were not clarified, the results of this study provide clear evidence directly linking welding fume-induced oxidative stress to activation of NF-κB and AP-1 in alveolar epithelial cells. In agreement with these findings, a similar mechanism involving metals, NF-κB activation and gene expression, which could be blocked using antioxidants, has been reported in human airway epithelial cells (BEAS-2B) exposed to ROFA (Quay et al., 1998).

The transcriptional regulation of IL-8 in lung epithelial cells involves not only NF-κB but the transcription factors NF-IL-6 and AP-1 (Mukaida et al., 1989; Roebuck et al., 1999; Li et al., 2002a; Zhu et al., 2003). As the soluble fraction of welding fumes significantly enhanced both NF-κB and AP-1-DNA binding, these results suggest that the increased IL-8 expression previously shown may involve both NF-κB and AP-1. In support of this hypothesis, a recent study by Li et al., (2002a) showed that maximal TNF-α-induced IL-8 expression in human bronchial epithelial cells (16 HBE14o-) required activation of both AP-1 and NF-κB. Likewise, a cooperative interaction between AP-1 and NF-κB has been demonstrated in respiratory syncytial virus-induced IL-8 gene expression in A549 cells (Mastronarde et al., 1998). The direct involvement of both NF-κB and AP-1 in welding fume-induced IL-8 expression however was not assessed in the present study.
The association between the MAPK signalling cascades and particle-associated transition metals was initially described by Samet et al., (1999). This study showed that metals present in/on air pollution particles activated the MAPKs, ERK, JNK and p38, resulting in the phosphorylation of MAPK-dependent transcription factors and IL-8 expression in a human bronchial epithelial cell line (BEAS). Similar studies have demonstrated that the metal-components of ROFA are responsible for the activation of the MAPK cascades in vitro in bronchiolar epithelial cells (Samet et al., 1999) and in vivo (Silbajoris et al., 2000). The results of the present study clearly demonstrate the involvement of the p38 MAPK in the pro-inflammatory effects of welding fumes as shown by the significant increase in the phosphorylation of p38 by both the whole and soluble fractions of all three welding fumes. The time-course of enhanced p38 phosphorylation, peaking at 2 hr, preceded the increased binding of transcription factors to DNA. This fits with the hypothesis that p38 MAPK plays an important role in the mechanisms involved in welding fume-induced inflammation. In addition, the fact that the washed particulate fractions of all three welding fumes failed to cause phosphorylation of p38 further supports the original hypothesis that the inflammatory effects of welding fumes were entirely attributable to the soluble fractions. In line with these findings, it has been suggested that MAPK activation by other particles such as ambient air pollution may involve metal-catalysed oxidant production or metal ion dysregulation of phosphatase function, or possibly elements of both mechanisms (Ghio et al., 1999b). Indeed, metals such as vanadium and arsenic are potent inhibitors of protein tyrosine phosphatases (PTPs); (Gordon, 1991; Samet et al., 1997; 1999). Thus it has been suggested that inhibition of phosphatases prolongs Tyr and possibly Ser/Thr phosphorylation and may potentially be a key event in the initiation and maintenance of metal-induced MAPKs activation (Samet et al., 1998). Alternatively, ROS have been shown to directly activate MAPKs (Torres et al., 2003, Fubini et al., 2003; Torres, 2003). These studies suggest two potential mechanisms through which welding fumes could initiate the MAPK signalling cascade: 1) by inhibiting PTPs, 2) via the generation of ROS. As all three welding fumes contained trace amounts of vanadium (Chapter 4) and significantly enhanced intracellular ROS levels in A549
cells (Chapter 3), the findings of this study strongly suggest that welding fumes active the MAPK via an oxidative stress-mediated mechanism.

There is growing evidence demonstrating cross-talk between MAPKs and transcription factors such as NF-κB and AP-1. For example extra-cellular stimuli such as UV, TNF-α and ROS have been shown to activate both NF-κB and AP-1 via MAPK phosphorylation (Chen et al., 2001b; Shi et al., 2002; Zhou et al., 2003a). TNF-α can activate both AP-1 and NF-κB in a MEKK-1-dependent fashion causing sequential phosphorylation of downstream kinases such as JNK, p38 and IKK (Winston et al., 1995; Meyer et al., 1996; Hagemann et al., 2001; Zhou et al., 2003a). The use of specific chemical inhibitors, dominant negative mutants and anti-sense RNA has provided a means of investigating the involvement of different MAPKs in transcriptional regulation and resultant gene expression (Rubinfeld et al., 2004; Kumar et al., 2003). The pyridinylimidazole compound, SB 203580, was originally prepared as an inhibitor for inflammatory cytokine synthesis and works via competitive binding in the ATP pocket (Young et al., 1997; Kumar et al., 1999; Lee et al., 1999; 2000). By using this specific p38 inhibitor, the results of this study identified a crucial role for p38 phosphorylation in welding fume-induced NF-κB and AP-1-DNA binding and resultant gene expression. In the presence of 10 μM SB 203580, transcription factor-DNA binding and IL-8 production were significantly reduced in A549 cells exposed to both the whole and soluble fractions of all three welding fumes. This highlights the potential importance of the MAPK signalling pathway in the pro-inflammatory effects of welding fume.

The importance of p38 activation on downstream effector pathways is demonstrated by its ability to up-regulate numerous proteins such as c-fos, c-jun, jun-B and activating transcription factor-2 (ATF-2);(Wesselborg et al., 1997; Chen et al., 2001b). Indeed, up-regulation of c-Jun causes increased binding to the TRE/AP-1 DNA response element and results in enhanced IL-8 and IL-6 gene expression (Munoz et al., 1996; Karin, 1996). Similarly, inhibition of p38 has been shown to block stress-induced c-Fos and c-Jun synthesis, AP-1 trans-activation and DNA binding, and IL-8 mRNA expression (Hazzalin et al., 1996; Jung et al., 2002; Swain
et al., 2002). Although p38 does not directly phosphorylate NF-κB (Wesselborg et al., 1997; Vanden Berghe et al., 1998), inhibition of the p38 pathway using specific chemical inhibitors, anti-sense RNA or dominant negative mutants, greatly attenuates NF-κB-dependent transcription of genes including TNF-α, IL-8, IL-6, GM-CSF and RANTES in numerous cell types in response various stimuli (Wesselborg et al., 1997; Matsumoto et al., 1998; Hashimoto et al., 2000a; 2000b; Nick et al., 1999; Bergmann et al., 1998).

In support of the data presented here, phosphorylation of p38 has been implicated as playing an important role in the pathogenicity of many other environmental particulates including diesel exhaust particles, crocidolite asbestos, PM and crystalline silica (Hashimoto, et al., 2000a; Swain et al., 2002; Ding et al., 1999; Reibman et al., 2002; Geist et al., 2000). Similar studies using SB 203580 have demonstrated a decrease in inflammatory gene expression, such as IL-8, in numerous cell types confirming the involvement of p38 MAPK in inflammatory responses (Feoktistov et al., 1999; Adams et al., 2001; Hashimoto et al., 2000b). For example, Kawasaki et al., (2001) showed that SB 203580 suppressed diesel-induced IL-8, GM-CSF and RANTES expression in human airway epithelial cells. Likewise Swain et al., (2004) found a reduction in crocidolite asbestos-induced AP-1-DNA binding in rat mesothelial cells in the presence of SB 203580.

In contrast to the inhibitory effect of SB 203580 on both NF-κB and AP-1-DNA binding reported in this study, other investigators have shown that the selective inhibition of p38 MAPK by SB 203580 affected neither DNA-binding nor nuclear translocation of NF-κB (Bergmann et al., 1998; Li et al., 2002; Vanden Berghe et al., 1998; Beyaert et al., 1996). It has been suggested that p38 and NF-κB pathways converge downstream at the level of NF-κB mediated trans-activation (Wesselborg et al., 1997; Jaspers et al., 2000). The reasons for the inhibitory effect on NF-κB-DNA binding reported in the present study are unclear. However, the concentration of inhibitor used, the treatment regime followed and the cell type may in part explain these anomalous results. In the study by Carter et al., (1999) which showed that SB 203580 had no affect on NF-κB-DNA binding, THP-1 cells were pre-treated for 1 hr
with 0.5 μM inhibitor then stimulated with LPS. Similarly, Nick et al., (1999) found 1 hr pre-treatment of neutrophils with 10 μM SB 203580 did not affect NF-κB-DNA binding. A notable difference between these studies and the present study was that alveolar epithelial cells were pre-treated for 1 hr with 10 μM SB 203580, then co-treated with welding fumes and the inhibitor for a further 4 hr. The continuous presence of the inhibitor may result in non-specific cross-inhibition of other protein kinases such as JNK (Nick et al., 1999), or other unrelated enzymes (Shi et al., 2002a). This may block the ability of p38 and other kinases to phosphorylate downstream substrates (Kumar et al., 1999; Young et al., 1997). In turn this could inhibit I-κB phosphorylation and subsequent NF-κB translocation and DNA binding.

The results of this chapter demonstrate the involvement of the redox-sensitive transcription factors NF-κB and AP-1 in the pro-inflammatory effects of welding fumes. The inhibition of transcription factor-DNA binding in the presence of the antioxidant NAC suggests that the effects of welding fumes are driven via an oxidant-mediated mechanism. Furthermore, the enhanced phosphorylation of p38 MAPK preceding transcriptional activation and the attenuated DNA-binding and reduced IL-8 production observed in A549 cells in the presence of the specific p38 inhibitor SB 203580, suggests that signalling via p38 MAPK plays a fundamental role in the regulation of this inflammatory cascade. More importantly, these responses could be entirely attributed to the soluble fraction supporting the original hypothesis that welding fume-induced inflammation is driven via soluble transition metals. Overall, the data presented in this chapter illustrates a potential mechanism through which welding fumes induce their inflammatory effects in vitro.
Chapter 6

The Inflammatory Effects of Welding Fumes *In Vivo*
6.1 Introduction

The data presented in Chapter 3 identified a pivotal role for the soluble transition metal fraction of welding fumes in inducing the release of the pro-inflammatory cytokine IL-8 in alveolar epithelial cells. Further investigations in Chapter 5 demonstrated this inflammatory response was mediated via an oxidative-stress mechanism involving phosphorylation of p38 culminating in activation of NF-κB and AP-1.

An in vitro cell culture system was adopted for the previous chapters of this thesis as it provided a useful model for investigating the molecular mechanisms involved in the pro-inflammatory responses induced by welding fume in type II pneumonocytes. The relative simplicity of in vitro models however raises questions regarding the ability to fully predict in vivo responses. Consequently, animal models have been widely accepted as more physiologically relevant models to investigate pathological responses and biological mechanisms.

The aim of this Chapter was to determine whether as in the in vitro model, the pro-inflammatory effects of welding fumes could be explained by the soluble transition metals. In addition, an attempt was made to assess the validity of utilising an in vitro model to investigate welding fume associated toxicity.
6.2 Analysis of Bronchoalveolar Lavage Fluid

6.2.1 Cellular Parameters of Lung Inflammation

To determine the inflammogencity of the various fractions of the welding fume NIMROD c276, the whole welding fumes, the soluble fraction, the washed particulate fraction or the soluble fraction pre-treated with the transition metal chelator, chelex-100 were instilled into the lungs of rats and the cellular content of bronchoalveolar lavage fluid (BALF) was examined. Previous studies have verified analysis of BALF as a means of characterising lung inflammation and injury (Beck et al., 1982; Henderson, 1984; Antonini et al., 1996). In addition, it has been suggested that alterations in the biochemical and cellular constituents of lavage fluid are predictive of pulmonary responses (Lindenschmidt et al., 1990; Cobben et al., 1999).

The acute inflammatory potential of the various fractions of welding fumes was assessed as the total number of inflammatory cells (neutrophils, macrophages and lymphocytes) in the BALF of rats 24 hr after instillation of welding fumes. Following 24 hr instillation, both the whole and soluble fractions of welding fumes induced a significant influx of inflammatory cells compared to saline-instilled animals (Figure 6.1.). The total inflammatory cell numbers for whole and soluble fractions were $4.43 \pm 0.38 \times 10^6$ and $4.01 \pm 0.29 \times 10^6$ (p<0.01; p<0.05 respectively) compared with $2.94 \pm 0.12 \times 10^6$ for the saline group. Furthermore, a significantly greater total cell count was observed in the BALF of animals instilled with the whole welding fumes compared to BALF from rats instilled with the washed particulate fraction ($4.43 \pm 0.38 \times 10^6$ vs $2.38 \pm 0.41 \times 10^6$ total cell number).

A crucial role for soluble transition metals in the pro-inflammatory effects of welding fume was demonstrated previously. Therefore to establish whether soluble transition metals were involved in welding fume-induced inflammation in vivo, the soluble fraction of welding fumes were pre-treated with the transition metal chelator, Chelex-100, prior to instillation. Twenty four hr after instillation markedly fewer total leukocytes (macrophages and neutrophils) were observed in the BALF following instillation of the chelated soluble fraction compared to animals instilled with the unchelated soluble fraction.
Figure 6.1. Total Number of Different Cell Types in BALF 24 hr after Instillation of Welding Fumes. Rats were intra-tracheally instilled with either saline, whole, soluble, washed particulate or chelated soluble fractions of welding fumes (250 µg/0.5 ml). Lungs were lavaged and cytospins prepared as described in section 2.20.2. Columns represent the mean of four animals per treatment and the bars represent ± S.E.M. Macrophage data: *p<0.05 compared to saline control. PMN data: §§§p<0.001 compared to saline control; ###p<0.001 whole v washed; φφφp<0.001 soluble v soluble chelated.
A classical characteristic of acute pulmonary inflammation is airspace neutrophilia. Therefore to determine the relative inflammatory potential of the various fractions of welding fumes the percentage of neutrophils present in the BALF were compared. The neutrophil influx induced by the whole and soluble fractions were 18.8 ± 3.5 % and 39.6 ± 1.2 % respectively compared with 2.9 ± 0.6 % in the saline control (Table 6.1). Notably there was no significance difference in percentage BALF neutrophils between the whole and soluble treatments (Table 6.1) confirming in vivo, that the inflammogenic potential of welding fumes resides within the soluble fraction. Moreover, as < 5 % neutrophils were seen in the BALF following instillation of the washed particulate fraction (core particulates) indicating that in the present study, welding fume particulates were essentially non-inflammogenic. A role for soluble transition metals was confirmed by the striking reduction in neutrophils in BALF following chelation of the soluble fraction (Table 6.1) with 4.3 ± 0.6 % neutrophils compared to 39.6 ± 1.2 % untreated soluble fraction.

<table>
<thead>
<tr>
<th>Welding Fume Fraction</th>
<th>Neutrophils %</th>
<th>Macrophages %</th>
<th>Lymphocytes %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>2.9 ± 0.8</td>
<td>97.0 ± 0.6</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>Whole</td>
<td>18.8 ± 3.5**</td>
<td>80.7 ± 3.6</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>Soluble</td>
<td>39.6 ± 1.2***</td>
<td>59.6 ± 1.4</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>Washed</td>
<td>4.7 ± 1.2</td>
<td>94.3 ± 1.2</td>
<td>0</td>
</tr>
<tr>
<td>Soluble Chelated</td>
<td>4.3 ± 0.6</td>
<td>96.4 ± 1.3</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>

Table 6.1. Cellular Analysis of BALF. Percentage of the different cell types present in BALF of four animals after 24 hr instillation of various welding fume fractions. Values were calculated following blind cellular counts of two separate cytopsins per animals, four animals per group, with a minimum of 300 cells counted per slide (Section 2.20.2). Values represent mean ± S.E.M. *** p<0.001 compared to saline control; ##p<0.01 whole v washed particulates; ###p<0.001 soluble v soluble chelated.
6.2.2 Markers of Pulmonary Injury in BALF of Rats Following 24 hr Instillation of Welding Fumes

Data from the previous section (6.2.1) illustrated an acute inflammatory response in rats instilled with either the whole or soluble fractions of welding fumes. To further identify the extent of welding fume-induced inflammation, various conventional indices of lung damage were assessed in the BALF. Measurement of total protein in lavage was used as an indication of epithelial permeability as well as cellular damage, whereas the level of the cytosolic enzyme, lactate dehydrogenase (LDH) was a direct measurement of cell membrane damage (Cobben et al., 1999). Total protein and LDH were measured in BALF of rats following 24 hr instillation with the various fractions of welding fumes. Levels of total protein present in lavage showed a similar pattern to that seen for neutrophils, with only instillation of the whole and soluble fractions of welding fumes inducing a significant increase in total protein compared to saline control instilled animals (p<0.01); (Figure 6.2). The consistent trend observed with both total protein and neutrophil influx was not apparent for LDH release with levels measured in the BALF following instillation with whole welding fumes remaining comparable with saline control. In contrast, a significant increase in LDH release was observed following instillation of the soluble fraction (p<0.001); (5.34 ± 0.26 soluble v 2.91 ± 0.24 saline control OD respectively); (Figure 6.3).

These data suggest that PMN influx and protein leakage into lavage involve mechanisms independent of direct cellular toxicity, as measured by LDH release and may be related to constituent metal bio-availability, but this was not further investigated. No evidence of lung injury or cellular damage was observed in the BALF following instillation with the washed particulate fraction compared to either the saline treatment or whole welding fumes (Figure 6.2 and 6.3). Similarly, levels of both total protein and LDH in BALF of animals instilled with the chelated soluble fraction were comparable to saline control (Figures 6.2 and 6.3), confirming a role for soluble transition metals in welding fume-induced lung inflammation and injury.
Figure 6.2. Total Protein Levels in BALF 24 hr after Instillation of Welding Fumes. Rats were intra-tracheally instilled with saline, whole, soluble, washed particulate or soluble-chelated fractions of welding fumes (250 μg/0.5 ml). Following 24 hr exposure rat lungs were lavaged and total protein was measured in primary lavage fluid. Columns represent the mean of four animals per group with samples from each animal analysed in triplicate. The bars show ± S.E.M.
Figure 6.3 LDH Levels in BALF 24 hr after Instillation of Welding Fume Fractions. Rats were intra-tracheally instilled with saline, whole, soluble, washed particulate or soluble chelated fractions of welding fumes (250 μg/0.5 ml). Following 24 hr exposure, rat lungs were lavaged and LDH was measured in primary lavage fluid. Columns represent the mean of four animals per group with samples from each animal analysed in triplicate. The bars show ± S.E.M.
Inflammation in response to inhaled particles and pathogens involves the migration and activation of inflammatory leukocytes, such as neutrophils and monocytes, into the lungs to facilitate particle clearance, stimulate cell proliferation and regenerate tissue (Sibille et al., 1990). A number of well-characterised cytokines such as TNF-α, IL-1β and IL-8 have been shown to play an important role in this inflammatory cell recruitment (Driscoll et al., 1993; Blanc et al., 1993; Luster et al., 1999). The specific chemotactic cytokines Macrophage Inflammatory Proteins-1 and -2 (MIP-1 and 2), which are secreted by rat alveolar macrophages, fibroblasts and epithelial cells have been identified as key components in the host defence mechanism (Driscoll et al., 1993). Indeed, MIP-2, the rodent homologue of IL-8 (Frevert et al., 1995; Driscoll et al., 1995), is a potent neutrophil chemo-attractant both in vivo and in vitro and appears to be a mitogen for epithelial cells. Increased expression of MIP-2 has been implicated in the development of inflammatory diseases in several animal models such as pulmonary inflammation following exposure to a number of stimuli including crystalline silica and transition metals (Duffin et al., 2001; Shi et al., 1999a; Dick et al., 2003; Chong et al., 2000; Tsai et al., 1996). Therefore, the levels of this chemokine were measured in BALF to determine the extent of inflammation induced by the various fractions of welding fumes.

In agreement with the neutrophil data, (section 6.2.1), instillation of the whole and soluble fractions of welding fumes induced a significant increase in MIP-2 protein (whole: 201.74 ± 8.23; soluble: 204.74 ± 11.34 MIP-2 pg ml⁻¹) compared to saline (37.2 ±12.9 pg ml⁻¹) (Figure 6.4). Levels of MIP-2 in BALF from animals instilled with the soluble chelated fraction were similar to saline control levels. An increase in MIP-2 was observed in the BALF from animals instilled with the washed particulate fraction, however this was significantly lower than the levels measured in BALF following exposure to the whole welding fume (p<0.001) (Figure 6.4). The positive correlation between MIP-2 protein and BALF neutrophils (correlation co-
efficient $r = 0.867; p<0.05$) supports a role for this cytokine in welding fume-induced leukocyte recruitment.

Figure 6.4 MIP-2 Protein Levels in BALF 24 hr after Instillation of Welding Fumes Rats were intra-tracheally instilled with saline, whole, soluble, washed particulate or soluble chelated fractions of welding fumes (250 µg/0.5 ml). Following 24 hr exposure rat lungs were lavaged and MIP-2 was measured in primary lavage fluid by ELISA (section 2.20.3). Columns represent the mean of four animals per group with samples from each animal analysed in triplicate. The bars show ± S.E.M.
6.4 Effect of Welding Fumes on NF-κB and AP-1 Nuclear Binding

The regulation and expression of several genes coding for inflammatory and immuno-regulatory proteins including the neutrophil chemokine MIP-2 involves NF-κB (Driscoll et al., 1998). Similarly, AP-1, which is comprised of a number of homodimeric complexes of the Jun family, or heterodimeric complexes of the Fos and Jun families, plays an important role in cellular proliferation, differentiation, transformation and apoptosis (Ding et al., 1999; Guo et al., 2002) It is has also been suggested that activation of AP-1 may be required for the induction of acute, cytokine-mediated inflammation (Guo et al., 2002).

Genomic cloning of the rat MIP-2 gene 5' flanking region identified consensus NF-κB and AP-1 binding sites (Shi et al., 1999b). A previous study by Driscoll et al., (2001) utilizing the pneumotoxic particles quartz and crocidolite asbestos, demonstrated increased MIP-2 expression was dependent on a single NF-κB consensus binding site. In addition, intra-tracheal instillation of freshly fractured crystalline silica caused increased AP-1 activation in AP-1-luciferase reporter transgenic mice (Ding et al., 1999). Moreover, data presented in this thesis demonstrated that the soluble fraction of welding fumes increased AP-1 and NF-κB-DNA binding in alveolar epithelial cells in vitro (section 5.3-5.4).

To elucidate the mechanisms of transcriptional activation in welding fume-induced pulmonary inflammation in vivo, electrophoretic mobility shift assays for NF-κB and AP-1 were performed using nuclear proteins isolated from BAL cells from animals exposed to the various welding fume treatments. In support of the MIP-2 protein data and the in vitro findings, only instillation of the whole or soluble fractions of welding fumes increased NF-κB-DNA binding in nuclear proteins from BAL cells compared to saline controls (p<0.05) (Figure 6.5A & B). Furthermore, there was no difference in transcription factor-DNA binding between whole and soluble treatments. The nuclear proteins isolated from BAL cells of animals instilled with washed particulate fractions, showed no increase in NF-κB-DNA binding compared to saline control and were significantly less than whole welding fume
treatments (p<0.01). Furthermore, chelation of the soluble fraction decreased NF-κB-DNA binding to saline control levels (Figure 6.5 A & B).

![Figure 6.5 NF-κB-DNA Binding in BAL Cells 24 hr after Instillation of Welding Fumes.](image)

A. Representative EMSA gels. + ve: TNF; S: Saline; W: Whole; Sol: Soluble; Wa: Washed particulate; CX: Soluble Chelated; nc: non-competitor; cc: cold-competitor. Numbers 1-10 represent different animals.

B. The histogram represents the mean of four experiments and bars show ± S.E.M.

Figure 6.5 NF-κB-DNA Binding in BAL Cells 24 hr after Instillation of Welding Fumes. Rats were intra-tracheally instilled with saline, whole, soluble, washed or soluble chelated fractions of welding fumes (250 µg/0.5 ml). Following 24 hr exposure, rat lungs were lavaged and nuclear extracts were prepared from BAL cells (Section 2.16). A. Representative EMSA gels. + ve: TNF; S: Saline; W: Whole; Sol: Soluble; Wa: Washed particulate; CX: Soluble Chelated; nc: non-competitor; cc: cold-competitor. Numbers 1-10 represent different animals. B. The histogram represents the mean of four experiments and bars show ± S.E.M.
A very similar pattern to that seen for NF-κB was observed for AP-1 (Figure 6.6 A & B) with a significant increase in AP-1-DNA binding in nuclear protein from BAL cells of rats instilled with whole or soluble fractions of welding fumes (p<0.01 compared to saline control). Again no marked difference was shown in AP-1 nuclear binding following instillation with either the whole or soluble fractions. In agreement with the previous data, AP-1-DNA binding was significantly greater after instillation of the whole fumes compared to the washed particulate fractions (p<0.01). Similarly, following chelation of the soluble fraction AP-1-DNA binding returned to basal levels (Figure 6.6 A & B), suggesting that soluble transition metals are involved in welding fume-induced inflammation at a transcriptional level.

Figure 6.6 A. AP-1-DNA Binding in BAL Cells 24 hr after instillation of Welding Fumes. Rats were intra-tracheally instilled with saline, whole, soluble, washed particulate or soluble chelated fractions of welding fumes (250 μg/0.5ml). Following 24 hr exposure, rat lungs were lavaged and nuclear extracts were prepared from BAL cells (Section 2.16). A. Representative EMSA gels S: Saline; W: Whole; Sol: Soluble; Wa: Washed particulate; CX: Soluble Chelated; nc: non-competitor; cc: cold-competitor.
Figure 6.6 B. AP-1-DNA Binding in BAL Cells 24 hr after instillation of Welding Fumes. Rats were intra-tracheally instilled with saline, whole, soluble, washed particulate or soluble chelated fractions of welding fumes (250 μg/0.5ml). Following 24 hr exposure, rat lungs were lavaged and nuclear extracts were prepared from BAL cells (Section 2.16). The histogram represents the mean of four experiments and the bars show ± S.E.M.
6.5 Discussion

*In vivo* the endogenous environment is highly complex due the presence of multiple cell types, their products, cross-talk between cell types and the potential for synergistic, additive or inhibitory interactions. This raises questions as to whether the molecular mechanisms underlying welding fume-induced inflammation demonstrated *in vitro* are representative of cellular events occurring *in vivo*. The aim of this chapter was to determine the role of transition metals in welding fume-induced lung inflammation *in vivo*. The hypothesis that soluble transition metals are primarily involved in welding fume-associated inflammation, derived from the *in vitro* experiments (Chapter 3), was addressed in *in vivo* studies to investigate possible correlations between *in vivo* and *in vitro* models.

Male Sprague-Dawley rats were instilled with either; whole welding fumes, the soluble fractions, the washed welding fume particulates or soluble chelated fractions from the welding fume NIMROD c276 and BAL inflammatory indices were assessed 24 hr following instillation. Although intra-tracheal instillation is less physiological than inhalation, it has been accepted as a technique that yields valuable information regarding the relative potential of particles to induce inflammation (Driscoll *et al.*, 1997; Henderson *et al.*, 1995; Taylor *et al.*, 2003). Furthermore, instillation has the advantage that a specific dose of fume can be delivered accurately to the lungs, without any potential for particle deposition in, or clearance from, the nasal cavity. More importantly, the design of this study, to determine the role of soluble transition metals, necessitated instillation of the soluble components alone.

Transition metals have been shown to drive the pro-inflammatory effects of many environmental particulates both *in vivo* and *in vitro* (Carter *et al.*, 1997; Kennedy *et al.*, 1998; Dreher *et al.*, 1997; Adamson *et al.*, 1999; Jiménez *et al.*, 2000). Indeed, the content, bio-availability and interactions of metals can greatly influence the inflammogenicity of particles. Dreher *et al.*, (1997) demonstrated that interactions between Fe, Ni and V and pH greatly influenced the severity and kinetics of lung injury induced by ROFA and soluble transition metals. In contrast, Ghio *et al.*, (1999b) identified the inflammatory effects of Provo PM *in vivo* was
related to the catalytically active metals present in both the soluble and insoluble fractions. There is also considerable evidence that the ultra-fine (uf) particles (<0.1 μm diameter) cause inflammatory responses both in vitro and in vivo (Ferin et al., 1992; Li et al., 1999b; Brown et al., 2001; Gilmour et al., 2004). The central hypothesis surrounding the inflammatory effects of uf particles is related to the greater surface area-to-mass ratio causing enhancement of surface-associated free radical production resulting in oxidative stress (reviewed by Donaldson et al., 2002a).

By isolating welding fumes into different fractions, the results of this study demonstrated that the pulmonary inflammation induced in vivo could be entirely attributed to the soluble fraction. Evidence to support this theory was shown by the lack of inflammation in animals instilled with the washed particulate fraction, absent of any soluble components, compared to whole welding fumes. Furthermore, no inflammatory response was evoked in the lungs of animals following instillation of the soluble fraction pre-treated with a transition metal chelator, thus confirming that soluble transition metals play a pivotal role in welding fume-induced lung inflammation. Similarly, the soluble metal content and the sulphate leachate of ROFA have been implicated as key factors in the development of lung injury and airway hyper-reactivity in rats (Gavett et al., 1997).

The aim of this study was to determine whether the relative potential of the soluble and insoluble (particulate) components of welding fumes to cause pro-inflammatory responses could be validated in a rat model of lung inflammation. Following instillation with both whole welding fumes and the soluble fractions, a significant influx of neutrophils into the lungs was observed. The greater number of neutrophils noted in animals instilled with the soluble fraction compared to the whole welding fumes may be related to the cytokine profiles shown in vitro (Chpt. 3 Fig.3.9; table 3.3; Appendix VI). Following 24 hr treatments, whole welding fumes caused a greater production of the anti-inflammatory cytokine, IL-10, than the soluble fraction. This may explain the slightly lower neutrophil numbers observed in the BALF of animals instilled with the whole welding fumes.
Neutrophilia was associated with increased markers of lung injury, including total protein in BALF which is a measure of epithelial permeability/oedema and cell damage. Surprisingly, only animals instilled with the soluble fraction of welding fumes showed signs of cellular damage and death, as determined by increased LDH levels in BALF. This result was somewhat unexpected, considering that the whole welding fume treatment contained the same concentration of metals as the soluble fraction. However, this anomalous result may be explained by the slightly higher chromium content present in the soluble fraction (Chapter 4).

Chromium exists in several oxidation states, the most common forms in biological systems being trivalent Cr(III) and hexavalent Cr(VI) (Costa et al., 2002). The data presented in Chapter 4 showed that chromium was the predominant metal present within this fume. It is postulated that correlations exist between the increased incidence of cancer in welders and exposure to Cr(VI)-containing welding fumes (Pasanen et al., 1986; Stern et al., 1986; Gray, 1987). This hypothesis is supported by both toxicological and epidemiological studies that demonstrate the pulmonary toxicity/carcinogenicity of Cr(VI) (Cohen et al., 1993; Costa et al., 2002; De Flora et al., 1990). Soluble hexavalent Cr is approximately 500-1000 times more toxic and carcinogenic than the trivalent form due to active cellular uptake by anionic transport systems and interactions with nascent reducing compounds such as cysteine, glutathione, NAD(P)H and ascorbic acid (Arslan et al., 1987; Cohen et al., 1993; DeFlora et al., 1989; Shi et al., 1999c). Following uptake, Cr(VI) is reduced to Cr(III); however this process results in the formation of highly cytotoxic and genotoxic reactive intermediates such a hydroxyl radicals (Shrivastava et al., 2002). Furthermore, although Cr(III) normally exists in a stable state, once intracellular, it is highly reactive and able to bind DNA and proteins (Costa et al., 2002). Consequently the greater levels of toxicity observed in animals following instillation of the soluble fraction of welding fumes may be the result of the higher content of soluble Cr in a hexavalent form.

Previous work from this laboratory has demonstrated the pro-inflammatory effects of a wide range of ultra-fines using the influx of neutrophils as the key
characteristic of pulmonary inflammation (Donaldson et al., 2002a). Although the welding fumes used in this thesis were comprised primarily of particles within this range, (<100 nm); (Appendix III) the data presented here found that washed welding fume particulates were essentially non-inflammogenic at the doses used. These results substantiate the in vitro data and suggest that inflammation in vivo is driven by constituents other than the particulate fractions of welding fumes.

To establish the role of soluble transition metals in the inflammation observed in vivo, the soluble fractions of welding fumes were pre-treated with the transition metal chelator, chelex-100. The results in previous chapters of this thesis demonstrated that chelex-100 effectively removed any soluble transition metals. As no inflammatory response was observed in animals instilled with the soluble chelated fraction, this confirmed a fundamental role for soluble transition metals in welding fume-induced inflammation, and more importantly fully supported the in vitro data. These findings are consistent with a recent study by Roberts et al., (2004) which reported no increase in lung damage or mortality of rats upon instillation of the chelated-soluble fraction of ROFA compared to sham control animals. Furthermore, many studies investigating the toxicity of other environmental particulates such as PM, ROFA and stone quarry dust have attributed the pneumotoxic effects to the soluble transition metal components (Frampton et al., 1999; Adamson et al., 1999; Dreher et al., 1997; Hetland et al., 2001).

The chemokine MIP-2 is a potent chemotactic protein mediating neutrophil recruitment to the lungs and other tissues (Frevert et al., 1995; Driscoll et al., 2001; Duffin et al., 2001). Studies using animal models of lung inflammation and injury have implicated MIP proteins (MIP-1 and 2) as important mediators of the lungs defence mechanism (Driscoll, 1994). Increased expression of MIP-2 has been associated with the development of pulmonary inflammation induced by a variety of stimuli such as quartz, ROFA and ozone (Duffin et al., 2001; Driscoll et al, 2001 Dye et al., 1999; Johnston et al., 2003). The elevated levels of MIP-2 protein in lavage following instillation of both the whole and soluble fractions of welding fumes are consistent with the neutrophilic chemotactic activity of this cytokine. In addition, approximately one third less MIP-2 was measured in the BALF of animals
instilled with soluble chelated fraction, providing clear evidence for the role of soluble transition metals in mediating inflammation. In line with these findings, transition metals such as Fe, V, Cu and Ni have all been shown to increase gene expression of this pro-inflammatory cytokine (Kodavanti et al., 1997; Pierce et al., 1996; Rice et al., 2001). Moreover, these results corroborate those of Molinelli et al., (2002) who demonstrated a reduction in lung damage, as measured by attenuation of both LDH and protein in the BALF of rats following instillation of chelex treated extracts of Utah PM. Although the magnitude of MIP-2 production in this study was lower than previously published for other particulates and metals (Pierce et al., 1996; Kodavanti et al., 1997; Dick et al., 2003), several factors could explain these finding. As MIP-2 expression precedes the influx of inflammatory cells, by measuring levels of MIP-2 protein and neutrophils simultaneously (24 hr after instillation), peak expression of MIP-2 may have been missed. Furthermore, differences between this study and others may be indicative of the form and concentrations of metals used and the expression of MIP-2 by cells other than BAL leukocytes (Pierce et al., 1996; Carter et al., 1997; Dye et al., 1999).

A recent study by Taylor et al., (2003) showed evidence to suggest that both the soluble and insoluble fractions of MMA-SS welding fumes were involved in welding fume induced-lung damage and inflammation. The data presented in the present study however does not support these findings, as no activity was found in the insoluble (washed particulate) fraction of welding fumes and the total activity was mimicked by the soluble fraction alone. A number of differences may explain these discrepancies. Firstly, welding fumes are a highly complex mixture of metal oxides where the type of welding and materials used determines the actual metal content (Lockey et al., 1988; Sferlazza et al., 1991; Yu et al., 2000). Therefore welding fume composition is likely to differ greatly between studies. Additionally, the concentration of welding fume used in this study (250 μg/0.5 ml) was 1/8 of that used by Taylor et al., (2003). Consequently, the high dose of insoluble particulate fraction instilled may have induced a localized particle effect (Tran et al., 2000) in the study by Taylor et al, (2003). Furthermore, the washed (insoluble) particulate fraction in the present study had a very low metal content and the washing process
itself would remove any surface-associated free radicals, thus rendering these particulates essentially non-inflammogenic.

Transition metals have been shown to play a fundamental role to the toxicity of many particle types (Donaldson et al., 1997; Gilmour et al., 1996; Lund et al., 1991). Several metals present in welding fumes such as Cr (Leonard et al., 2000; Ye et al., 1999) and Fe are known to undergo redox-cycling resulting in the production of ROS (Stohs et al., 1995). However, the mechanisms involved in leukocyte influx and cytotoxicity of welding fumes are poorly understood.

It is well established that NF-κB and AP-1 are involved in the particle-induced expression of pro-inflammatory genes such as MIP-2, IL-6 and IL-8 (Rahman et al., 2000a; Schins et al., 2000; Shi et al., 1999b). In line with the neutrophil and MIP-2 data, increased DNA binding to both NF-κB and AP-1 was observed in the nuclear extracts of BAL cells isolated from animals instilled with the whole and soluble fractions of welding fumes. Furthermore, the lack of DNA binding of both transcription factors noted in animals instilled with either the washed particulate fractions or soluble chelated fractions, support the in vitro findings and suggest a potential signalling mechanism by which welding fumes induce inflammation. In accord with these findings Chong et al., (2000) showed that the elevated MIP-2 mRNA expression was accompanied by increased NF-κB-DNA binding activity in BAL cells isolated from a rat model of vanadium-induced pulmonary inflammation. A recent study by Zhou et al., (2003b) identified a synergistic interaction between iron and soot particles caused oxidative stress resulting in a significant increase in IL-1β and activation of NF-κB in rats. In addition, it has recently been suggested that the MIP-2 promoter could be synergistically activated by NF-κB and c-Jun transcription factors in LPS-induced pulmonary inflammation (Kim et al., 2003a) illustrating the potential cross-talk interactions that may be involved in welding fume-induced MIP-2 production.

Although the exact mechanisms of NF-κB activation were not investigated, they could involve tyrosine phosphatase inhibition resulting in the accumulation of
phosphoprotein and subsequent IκB phosphorylation, thus enabling the cytoplasmic to nuclear translocation of NF-κB (Samet et al., 1997). This pathway has been linked to ROFA-, Ni- and Cu-induced NF-κB activation (Goebeler et al., 1995; Samet et al., 1997; Kennedy et al., 1998). Alternatively, the soluble transition metal components and/or the ROS they produce have been shown to cause transcription factor activation and expression of pro-inflammatory cytokines, key events in the initiation of inflammation (Quay et al., 1998; Shi et al., 1999b; Chong et al., 2000).

The results of this chapter demonstrated that the acute neutrophilic inflammation following instillation of welding fumes was mediated by local production of the pro-inflammatory cytokine MIP-2 via the activation of NF-κB and AP-1. Furthermore, chelation of the soluble fraction attenuated both NF-κB and AP-1-DNA binding confirming the role of soluble transition metals in welding fume-induced pulmonary inflammation and entirely supporting the in vitro data. These findings support those of Quay et al., (1998) who demonstrated that transition metals present in ROFA cause increased cytokine production via an NF-κB mediated mechanism. Indeed, metals present in welding fumes such as Co, Ni and Cr have been reported to activate NF-κB and AP-1 in numerous cell types (Shi et al., 1999b; Chen et al., 2002; Goebeler et al., 1995; Zou et al., 2001; Barchowsky et al., 2002; Ding et al., 2000; Kaltreider et al., 1999). To date, this is the first known study demonstrating that the activation of NF-κB and AP-1 by soluble transition metals are critical events in the molecular signalling mechanisms leading to welding fume-induced inflammation in vivo. Furthermore, these data entirely support the results obtained in vitro, confirming the validity of A549 cells as a model for investigating particle-induced inflammation.
Chapter 7

The Pro-Inflammatory Effects of Diesel Exhaust Particles
: A Comparative Study
7.1 Introduction

Epidemiological studies have consistently reported an association between the concentration of ambient particulate matter and increased cardiopulmonary morbidity and mortality (Dockery et al., 1993; Pope et al., 1991; 1995; 1999b). Considerable research has focused on delineating the potential mechanisms responsible for these adverse affects. Numerous studies have demonstrated an important role for particle size, in terms of surface area, reactivity and ability to penetrate to the lower regions of the lung (Peters et al., 1997; Donaldson et al., 2001b). It has been shown that particles in the ultrafine (uf) range (<0.1 μm) induce a greater inflammatory response than their larger counterparts (Ferin et al., 1992; Li et al., 1999b; Brown et al., 2001). Particle composition, which encompasses a number of potentially active components including organics, endotoxins and transition metals have also been implicated as playing a critical role in particle toxicity (Li et al., 2003; Schins et al., 2004; Becker et al., 2003; Dye et al., 1999; Kodavanti et al., 1997).

Increased use of diesel engines in past 30 years has meant that diesel exhaust particles (DEP) are now a main component of fine-particulate matter (PM$_{2.5}$) in urban areas (Bonvallot et al., 2001; Takizawa et al., 2003), yet their real impact on air quality has not been fully elucidated. DEP have been shown to be highly inflammogenic and potentially carcinogenic in human and animal models in addition to in vitro culture systems (Boland et al., 1999; Ma et al., 2002; Verheyen et al., 2004; Pohjola et al., 2003). Indeed, DEP and whole diesel exhaust have been linked to lung cancer, pulmonary fibrosis, chronic alveolitis, bronchitis, airway hyper-responsiveness and inflammation (McClellan, 1987; Ichinose et al., 1995; Sagai et al., 1996; Sydbom et al., 2001).

DEP have a complex structure characterised by an inert elemental carbonaceous core with a large surface area to which various chemicals are adsorbed. These include organic compounds such as PAHs, quinones, aldehydes and aliphatic hydrocarbons (Schuetzle et al., 1981; Sagai et al., 1996; Anderson et al., 1998), as
well as sulphates and heavy metal ions. It has been suggested that DEP-induced lung injury may due to increased ROS production (Sagai et al., 1993; 1996) which trigger signalling mechanisms resulting in enhanced expression of pro-inflammatory genes culminating in pulmonary inflammation and enhanced allergic responses (Sagai et al., 1993; Hashimoto et al., 2000a; Whitekus et al., 2002; Baulig et al., 2003). PAHs and their oxygenated derivatives, quinones, undergo redox cycling, generating ROS in target cell populations such as macrophages and epithelial cells (Hiura et al., 1999; 2000; Nel et al., 1998; Squadrito et al., 2001). Similarly, surface-adsorbed reactive metals have been shown to generate cytotoxic reactive oxygen and nitrogen species and activate redox-sensitive signalling cascades leading to inflammation (Sagai et al., 1996; Boland et al., 1999). As such, the relative importance of these various components in mediating the biological effects of DEP is the topic of much research (Boland et al., 1999).

The aim of this chapter was to identify which components of diesel exhaust particles i.e. the soluble, particulate, metals and/or organics fractions caused an increased expression of the pro-inflammatory cytokine IL-8 in alveolar epithelial cells.
7.2 Diesel Exhaust Particle Morphology

Transmission Electron Microscopy (TEM) was used to determine the morphology of National Institute of Standard Technology (NIST) standard reference material diesel exhaust particles (SRM 2975). The TEM image shown in Figure 7.1 A, illustrates the uniform nature of DEP, with all single particles having a spherical appearance. The similarities between DEP and ufCB, a reference ultra-fine particle are shown in Figure 7.1 B, confirming that the single DEP are within the uf range (<100 nm diameter), but larger than ufCB. A common characteristic of DEP is the chain-like aggregates, formed following the combustion process. The NIST reference diesel is comprised primarily of these aggregates typical of classical DEP (Park et al., 2003).

Figure 7.1 Transmission Electron Micrographs of Diesel Exhaust Particles and Ultra-fine Carbon Black. The images represent particles at a concentration of 1 mg ml⁻¹. A. DEP. B. ufCB. Images are shown at a magnification of x 35,000. Arrows indicate single particles.
7.3 The Effect of Diesel Exhaust Particles on the Metabolic Activity of Alveolar Epithelial Cells

DEP are highly complex particles containing a number of mutagenic, carcinogenic and toxic compounds (Kagawa, 2002; Ma et al., 2002; Verheven et al., 2004). It is well documented that DEP induce oxidative stress in bronchial epithelial cells, which can result in an enhanced inflammatory response, protein modification and DNA damage (Kumagai et al., 1997; Li et al., 2002b; Dybdahl et al., 2003). However, there is limited information regarding the effects of DEP on alveolar epithelial cells. To determine the whether DEP affect the survival and proliferation of alveolar epithelial cells, the metabolic activity of A549 cells was assessed using the MTT assay.

Cells were exposed to DEP at concentrations ranging from 2-250 μg ml⁻¹ for various time points and the metabolic activity was determined by the conversion of the tetrazolium salt (MTT) to formazan (Mosmann, 1983). DEP had no effect on the metabolic activity of alveolar epithelial cells at all concentrations tested following 24 hr exposure (Figure 7.2). In fact, there was a slight increase in the conversion of MTT to formazan following 24 hr exposure to 2 μg ml⁻¹ DEP compared to control (110.7 ± 2.01 % v 100 % Control). This suggests that DEP may enhance A549 cellular metabolism or proliferation.
Figure 7.2. Effect of Diesel Exhaust Particles on the Metabolic Activity of Alveolar Epithelial Cells. A549 cells were exposed to DEP ranging in concentration from 2-250 μg ml⁻¹ over a 24 hr time course. The metabolic activity was determined by the reduction in MTT following treatments. The graph represents the mean of three independent experiments conducted in triplicate and values are expressed as percent control (untreated cells 0 μg ml⁻¹). The bars represent ± S.E.M.
7.4 Cytotoxicity of Diesel Exhaust Particles

It has been reported that DEP causes apoptosis and necrosis in a variety of different cell types, including bronchial epithelial cells; NHBE and 16HBE14o-, and promyelocytic cells HL-60 cells (Matsuo et al., 2001; Boland et al., 1999; Matsuo et al., 2003). The results from previous section suggest that DEP (SRM 2975) are not toxic to A549 alveolar epithelial cells. To confirm cell viability, the release of LDH into culture media was measured as an indicator of plasma membrane damage. As the relative contribution of the various components i.e. the particulate fractions or soluble components, in the toxicity of DEP remains controversial, A549 cells were exposed to whole DEP, the soluble fractions or washed diesel particulates which contained no soluble components, at concentrations ranging from 2-250 μg ml$^{-1}$ for 24 hr. Following exposure to whole DEP, only at the highest concentration (250 μg ml$^{-1}$) was any significant degree of cell death observed (p<0.05);(Figure 7.3). When cells were exposed to either the soluble or particulate fractions alone, this significance was lost suggesting an additive effect between the soluble and particulate fractions in DEP-induced toxicity. As minimal toxicity (<10 %) was only noted at the highest concentration of 250 μg ml$^{-1}$, this indicated that the DEP used in this study was essentially non-toxic to alveolar epithelial cells.
Figure 7.3. Toxic Effects of Diesel Exhaust Particles on the Alveolar Epithelial Cells. A549 cells were exposed to whole DEP, the soluble fractions or washed diesel particulates at concentrations ranging from 2-250 μg ml⁻¹ for 24 hr. LDH release was measured as a marker of cytotoxicity. Values are expressed as % total cellular LDH from cell lysates obtained by treatment with 0.1% Triton X-100. The graph represents the mean of three independent experiments conducted in triplicate and values are expressed as percent control (untreated cells 0 μg ml⁻¹). The bars represent ± S.E.M. *p<0.05 compared to control values.
7.5 The Effect of Diesel Exhaust Particles on IL-8 Gene Expression in Alveolar Epithelial Cells

It has been well documented that DEP can evoke an enhanced inflammatory response in both human and animal models as well as in various in vitro culture systems (Nel et al., 1998; Bonvallot et al., 2001; Bommel et al., 2003). To determine the pro-inflammatory effects of DEP on alveolar epithelial cells, reverse transcriptase PCR was used to investigate changes in steady-state IL-8 gene expression. As data from section 7.4 suggested an additive effect between the soluble and particulate fractions in the toxicity of DEP, A549 cells were exposed to whole DEP, the soluble fractions or the washed particulate fractions to verify the potential inflammogenicity of the various components of DEP. Following 6 hr exposure, all fractions of DEP enhanced IL-8 gene expression in a dose-dependent manner reaching significance at a concentration of 250 μg ml⁻¹ (Figure 7.4) (p<0.05 whole and soluble DEP; p<0.01 washed DEP). There was no significant difference in IL-8 mRNA expression between the various fractions of diesel (whole DEP 0.86 ± 0.05; soluble DEP 0.89 ± 0.01; washed DEP 1.05 ± 0.09 ratio IL-8:GAPDH). In contrast to the toxicity data, there was no additive or synergistic effect between the soluble and particulate fractions.
Figure 7.4. The Effect of Diesel Exhaust Particles on IL-8 Gene Expression in Alveolar Epithelial Cells. A549 cells were treated with the whole, the soluble or the washed particulate fractions of DEP at concentrations from 63-250 μg ml⁻¹ for 6 hr. RNA was isolated and IL-8 mRNA quantified by RT-PCR (sections 2.10-2.12). A. A representative PCR gel showing IL-8 and GAPDH. The arrows show the size of the bands. C: Control; TNF: TNF-α (10 ng ml⁻¹). B. The histogram represents the mean of four separate experiments conducted on pooled triplicate samples. The values are expressed as the ratio of IL-8:GAPDH. *p<0.05; **p<0.01 compared to control.
7.6 The Effect of Diesel Exhaust Particles on IL-8 Protein release by Alveolar Epithelial Cells

The result in section 7.5 showed that the increased IL-8 mRNA expression in alveolar epithelial cells was caused by both the soluble and particulate fractions of DEP. To determine whether the enhanced levels of mRNA resulted in increased protein production, levels of IL-8 protein were measured following 24 hr exposure to various concentrations of the different DEP fractions. All fractions of DEP caused a dose-dependent increase in IL-8 protein release following 24 hr exposure (Figure 7.5). However, for both whole DEP and the washed particulate fractions, a dose-dependent decreased in detectable IL-8 was observed at concentrations greater than 15 μg ml⁻¹. The levels of IL-8 were comparable to control at the highest concentrations (250 μg ml⁻¹); (whole DEP 111.81 ± 16.71, washed particulate fractions 110.28 ± 11.21 IL-8 % Control). A similar effect was shown with the soluble fractions. The reduction in IL-8 was initially observed at a concentration of 31 μg ml⁻¹, but levels of IL-8 still remained significantly greater than control levels even at the highest concentration (250 μg ml⁻¹; p<0.05) (Figure 7.4 B). At the lower concentrations, 2-15 μg ml⁻¹, both the soluble fractions and the washed particulate fractions induced similar levels of IL-8 protein production in A549 cells (soluble 183.32 ± 12.75 v washed particulate 198.81 ± 8.14 : IL-8 % control at 15 μg⁻¹). At concentrations above 15 μg ml⁻¹ the soluble fractions caused a significantly greater increase in IL-8 protein production than either the whole DEP or the washed particulate fractions (p<0.05).
Figure 7.5. Effect of Diesel Exhaust Particles on IL-8 Protein Production by Alveolar Epithelial Cells. A549 cells were exposed to whole, soluble or washed particulate fractions of DEP at concentrations ranging from 2-250 μg ml⁻¹ for 24 hr and IL-8 protein was measured in the supernatant. The values are expressed as percent control (untreated cells 0 μg ml⁻¹). The histogram represents the mean of three separate experiments conducted in triplicate and the bars represent ± S.E.M. *p<0.05; **p<0.01; ***p<0.001 compared to control. # p<0.05 soluble v whole DEP; $p<0.05$ soluble v washed particulates.
Although DEP induced a significant increase in IL-8 mRNA at concentrations where IL-8 protein remained at basal levels (Figure 7.4-7.5), previous work from this laboratory has demonstrated that cytokines can be adsorbed onto the surfaces of ultra-fine particles, such as ufCB (Kim et al., 2003c). Thus the lack of IL-8 protein measured following exposure to both the whole and washed particulates of DEP may be due IL-8 adsorption onto the particle surfaces producing an artificial, yet appreciable decrease in measurable IL-8 in the cell supernatant. To test this hypothesis, various concentrations of whole DEP, soluble and washed particulate fractions of DEP were incubated with recombinant IL-8 at concentrations of 100, 400 and 800 pg ml⁻¹ at 37 °C and the remaining IL-8 concentrations after 24 hr were compared to untreated standards. IL-8 adsorption occurred at all concentrations of whole DEP tested, as illustrated by the significant reduction in optical density (OD) compared to the standards (Figure 7.6 A). Similar results were shown for both the soluble and washed particulate fractions (Figures 7.6 B & C respectively), although adsorption was only found at the highest concentration of IL-8 (800 pg ml⁻¹). These results confirm that IL-8 protein is adsorbed onto DEP and provides an explanation for the dose-dependent decrease in protein levels shown previously. The fact that a reduction in OD occurred following incubation with the soluble fractions remains unexplained as there were no particles present for adsorption to take place. However it could be the result of soluble components of DEP causing a conformational change in the IL-8 antibody, thus reducing the binding affinity to the IL-8 capture antibody.
Figure 7.6. Adsorption of IL-8 Protein by Diesel Exhaust Particles. Whole, soluble or washed particulate fractions of DEP at concentrations ranging from 63-250 µg ml⁻¹ were incubated with various concentrations of recombinant IL-8 antibody for 24 hr at 37 °C. Optical density (OD) was measured at dual wavelengths 450/570 nm. Adsorption was regarded as a significant deviation from known IL-8 standard concentrations. A. Whole DEP B. Soluble Fractions C. Washed Particulate DEP
7.7 The inflammatory Effects of Diesel Exhaust Particles In Vivo

It is well established that instillation of diesel exhaust particles cause an enhanced inflammatory response in various animal models (Ichinose et al., 1995; Sagai et al., 1996; Miyabara et al., 1998; Murphy et al., 1998; Ghio et al., 2000). To confirm the inflammatory effects of diesel observed in the alveolar epithelial cell line A549, rats were instilled with the same DEP (SRM 2975) for 6 hr and the bronchoalveolar lavage fluid (BALF) was analysed for leukocyte influx and chemotactic mediators. Figure 7.6.1 shows the even distribution of DEP throughout the lungs of rats.

![Saline Control and Diesel-Instilled Rat Lungs](Image)

*Figure 7.6.1. Rat lungs following 6 hr Instillation of either Saline or DEP
Arrows show the presence of DEP.*

(This study was carried out as a pilot study in collaboration with Prof. D McQueen, Department of Neuroscience, University of Edinburgh, to determine the short-term neurological effects of diesel soot (data not reported in this thesis).

Following 6 hr instillation of DEP (250 μg ml⁻¹), there was a significant leukocyte influx into the lungs, with a total inflammatory cell count (macrophages and neutrophils) of $4.03 \pm 0.91 \times 10^6$ ml⁻¹ compared to $1.43 \pm 0.15 \times 10^6$ ml⁻¹ in saline-instilled animals. Furthermore, a third of the total cell population in diesel-instilled animals were neutrophils ($1.36 \pm 0.53 \times 10^6$ ml⁻¹) indicating acute pulmonary inflammation (Figure 7.6.2 A). Representative cytospin images show the presence of neutrophils, characterised by their multi-lobed nuclei and DEP-loaded...
macrophages from a diesel-instilled animal. A comparative cytospin from a saline instilled animal which lacks any neutrophils is shown in Figure 7.6.2.B.

![Graph showing total leukocytes in BALF](image)

**Figure 7.6.2 A. Total Leukocytes in BALF.** Differential cell counts were performed as described in section 2.20.2. Five animals were analysed per group. The values are expressed as total number of cells per ml BALF ± S.E.M. *p<0.05 compared to saline control.

![Images of cytospins](images)

**Figure 7.6.2 B. Representative Cytospins of BALF Cells.** Cytospins were prepared as described section 2.20.2. Figure (a) shows the presence of macrophages in the BALF of saline instilled animals. Figure (b) illustrates the presence of multi-lobular neutrophils plus DEP-loaded macrophages in the BALF of diesel-instilled animals. Images are taken at x 100 magnification.
As a potent neutrophil chemo-attractant both *in vivo* and *in vitro*, Macrophage Inflammatory Protein-2 (MIP-2), the rodent homologue of human IL-8 (Frevert *et al.*, 1995; Driscoll *et al.*, 1995) has been implicated in the development of pulmonary inflammation, recruiting inflammatory cells into the site of injury. In line with the data obtained for neutrophil influx, elevated levels of MIP-2 were measured in the primary BALF of animals instilled with DEP confirming a role for this pro-inflammatory cytokine in DEP-induced inflammation (Figure 7.6.3).

![Figure 7.6.3. MIP-2 Protein in BALF. MIP-2 levels were measured in the primary BALF of rats following the procedure describe in Section 2.20.3. The histogram represents the mean of five animals per group with samples analysed in triplicate. The values are expressed as MIP-2 pg ml⁻¹ ± S.E.M. **p<0.01 compared to saline control.](image-url)
7.8 The Role of Organics in the Pro-Inflammatory Effects of Diesel Exhaust Particles in Alveolar Epithelial Cells

It is well documented that DEP exert pro-inflammatory and pro-oxidative effects in airway epithelial cells (Abe et al., 2000; Li et al., 2002b). A number of studies have related these effects to the redox-cycling PAHs and quinones, which generate ROS (Kumagai et al., 1997), thereby altering the cellular redox status and activating signalling cascades, culminating in inflammatory gene expression (Li et al.; 2002b; Hashamoto et al., 2000a; Ma et al., 2004). To investigate the role of the organic fractions of DEP in the induction of IL-8 in alveolar epithelial cells, DEP were baked at 500 °C for 24 hr to remove any organic components. Cells were then treated with either baked DEP or whole standard DEP at concentrations ranging from 2-63 µg ml⁻¹ for 24 hr and IL-8 protein was measured. A role for the organic fractions of DEP on IL-8 production was demonstrated by the absence of a significant IL-8 protein release following exposure to baked DEP compared to whole, standard DEP (Figure 7.7). Exposure to baked DEP at concentrations of 15 and 31 µg ml⁻¹ showed a 1.5- and 1.4-fold reduction (p<0.01) in IL-8 release compared to the standard DEP. At the higher concentrations tested (≥ 63 µg ml⁻¹) no difference was noted between treatments but this lack of response was most probably due to IL-8 adsorption (shown previously, section 7.6).
Figure 7.6. Role of Organics in IL-8 Production in Alveolar Epithelial Cells. A549 cells were exposed to either whole DEP or baked DEP at concentrations ranging from 2-63 µg ml$^{-1}$ for 24 hr and IL-8 protein was measured in the supernatant. The histogram represents the mean of three experiments conducted in triplicate and the bars represent ± S.E.M. *p<0.05; **p<0.01 compared to control (untreated cells 0 µg ml$^{-1}$); $$$p<0.01$ (whole) standard DEP compared to baked DEP.
7.9 The Role of Soluble Transition Metals in the Pro-Inflammatory Effects of Diesel Exhaust Particles in Alveolar Epithelial Cells

The results of section 7.7 confirmed an important role for the organic components of DEP in driving the pro-inflammatory response. However, a trend towards increased IL-8 protein was still noted following exposure to the baked DEP in comparison to control levels (1.4-fold increase compared to control levels at 15 µg ml⁻¹), suggesting other components of DEP contribute to the pro-inflammatory effects. In addition to organics and sulphates, DEP also contains trace heavy metals such as iron and copper (Ichinose et al., 1997). These metals are known to undergo redox-cycling, causing enhanced free radical activity resulting in cellular damage, lung injury and inflammation (Halliwell et al., 1985; Stohs et al., 1995). To investigate whether soluble transition metals were involved in DEP-induced inflammation, the soluble fractions of DEP were pre-treated for 4 hr with the non-specific transition metal chelator chelex-100. Cells were then exposed to either the soluble fractions or the soluble-chelated fractions and IL-8 protein was measured in the cell supernatant after 24 hr. A dose-dependent increase in IL-8 was observed following exposure to the soluble fractions of DEP (p<0.001);(Figure 7.8). However, following pre-treatment of the soluble fractions with the metal chelator, IL-8 release was significantly attenuated with no significant difference from control, although there was a trend. A 1.6-fold reduction in IL-8 (p<0.05) was noted between soluble and chelated soluble fractions of DEP at 31 µg ml⁻¹. Taken together with the results shown in section 7.8, these data suggest that both the organic and soluble transition metals components are involved in the pro-inflammatory effects of DEP.
Figure 7.8. Role of Soluble Transition Metals on IL-8 production in Alveolar Epithelial Cells. The soluble fractions of DEP were pre-treated with Chelex-100 for 4 hr to remove any transition metals. A549 cells were then exposed to the Chelex treated soluble fractions (soluble chelated) or untreated soluble fractions at concentrations ranging from 8-63 µg ml⁻¹. IL-8 protein was measured in the supernatant following 24 hr exposure. The histogram represents the mean of three experiments conducted in triplicate and the bars represent ± S.E.M. **p<0.01; ***p<0.001 compared to control (untreated cells 0 µg ml⁻¹); $p<0.05; $Sp<0.01 soluble fractions compared to soluble chelated fractions.
7.10 Production of Free Radicals by Diesel Exhaust Particles

One of the main hypotheses regarding the inflammatory and immunological effects of diesel exhaust particles involves the formation of ROS and RNS (Sagai et al., 1993; Ichinose et al., 1995; Lim et al., 1998; Kumagai et al., 1997). To ascertain the oxidant capacity of the various diesel fractions, the super-coiled DNA plasmid assay was employed. The principle of the assay is that any particle-derived hydroxyl radicals will damage the DNA by “nicking” the super-coiled plasmid, causing it to unwind into a relaxed coil. Further damage resulting in linearization and eventually fragmentation indicates greater hydroxyl radical production (Donaldson et al., 1995).

The various fractions of DEP were incubated with the DNA plasmid, \( \phi \) X174 RF, at 37 °C for 8 hr and then resolved on an agarose gel. To determine whether DEP requires the presence of a reductant to initiate free radical generation, \( \text{H}_2\text{O}_2 \) was added to DEP samples. In addition, to establish whether the organic and transition metal components were involved in DEP-induced free radical production, baked DEP, absent of organic components, and chelated DEP, absent of any transition metals were also co-incubated with the plasmid. DEP caused a significant depletion of the super-coiled plasmid DNA (lane 4), (62.8 ± 5.8 \% depletion super-coiled DNA) compared to DNA control values. Neither the soluble fractions (lane 5) nor the washed particulate fractions alone (lane 6) had any marked effect, suggesting both fractions are involved in the generation of free radicals (Figure 7.9). When DEP were co-incubated with \( \text{H}_2\text{O}_2 \), only partial relaxation of the super-coil was noted (38.3 ± 2.4 \% depletion) compared to DNA alone (35.2 ± 4.3 \% depletion) indicating that the complex surface chemistry of DEP does not require the addition of reductants to initiate oxidant activity. In the absence of the organic and metal components, DEP failed to cause any significant plasmid damage, (baked DEP 28.8 ± 4.9 and chelated DEP 27.3 ± 2.9 vs DNA alone 35.2 ± 4.3 \% depletion) suggesting these components play a critical role in the oxidant activity of DEP.
Figure 7.9 Free Radical Activity of Diesel Exhaust Particles. Various fractions of DEP were incubated with 290 ng φ X174 RF plasmid DNA for 8 hr and the three plasmid forms were separated by electrophoresis and analysed by densitometry. Figure A shows a representative DNA plasmid gels with lanes 1-3 controls, 1: DNA plasmid; 2: Linear control; 3: Positive particle control, ROFA. Lanes 4-6 various fractions of DEP. 7: DEP with H2O2, Lanes 8-9: DEP without organic (8) or metal components (9). B. The histogram represents the mean of three experiments expressed as % depletion of total super-coiled DNA ((Relaxed + Linear)/Total DNA x 100). Bars represent ± S.E.M. **p<0.01; ***p<0.001 compared to DNA control.
7.11 The Role of Reactive Oxygen Species in the Pro-Inflammatory Effects of Diesel Exhaust Particles

The results of section 7.10 demonstrated the production of free radicals by DEP as shown by the depletion of super-coiled DNA plasmid. Furthermore, in the absence of both the organic and transition metals components of DEP no free radical activity was observed. It was therefore proposed that the enhanced IL-8 expression shown previously involved an oxidative stress-mediated mechanism via organic and/or metal-derived ROS. A range of antioxidants were used to determine the possible contribution of ROS in DEP-induced inflammation. The specific hydroxyl radical (OH) scavenger, mannitol was used to clarify a role of the OH radicals (Stoeh et al., 1995; Halliwell et al., 1995), whereas the thiol antioxidant, N-Acetyl-L-Cysteine (NAC), was used to investigate whether an oxidative-mediated mechanism was involved in DEP-induced inflammation (Morcillo et al., 1999). Alveolar epithelial cells were co-incubated with mannitol or NAC and either whole DEP, the soluble fractions or the washed particulate fractions for 24 hr and IL-8 protein production was measured. In the presence of mannitol, a significant reduction in IL-8 release was shown following exposure to the soluble fractions of DEP at concentrations of 15 and 31 μg ml⁻¹ (p<0.05) (Figure 7.10). In contrast, mannitol appeared to have no marked effect on either whole DEP or particulate fraction-induced IL-8 production.
Figure 7.10. Effect of Mannitol on DEP-Induced IL-8 production in Alveolar Epithelial Cells. A549 cells were treated with whole DEP, the soluble fractions or the washed particulate fractions of DEP (15-63 µg ml⁻¹) with or without mannitol (5 mM) for 24 hr and IL-8 protein was measured in the supernatant. The histogram represents the mean of three experiments conducted in triplicate and the bars represent ± S.E.M. *p<0.05; **p<0.01; ***p<0.001 compared to control (untreated cells 0 µg ml⁻¹); $p<0.05$ untreated DEP compared to co-treatments of mannitol and DEP.
The role of ROS in the pro-inflammatory effects of DEP was confirmed by the attenuated IL-8 release from A549 cells co-incubated with NAC. A significant reduction in IL-8 production was observed for all components of DEP, that is whole DEP, the soluble and the washed particulate fractions, at 31 µg ml\(^{-1}\) in the presence of 5 mM NAC (p<0.05); (Figure 7.11). Overall, these data implicate the involvement of oxidative-mediated pathways in the pro-inflammatory effects of DEP in alveolar epithelial cells.

![Figure 7.11 Effect of NAC on DEP-Induced IL-8 production in Alveolar Epithelial Cells.](image)

A549 cells were treated with the whole DEP, the soluble or the washed particulate fractions at concentrations ranging from 15-63 µg ml\(^{-1}\) with or without NAC (5 mM). IL-8 protein was measured in the supernatant following 24 hr exposure. The histogram represents the mean of three experiments conducted in triplicate and the bars represent ± S.E.M. **p<0.01; ***p<0.001 compared to control (untreated cells 0 µg ml\(^{-1}\)); $ p<0.05$ untreated DEP compared to NAC treated components.
Comparative Effects of Welding Fumes and Diesel Exhaust Particles on Alveolar Epithelial Cells

One of the principal aims of this thesis was to investigate the toxicological properties of particulate matter from occupational and ambient environments. More specifically the aim was to establish the relative contribution of transition metals to particle-induced inflammation. To this end, the pro-inflammatory effects of welding fumes, a metal-rich particle produced in high concentrations in occupational setting and known to cause pulmonary inflammation were compared to DEP, a major component of ambient PM with a low metal content, and also associated with enhanced inflammatory and immunological effects. A summary of the data is shown in Table 7.1.

Initially, the relative toxicity of the two particle types was compared. As shown in table 7.1 welding fumes were significantly more toxic to alveolar epithelial cells (A549) than DEP at all concentrations and time-points examined. The dose required to cause 25 % cell death (TD 25) for welding fumes ranged from 70-117.5 μg ml$^{-1}$, whereas DEP failed to induce this degree of cell death at any concentrations tested (2-250 μg ml$^{-1}$). A maximum of 5 % cytotoxicity (TD 5) was observed following 24 hr exposure to DEP at a concentration of 250 μg ml$^{-1}$, whereas the TD 5 for welding fumes ranged from 12.9 - 21.5 μg ml$^{-1}$ at the same time point. A similar pattern was observed for metabolic activity and cellular proliferation as determined by the MTT assay, with welding fumes causing a significant dose- and time-dependent decrease in the metabolic activity of A549 cells. In contrast, there was no difference between the metabolic activity of A549 cells exposed to DEP and control cells at any time points or concentrations tested. In fact, DEP appeared to cause a slight increase in cellular proliferation at the 24 hr time point although this affect was not significant. These data highlight the first notable difference between the particle types, namely that welding fumes were markedly more toxic to alveolar epithelial cells than DEP.
<table>
<thead>
<tr>
<th>PROPERTY / CELLULAR EFFECT</th>
<th><strong>DIESEL EXHAUST PARTICLES</strong></th>
<th><strong>WELDING FUMES (NIMROD c276)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SIZE</strong></td>
<td>&lt;0.1 μm</td>
<td>≤0.1 μm</td>
</tr>
<tr>
<td><strong>COMPOSITION</strong></td>
<td>Carbon core, organics (i.e. PAHs, quinones), heavy metals</td>
<td>Metal oxides (Zn, Cr, Mn, V)</td>
</tr>
<tr>
<td><strong>EXPOSURE STANDARD</strong></td>
<td>50 μg m⁻³</td>
<td>OEL: 5 mg m⁻³</td>
</tr>
<tr>
<td><strong>THRESHOLD TOXICITY</strong> (LDH)</td>
<td>*(&gt; 250 μg ml⁻¹)</td>
<td>****(&gt; 63 μg ml⁻¹)</td>
</tr>
<tr>
<td><strong>THRESHOLD DOSE (TD 25)</strong></td>
<td>&gt; 250 μg ml⁻¹</td>
<td>70-117.5 μg ml⁻¹</td>
</tr>
<tr>
<td><strong>THRESHOLD DOSE FOR REDUCTION IN METABOLIC ACTIVITY (MTT)</strong></td>
<td>&gt; 250 μg ml⁻¹</td>
<td>****(&gt; 63 μg ml⁻¹)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>THRESHOLD IL-8 PRODUCTION</strong></th>
<th><strong>Whole</strong></th>
<th><strong>Soluble</strong></th>
<th><strong>Washed</strong></th>
<th><strong>Whole</strong></th>
<th><strong>Soluble</strong></th>
<th><strong>Washed</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(15 μg ml⁻¹)</strong></td>
<td>***</td>
<td>***</td>
<td>**</td>
<td>***</td>
<td>***</td>
<td>&gt; 250 μg ml⁻¹</td>
</tr>
<tr>
<td><strong>(≥31 μg ml⁻¹)</strong></td>
<td>NA</td>
<td>§</td>
<td>NA</td>
<td>NA</td>
<td>§§§</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Metal chelation</strong></td>
<td>NA</td>
<td>§</td>
<td>NA</td>
<td>NA</td>
<td>§§§</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Removal of Organics</strong></td>
<td>##</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Antioxidants:</strong></td>
<td>$</td>
<td>$</td>
<td>$</td>
<td>$</td>
<td>$</td>
<td>$</td>
</tr>
<tr>
<td><strong>NAC</strong></td>
<td>NE</td>
<td>$</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td><strong>Mannitol</strong></td>
<td>NE</td>
<td>$</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
</tr>
</tbody>
</table>

Table 7.1 Summary of Data showing the comparisons between Diesel Exhaust Particles and Welding Fumes. Significance is shown as *p<0.05; **p<0.01; ***p<0.001 compared to control (untreated cells 0 μg ml⁻¹); ##p<0.01 baked DEP compared to standard DEP; §p<0.05; §§§p<0.001 chelated soluble fractions compared to untreated soluble fractions. $p<0.05; $$$p<0.001 co-incubation with antioxidants compared to untreated fractions. NA; Non-Applicable. NE; No Effect. OEL: Occupational Exposure Limit.

To establish the pro-inflammatory effects of the different fractions of both particle types, A549 cells were exposed to the whole particulates, which contained soluble components and particulates, the soluble fractions alone or the particulate fraction which had been washed thoroughly to remove any soluble components (washed particulates). As shown in table 7.1, the pro-inflammatory activity of welding fumes existed entirely within the soluble fraction, with the particulate
fraction having no inflammatory potential. In contrast, the pro-inflammatory effects of DEP were driven by both the soluble and particulate fractions.

To further identify the potential components within the different fractions causing the enhanced IL-8 production, various interventions were employed. To investigate whether soluble transition metals were involved in the pro-inflammatory effects of welding fumes and DEP, samples were co-incubated with the metal chelator Chelex-100 for 4 hr. Pre-treatment of welding fumes with Chelex-100 completely abrogated the IL-8 production. Although the concentration of transition metals is significantly lower in DEP than in welding fumes, the marked reduction in IL-8 (p<0.05) noted in cells exposed to chelex-treated soluble DEP compared to soluble DEP, suggests that soluble transition metals were involved in the pro-inflammatory effects of DEP. Levels of IL-8 following treatment with chelex-treated DEP however still remained 3-fold greater than control levels, whereas, IL-8 production following exposure to chelex-treated welding fumes returned to basal levels. These results demonstrated that the pro-inflammatory effects of welding fumes were entirely attributable to the soluble transition metals, whilst, other components such organics may be involved in DEP-induced inflammation. The role of the organic fractions of DEP in the enhanced inflammatory response was confirmed by the attenuated IL-8 production seen in A549 cells exposed to baked DEP, where the volatile organic components were depleted compared to standard DEP (p<0.01); (Table 7.1).

A common theory surrounding particle-associated inflammation involves the production of ROS and RNS. The involvement of ROS in the pro-inflammatory effects of welding fumes and DEP was confirmed by the significant damage to supercoiled DNA and the attenuated IL-8 release from cells co-incubated with the thiol antioxidant NAC. A 1.5- and 2-fold reduction in IL-8 was observed for all fractions of DEP and the soluble components of welding fumes respectively following co-incubation with 5 mM NAC.
Diesel exhaust particles (DEP) are a major component of ambient air pollution in urban areas (Takizawa et al., 1999). Epidemiological and toxicological studies have consistently reported enhanced inflammatory and immunological effects following exposure to DEP. Indeed, DEP have been shown to act as an adjuvant for allergic sensitization and it has been suggested that DEP may play a role in the exacerbation of allergic airway diseases such as asthma and rhinitis (Peterson et al., 1996; U.S. EPA, 2002). Increased expression of the allergic antibody, IgE, \textit{in vitro} and \textit{in vivo} and augmentation of both IgE and IgG production in human volunteers have been reported following exposing to DEP (Muranaka et al., 1986; Diaz-Sanchez et al., 1994; Pandya et al., 2002; Mastrangelo et al., 2003). In addition, previous studies have suggested that DEP can directly stimulate airway epithelial cells to produce inflammatory mediators such as IL-8, GM-CSF and soluble ICAM-1, thus facilitating pulmonary inflammation (Steerenberg et al., 1998; Boland et al., 2000; Madden et al., 2003; Baulig et al., 2003).

A number of organic compounds that are adsorbed onto the surface of DEP, such as PAHs, quinones and aldehydes, are mutagenic, carcinogenic and inflammogenic (Cohen et al., 1999). Much of the work surrounding DEP toxicity has therefore focused on identifying which components of DEP cause these adverse health effects (Kagawa, 2002). Few studies have, however, addressed whether transition metals, which are involved in the inflammatory effects of numerous other particle types, are involved in the pathogenicity of DEP. Furthermore, there is conflicting evidence regarding the relative importance of the soluble and insoluble components of DEP and the role of the carbonaceous core in the activity of DEP (Lovik et al., 1997; Samet et al., 2000). The aim of this chapter was to examine which components of DEP were responsible for the toxicological and pro-inflammatory effects of DEP on alveolar epithelial cells. In addition, the role of the soluble transition metal content of DEP was examined for comparison with the pro-inflammatory effects of welding fumes.
In terms of morphology and composition, DEP and welding fumes differ considerably, however they can both be classified as ultra-fine (uf) particles (Aksellson et al., 1976; Boland et al., 1999; Kawaga, 2002), and are therefore readily inhaled, penetrating to the distal regions of the lungs (Li et al., 2002b; Hirano et al., 2003; Subramaniam et al., 2003). As such, alveolar type II cells represent a potential target for the deleterious effects of uf particles and thus are a relevant cell type with which to study the toxicological and inflammatory effects of these particle types (Marano et al., 2002).

The toxicity of DEP on airway cells has been well studied, yet there is limited information regarding their toxic effects on alveolar epithelial cells with the majority of studies examining the effects on bronchial epithelial cells and alveolar macrophages (Hiura et al., 1999; 2000; Matsuo et al., 2003). The results shown here suggest that the DEP used in this study (SRM 2975) are essentially non cytotoxic towards alveolar epithelial cells over a range of concentrations (2-250 µg ml⁻¹) and time-points (2-24 hr), only causing a significant (p<0.05) increase in LDH release at a very high concentration (250 µg ml⁻¹). Further investigation suggested that the membrane damage causing the release of LDH was due to the additive effects of both the soluble and particulate fractions of DEP. In addition, DEP did not compromise the metabolic activity of A549 cells as illustrated by the consistent conversion of tetrazolium to formazan at all concentrations tested confirming mitochondrial dehydrogenase activity (Mossman et al., 1983). In support of these findings, Don Porto et al., (2001) reported no significant toxicity in either A549 cells or human monocytic THP-1 cells exposed to the similar standard reference DEP (SRM 1650). Likewise, Baulig et al., (2003) found that DEP did not effect the viability of human bronchial epithelial cells, 16HBE14o- as determined by PI staining, suggesting these SRM diesel particles (2975 and 1650) are essentially non-toxic to certain cell types.

The lack of cytotoxicity shown by DEP to alveolar epithelial cells is in complete contrast to welding fumes which caused a dose- and time-dependent increase in LDH release and decrease in cellular metabolism following exposure to similar concentrations (1-250 µg ml⁻¹); (Chapter 3 sections 3.3-3.4). Indeed, the
threshold concentration at which 5 % cell death occurred, (TD 5), was 4-fold lower for welding fumes than for DEP. Similarly, the concentration of DEP required to cause a 5 % decrease in the metabolic activity of A549 cells was 8-fold lower than for welding fumes. It is important to note that the occupational exposure limit (OEL) for welding fumes is set at 5 mg m$^{-3}$ over an 8 hr period, whereas the national air quality standard for PM$_{10}$ of which DEP are a major component is 50 µg m$^{-3}$ over a 24 hr period. The results shown here, however, indicate that per unit mass, the welding fumes used in this study were significantly more toxic than the DEP. Nevertheless, evidence from welders suggests that exposures at these high concentrations have few major long term health effects with the most frequently occurring respiratory illness being metal fume fever (MFF) (Lockey et al., 1988). This apparent discrepancy between the high particle toxicity of welding fumes but little adverse health effects, verses the well documented adverse effects of PM$_{10}$ may be explained by susceptible individuals who succumb to enhanced levels of PM$_{10}$ yet are not present in occupational environments where welding fumes are produced. Alternatively, it might also be an in vitro phenomenon due to the absence of metal-binding proteins in vitro whereas the lung lining fluid is replete with metal-binding proteins which render the transition metals non-redox active.

An essential feature of particle-induced pulmonary inflammation is the release of pro-inflammatory cytokines from alveolar macrophages and epithelial cells, resulting in leukocyte recruitment. Studies have reported enhanced IL-8 expression following exposure to DEP in a variety of cell types including human airway epithelial cells, the bronchial epithelial cell lines BEAS-2B and BET-1A and alveolar macrophages (Abe et al., 2000; Hashimoto et al., 2000a; Kawasaki et al., 2001; Li et al., 2002b). The results of this study found a similar significant increase in IL-8 mRNA and protein production in alveolar epithelial cells following exposure to DEP confirming the validity of the A549 cell line as a model to investigate the pro-inflammatory effects of DEP. Furthermore, both the soluble and the washed particulate fractions caused an enhanced release of IL-8 demonstrating that both components were involved in the pro-inflammatory effects of DEP. However, due the adsorption of IL-8 protein onto the surfaces of DEP it was difficult to establish
whether any potential additive or synergistic interactions were involved in the increased IL-8 production.

The in vivo data showing an acute inflammatory response in rat lungs following 6 hr instillation of DEP confirms the inflammogenicity of DEP. In line with these results, a recent study by Singh et al., (2004) showed an acute inflammation in mice following instillation of the same SRM 2975 DEP. Similar finding have been reported in healthy human subjects who were found to develop airway inflammation following an acute exposure to freshly generated DEP (Salvi et al., 2000; Holgate et al., 2003a; Stenfors et al., 2004). This response was characterized by increased IL-8 mRNA expression in the bronchial mucosa and protein in lavage fluid, which preceded airway neutrophilia and lymphocytosis and resulted in up-regulation of the endothelial adhesion molecules.

Inflammatory responses have been reported for welding fumes as illustrated by the study of Blanc et al., (1993) who found enhanced levels of polymorphonuclear leukocytes and elevated concentrations of inflammatory mediators such as TNF-α, IL-1β and IL-8 in the BALF of workers following exposure to zinc oxide welding fumes. The results presented here found that welding fumes and DEP caused enhanced expression of the pro-inflammatory cytokine IL-8 in alveolar epithelial cells. When comparing the relative potency of the different particle fractions, IL-8 production was 3.6-fold greater in cells exposed to the soluble fraction of welding fumes compared to the equivalent soluble fraction of DEP. In contrast, exposure to the washed particulate fractions of DEP caused a 2.2-fold greater production of IL-8 than for welding fume particles. There is considerable debate regarding the contribution of the soluble and insoluble components of DEP and the relative importance of the carbon core in DEP-induced inflammation. The data shown here suggests that the pro-inflammatory effects of DEP reside in both the soluble and particulate fractions. In accord, a recent study by Ma et al., (2002) suggested that DEP-induced oxidative stress is mediated via a similar dual effect of the particulate and organic components of DEP. In contrast to DEP, the inflammogenicity of welding fumes was found to be entirely attributable to the soluble fractions.
DEP consist of carbon core with a large surface area to which many different chemical compounds are absorbed. These compounds include organics such as PAHs and their oxygenated derivatives, nitrates, sulphates and transition metals (Sagai et al., 1996). Removing the organic fractions of DEP caused a significant reduction in IL-8 production (p<0.01) compared to standard DEP, confirming the involvement of organics in the pro-inflammatory effects DEP. In support of these results previous studies have demonstrated that the organic extracts of DEP replicate the pro-inflammatory and pro-oxidative effects of composite DEP (Kawasaki et al., 2001; Li et al., 2002b). Indeed, it has been shown that aromatic and polar chemical groups enriched for PAHs and quinones can directly reproduce the inflammation induced by crude DEP or an organic extract (Li et al., 1996; 2002b; Kawasaki et al., 2001; Baulig et al., 2003). It is important to note that in the present study, levels of IL-8 induced by baked DEP, depleted of any organics were still greater than basal levels (147.9 ± 14.3 v 100 IL-8 % control), indicating that other components are involved in driving these pro-inflammatory effects.

The pro-inflammatory effects of DEP have been well-documented (Li et al., 2002b; Baulig et al., 2003). However the exact contribution of transition metals in these effects of DEP remains relatively unknown. The transition metal content of a variety of particle types including PM$_{10}$, ROFA and asbestos is known to play a central role in their inflammogenicity (Kamp et al., 1995; Gilmour et al., 1997; Adamson et al., 1999; Shukla et al., 2003c). The attenuated IL-8 production from alveolar epithelial cells exposed to a metal-chelated fraction of DEP reported in this study provides clear evidence for the role of soluble transition metals in the pro-inflammatory effects of DEP. These findings corroborate those of Mudway et al., (2004) who reported that the oxidative activity of diesel exhaust (DE) was comparable to ROFA, a transition metal-rich particle. In addition, DTPA, a metal chelator capable of binding and inactivating a range of transition metals, largely protected against DE-induced depletion of ascorbic acid and glutathione in synthetic lung lining fluid. Likewise, Han et al., (2001) suggested that a Fenton-like mechanism was involved in DEP-induced OH generation in mice. Co-treatment with the iron chelator, desferrioxamine, decreased the Electron Spin Resonance (ESR)
decay rate in a dose-dependent manner demonstrating the involvement of transition metals.

The data presented previously highlighted a pivotal role for soluble transition metals in the inflammogenicity of welding fumes as illustrated by the complete abrogation of IL-8 production in A549 cells exposed to chelex-treated fumes. Similarly, soluble transition metals were also implicated in the pro-inflammatory effects of DEP as shown by the reduction in IL-8 release following pre-treatment of the soluble fraction of DEP with chelex-100. The extent to which metals were involved in the pro-inflammatory effects of DEP was however significantly lower than for welding fumes with a 33 % reduction in IL-8 noted for chelated-DEP compared to the 50 % reduction in the equivalent welding fume fraction. These data indicate that complex interactions between the organic and metal components of DEP dictate the inflammatory potential of DEP, with both components contributing to the overall effect. This interaction between the organic components and transition metals may relate to their involvement in quinone cycling (Bolton et al., 2000). The enzymatic (P450/P450 reductase), and non-enzymatic redox-cycling of quinones with their corresponding semi-quinone radicals, results in the formation of superoxide anions. In the presence of transition metals like iron or reductants such as H₂O₂, these superoxide radicals dismutate to form hydroxyl radicals (O'Brien, 1991; Monks et al., 1992). This interaction between the organic components and transition metals of DEP illustrates a potential mechanism through which DEP may induce its pro-inflammatory effects. This hypothesis is supported by the lack of free radical activity from DEP in the absence of either organic components or transition metals.

The biological mechanisms involved in particle-associated morbidity and mortality are partially understood with mechanistic data suggesting that oxidative stress is a key biological event in the causation of PM-induced adverse health effects (Nel et al., 1998; Whitekus et al., 2002; Donaldson et al., 2000; 2001a). Indeed, ROS have been identified as important mediators of pulmonary disorders, including asthma, ARDS, COPD and pulmonary inflammation and fibrosis caused by asbestos,
silica and quartz (Jarjour et al., 1994; Gonzalez et al., 1996; Langen et al., 2003; Kamp et al., 1992; Vallyathan et al., 1998). A common feature of DEP and welding fumes is their ability to generate reactive oxidants as illustrated by the significant damage to super-coiled plasmid DNA, indicative of hydroxyl radical activity. Transition metals and organics in DEP stimulate ROS production in various cell types such as epithelial cells and macrophages (Vogl et al., 1989; Ball et al., 2000; Wilson et al., 2002; Siegel et al., 2004). The lack of damage to plasmid DNA caused by DEP in the absence of organic components supports the hypothesis that the pro-oxidative effects DEP are related to the presence of redox-cycling organic compounds (Li et al., 2002b; Kawasaki et al., 2001; Squadrito et al., 2001) and that DEP exerts many of its toxic effects through the imposition of oxidative stress (Mudway et al., 2004). For example, the organic extracts of DEP induce superoxide production in lung microsomes via NADPH-dependent reductase and also through inner membrane damage of the mitochondria (Schuetzle et al., 1981; Kumagai et al., 1997; Li et al., 2002b). Likewise, quinones generated in the lung during enzymatic conversion of PAHs by cytochrome P450 1A1 have been shown to drive the biological activity of methanol extracts of DEP (Kumagai et al., 1997; Penning et al., 1999; Takano et al., 2002). Benzo[a]pyrene, one of the putative chemicals in benzene-extracted DEP, was shown to replicate DEP-induced IL-8 expression and NF-κB activation in the human bronchial epithelial cell line BEAS-2B and normal human airway epithelial cells (Kawasaki et al., 2001). These effects were suppressed by the addition of antioxidant NAC and the NFκB inhibitor PDTC implicating an oxidative mediated-mechanism was involved.

A similar redox-cycling mechanism is involved in metal-induced ROS production. In reducing environments, such as the lung lining fluid (Kelly et al., 1996; Greenwell et al., 2002), metals can undergo Fenton-like reactions, resulting in generation of ROS. ROS can then directly or indirectly act as signalling molecules causing the activation of NF-κB and AP-1 leading to inflammatory gene expression.

The results shown here suggest that a common signalling mechanism involving ROS may regulate the inflammatory effects of both DEP and welding fumes. In the presence of the antioxidant N-acetyl-L-cysteine (NAC), IL-8
production was significantly attenuated for all fractions of DEP and welding fumes. In line with these findings, Hashimoto et al., (2000a) reported inhibition of p38 MAPK-dependent IL-8 and RANTES expression in the presence of NAC in human bronchial epithelial cells (BEC). As a cysteine derivative, NAC is a precursor for glutathione synthesis, and thus may lower IL-8 production by increasing the GSH/GSSG ratio, blocking oxidant-induced signalling mechanisms involved in IL-8 gene transcription. Alternatively, upon entering the cells NAC may directly scavenge ROS preventing oxidant-induced IL-8 expression. The direct contribution of organically-derived compared to metal-derived oxidants in DEP-induced IL-8 release remains unclear. However, there is strong evidence to support the involvement of both components as demonstrated by the lack of damage to super-coiled DNA plasmid in DEP devoid of either organics or transition metals. It has been suggested that the generation of ROS by standard diesel particulates SRM 1650 involves transition metals (Ball et al., 2000). Likewise, Baulig et al., (2003) detected an increase in DCF fluorescence intensity in nasal and bronchial epithelial cells exposed to native SRM 1650 DEP and an organic DEP-extract, indicating increased intracellular ROS production via the organic components. There is substantial evidence to suggest that \( \cdot \text{OH} \) are the main reactive oxidants generated by DEP. However, the fact that mannitol, a specific \( \cdot \text{OH} \) scavenger, only decreased IL-8 production induced by the soluble fractions of DEP indicates that the pro-inflammatory effects of DEP are driven by other free radicals such as superoxide anions. Furthermore, as mannitol is an extra-cellular antioxidant, the soluble fractions must generate extra-cellular ROS implicating an alternative mechanism is involved in the inflammatory effects of soluble DEP components.

Overall, the results of this chapter suggest that both the soluble and insoluble components of DEP were required to induce the maximal pro-inflammatory response in alveolar epithelial cells. Moreover, both organic compounds and soluble transition metals were involved in these pro-inflammatory effects and it is postulated that this interaction may relate to the generation of hydroxyl radical generation via quinone cycling. In contrast, the inflammatory effects of welding fumes were entirely attributed to the soluble transition metals,
highlighting an important difference in the initiation of ambient and environmental particulate-induced inflammation. It is important to note that there are similarities between DEP and welding fumes, such as their ability to generate ROS. This suggests that although the relative contribution of soluble and insoluble components and transition metals differs greatly between particle types, both cause oxidative stress in alveolar epithelial cells culminating in that activation of signalling cascades and up-regulation of pro-inflammatory gene transcription (Figure 7.12).

Figure 7.12 Schematic diagram illustrating the potential mechanisms involved in DEP and welding fumes induced inflammation.
Chapter 8

Summary & Future Work
8.1 Summary & Future Work

The aims of this thesis were to determine the importance of transition metals in the pathogenicity of an occupational and an environmental particulate. The hypothesis that soluble transition metals drive the pro-inflammatory effects of the two particle types, welding fumes and diesel exhaust particles, was addressed in *in vitro* and *in vivo* models of pulmonary inflammation. In addition the molecular signalling mechanisms involved in welding fume-induced inflammation were investigated.

Welding fumes are complex highly metal-rich particles produced during the welding process. The composition of welding fumes is highly variable, with factors such as the filler wire, fluxes and electrodes as well as the welding technique influencing the fume composition (Lockey *et al.*, 1988; Antonini, 2003). As a result, welding fumes also differ in their inherent toxicity and inflammogenicity (Sferlazza *et al.*, 1991; Antonini *et al.*, 2004b). These differences were highlighted in the present study by using three compositionally different welding fumes, two nickel-based fumes; NIMROD 182, NIMROD c276 and a cobalt-based fume; COBSTEL 6, which varied in their toxicity and inflammatory activity towards alveolar epithelial cells (Sections 3.3-3.4, 3.8). Although the welding fumes used in this study were largely comprised of ultra-fine particulates (Section 3.2, Appendix III), the particulate (insoluble) fraction of welding fumes appeared to be essentially non-inflammogenic with the inflammatory effects of welding fumes being entirely attributable to the soluble components (Sections 3.7-3.8; 6.2.1-6.3), confirming the original hypothesis. The lack of activity in welding fume particulates remains unclear, as previous studies have shown that ultra-fine particles of essentially inert material can enhance pro-inflammatory cytokine gene expression *in vitro* and induce pulmonary inflammation *in vivo* (Brown *et al.*, 2001). One possible explanation may be related to the age of the welding fumes, with samples collected and stored over a three year period. Freshly generated welding fumes are highly reactive and cause a greater inflammatory response in rats than “aged” fumes (Antonini *et al.*, 1998). Consequently, the age of the welding fumes used in the present study may have
contributed to their lack of activity. By obtaining freshly generated welding fumes the actual inflammatory potential of the particulate components could be clarified.

Epidemiological studies have consistently reported a higher incidence of pulmonary disorders amongst welders than the general population (Lockey et al., 1988; Sferlazza et al., 1991). Many of these adverse health effects such as metal fume fever (MFF) are characterised by an acute inflammatory response (Van Pee et al., 1998; Antonini et al., 2003). The present research has shown that welding fumes enhance the release of the pro-inflammatory cytokine IL-8 in alveolar epithelial cells and cause acute inflammation in rat lungs (Sections 3.7-3.8; 6.2.1-6.3). Moreover, these effects were entirely attributed to the soluble transition metal contents as demonstrated by the lack of inflammation following pre-treatment of the soluble fraction of welding fumes with a transition metal chelator (Sections 6-2-6.3). Metal analysis of welding fumes by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) identified chromium as the predominant metal present in the soluble fraction of all three welding fumes (> 80 %) (Section 4.2). Further investigation demonstrated an almost a linear relationship between chromium concentration and IL-8 production (Section 4.3.1). Attempts to mimic the effects of soluble welding fume fractions with equivalent concentrations of chromium compounds failed to reproduce the same responses (Section 4.3.2). However, the combination of chromium and cobalt compounds evoked a similar level of IL-8 production as the soluble fraction of COBSTEL 6, indicating complex metal-metal interactions may be involved in the pro-inflammatory effects of welding fumes (Section 4.3.2). To further investigate the metal(s) involved in welding fume-induced inflammation, ICP-MS analysis of welding fumes to include a wider spectrum of metals, such as fluorine and silica, which are generated in the welding process, (Antonini, 2003), may provide some insight into other potential candidates involved in welding fume-induced inflammation. Metal speciation of the principal metals present in welding fumes by high pressure liquid chromatography (HPLC) directly coupled to an Inductively Coupled Plasma Time of Flight Mass Spectrometer (ICP-TOFMS) would ensure that the metal compounds used to mimic the effects of welding fumes were selected to produce similar metal ions when in solution. Identifying the specific metals
responsible for the pathogenicity of welding fumes may provide a potential target for chemical manipulations as a therapeutic intervention to reduce the adverse health effects associated with welding fumes.

Metal fume fever (MFF) is the most frequently described illness associated with welding fume exposure (Graeme et al., 1998; Antonini et al., 2003). It is suggested that the acute inflammatory response associated with MFF involves a cytokine or cytokine-like network (Blanc et al., 1991; 1993). The molecular signalling mechanisms involved in the inflammatory effects of welding fumes however, remain unknown. The present research has shown that the soluble fraction of welding fumes deplete the antioxidant GSH and increase intracellular ROS resulting in oxidative stress in alveolar epithelial cells (Sections 3.11-3.13). In turn, this triggers a signalling cascade involving phosphorylation of p38 MAPK resulting in increased NF-κB and AP-1-DNA binding and culminating in pro-inflammatory gene expression (Sections 5.2-5.8; 3.6-3.8). The inhibition of transcription factor-DNA binding and resultant IL-8 production in the presence of the antioxidant N-acetyl-L-cysteine (NAC) confirms the involvement of ROS (Section 5.5). It is still unclear whether the soluble fractions of welding fumes, and/or welding fume-generated ROS cause signalling via other MAPK pathways that are also known to activate NF-κB and AP-1. In addition, how the soluble fractions of welding fumes interact with the cell surface to transmit signals leading to activation of MAPK remain unknown, although there is published evidence to suggest the involvement of the epidermal growth factor (EGF) receptor in the initiation of metal-induced signalling events (Samet et al., 2003; Wu et al., 1999; 2002). Investigating phosphorylation and activation of other MAPKs by Western blot analysis and kinase activity assays would ascertain whether other signalling pathways are involved in the pro-inflammatory effects of welding fumes. Over-expression of these kinases using dominant negative constructs, specific inhibitors and/or anti-sense RNA would confirm whether particular signalling pathways leading to NF-κB and/or AP-1 activation, or redundancy of cross-talk between pathways are responsible for the enhanced transcriptional activation induced by welding fumes. Identification of membrane receptors that interact with the soluble fractions of welding fumes would
help delineate the signalling cascades involved in the activation of NF-κB and AP-1 and may provide important information regarding potential therapeutic interventions for welding fume-induced inflammation.

The present study investigated the potential signalling mechanisms involved in the pro-inflammatory effects of welding fumes in acute models of pulmonary inflammation. Although this provided invaluable information regarding the initiation of welding fume-induced inflammation, the apparent tolerance that develops after successive days of fume exposure was not addressed. As a result, the mechanisms involved in welding fume tolerance remain elusive. Attempts to produce a tolerance to welding fumes in cell cultures proved unsuccessful, with cells dying either via welding fume-induced toxicity or by necrosis due to the extended time-course of the studies (data not shown). Assessing the molecular and cellular changes in the BALF and blood of welders throughout their working week and during a period of recovery would represent the ideal model with which to investigate the physiological processes involved in the tolerance towards welding fumes. Alternatively, a rat inhalation study would provide a relevant model with which to study the mechanisms involved in welding fume tolerance. Replicating the working environment of welders by exposing rats to physiologically relevant concentrations of welding fumes in inhalation chambers over the same time periods as for workers would also enable the progressive cellular and molecular events involved in welding fume tolerance to be investigated.

The adverse health effects associated with ambient air pollution particulates are well established with enhanced levels of PM exacerbating pre-existing pulmonary conditions in susceptible individuals (Pope et al., 1995; 1996; MacNee et al., 1999). Diesel exhaust particles (DEP) are a major component of ambient air pollution in urban environments (HEI, 1995; Bonvallot et al., 2001). DEP is known to enhance pulmonary inflammation causing activation of multiple signalling pathways and up-regulating numerous inflammatory mediators (Ohtoshi et al., 1998; Boland et al., 1999; Abe et al., 2000; Hashimoto et al., 2000; Baulig et al., 2003). The size of the particle carbon core (<0.1 μm) provides a large surface area for
adsorption of organic compounds, sulphates and metals (Boland et al., 1999). Although there is much debate regarding the relative importance of the carbonaceous core in DEP-induced inflammation, the results of the present study suggest both the soluble and particulate components are required to induce the maximal pro-inflammatory response (Section 7.6). These data suggest a potential additive effect between the soluble and particulate fractions of DEP. However the adsorption of IL-8 protein onto the surface of the DEP made any synergistic or additive interactions difficult to establish. To investigate this further, measuring the inflammatory response in rats instilled with either the soluble or the particle fractions of DEP (or CB as a surrogate for the carbon core of DEP) and comparing the leukocyte influx to that induced by standard (whole) DEP would determine whether any synergism or additive effects were involved in DEP-induced inflammation.

Due to the high content and potentially carcinogenic nature of the organic components of DEP, much work has focussed on assessing the pathogenicity of these organic compounds (Diaz-Sanchez, 1997; Ma et al., 2002; Takano et al., 2002). The attenuated IL-8 production in alveolar epithelial cells following exposure to DEP devoid of any organic components identified a role for organics in the pro-inflammatory effects of DEP in alveolar epithelial cells (Section 7.8). However, whether the technique used to remove the organic components effectively eliminated all the adsorbed PAHs is unknown. As the DEP used in this study were a standard reference material (SRM 2975) the concentration and types of PAHs present on these particles are known (Appendix I). Liquid chromatography with Mass Spectrometry (LC-MS) over a wide mass range could be used to determine whether or not hydrocarbons were still present on the baked DEP and validate the data presented in this thesis.

Transition metals have been identified as key components initiating the inflammatory response associated with numerous environmental particulates (Dreher et al., 1997; Zhang et al., 1998a; Becher et al., 2001; Schwarze et al., 2002; Donaldson et al., 2003). There is, however, limited evidence to suggest a role for transition metals in the pro-inflammatory effects of DEP. The present studies found
that DEP-associated transition metals do contribute to the pro-inflammatory effects of DEP as shown by the attenuated IL-8 response from alveolar epithelial cells exposed to DEP pre-treated with the transition metal chelator, chelex-100 (Section 7.9). Although removal of either the organic components or soluble transition metals reduced IL-8 production, it was evident from these studies that neither component was solely responsible for the up-regulation of IL-8 in alveolar epithelial cells. To further investigate potential interactions between the organic and metal components, future studies could involve identifying the predominant metals present in DEP to perform co-stimulatory studies using various combinations of organics and metals to try and replicate the inflammatory effects of DEP.

8.2 Summary

Transition metals are an important component of particulate air pollution in both occupational and ambient environments. The data presented in this thesis demonstrated that soluble transition metals present in welding fumes and diesel exhaust particles cause the increased expression of the pro-inflammatory cytokine IL-8 in type II alveolar epithelial cells. Although the relative contribution of soluble transition metals the inflammogenicity of the two particle types differs, a characteristic feature of both welding fumes and DEP is their ability to generate reactive oxygen species which activate oxidant-mediated signalling mechanisms culminating in pro-inflammatory gene expression.
Reference List


Appendices
## Appendix I  Reference Concentrations for Selected PAHs in SRM 2975

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mass Fraction (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Methyphenanthrene</td>
<td>0.89 ± 0.11</td>
</tr>
<tr>
<td>2-Methyphenanthrene</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>3-Methyphenanthrene</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>4- and 9-Methyphenanthrene</td>
<td>0.44 ± 0.09</td>
</tr>
<tr>
<td>1,2-Dimethyphenanthrene</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>1,6-1,7-,2,5-, and 2,9- Dimethyphenanthrene</td>
<td>0.57 ± 0.08</td>
</tr>
<tr>
<td>1,8-Dimethyphenanthrene</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>2,6-Dimethyphenanthrene</td>
<td>0.25 ± 0.05</td>
</tr>
<tr>
<td>2,7-Dimethyphenanthrene</td>
<td>0.23 ± 0.05</td>
</tr>
<tr>
<td>3,6-Dimethyphenanthrene</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td>Anthracene</td>
<td>0.038 ± 0.008</td>
</tr>
<tr>
<td>Benzo[ghi]fluoranthene</td>
<td>10.2 ± 0.5</td>
</tr>
<tr>
<td>8-Methylfluoranthen</td>
<td>0.068 ± 0.004</td>
</tr>
<tr>
<td>1-3-, and 7-Methylfluoranthen</td>
<td>0.53 ± 0.03</td>
</tr>
<tr>
<td>2-Methylpyrene</td>
<td>0.04 ± 0.008</td>
</tr>
<tr>
<td>4-Methylpyrene</td>
<td>0.022 ± 0.005</td>
</tr>
<tr>
<td>Benzo[c]phenanthrene</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>Benzo[a]phenanthrene</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>Benzo[b]phenanthrene</td>
<td>11.5 ± 3.6</td>
</tr>
<tr>
<td>Perylene</td>
<td>0.054 ± 0.009</td>
</tr>
<tr>
<td>Indeno[1,2,3-cd]pyrene</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Indeno[1,2,3-cd]fluoranthen</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Dibenz[a,j]anthracene</td>
<td>0.37 ± 0.07</td>
</tr>
<tr>
<td>Dibenz[a,c]anthracene/Dibenz[a,h]anthracene</td>
<td>0.52 ± 0.08</td>
</tr>
<tr>
<td>Pentaphene</td>
<td>0.038 ± 0.007</td>
</tr>
<tr>
<td>Benzo[b]chrysene</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>Picene</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Coronene</td>
<td>1.1 ± 0.2</td>
</tr>
</tbody>
</table>

---

*Each set of results is expressed as the reference value ± the expanded uncertainty. The expanded uncertainty defines a range that contains the estimate of the true value at a level of confidence of approximately 95%.

b GC/MS (Ia) on 5% phenyl-substituted methylpolysiloxane phase after PFE with toluene:methanol mixture.

c GC/MS (II) on 5% phenyl-substituted methylpolysiloxane phase after PFE with DCM.

d GC/MS (III) on 5% phenyl-substituted methylpolysiloxane phase after Soxhlet extraction with DCM.

e GC/MS (IVa) on 5% phenyl-substituted methylpolysiloxane phase after PFE with DCM.

f GC/MS (IVb) on 50% phenyl-substituted methylpolysiloxane phase of same extracts as GC/MS (IVa).

g LC-FL of isomeric PAH fractions after Soxhlet extraction with DCM.

h GC/MS (IVc) on a smectic liquid crystalline phase of same extracts as GC/MS (IVa).

i GC/MS (Ib) on 50% phenyl-substituted methylpolysiloxane phase of selected extracts from GC/MS (Ia).

National Institute of Standards & Technology Certificate of Analysis (SRM® 2975)
Appendix II  Reagents and Buffers

RT-PCR

*Sterile Rnase-Free Water (DEPC)*
0.2% (w/v) diethyl pyrocarbonate (DEPC) in distilled/deionised water
  - Solution prepared and mixed overnight and then autoclaved to inactivated DEPC.

All reagents for RT-PCR were supplied by Promega, Southampton, UK

<table>
<thead>
<tr>
<th><strong>RT-Buffer</strong></th>
<th><strong>PCR Mix</strong></th>
<th><strong>6 X Loading Dye</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM Tris-HCl</td>
<td>10 mM Tris-HCl (pH 9.0)</td>
<td>10 mM Tris-HCl (pH 7.5)</td>
</tr>
<tr>
<td>20 mM DTT</td>
<td>50 mM KCl</td>
<td>15 % Ficoll® 400</td>
</tr>
<tr>
<td>20 µg ml⁻¹ oligo(dT),</td>
<td>0.1% Triton X-100</td>
<td>0.03 % bromophenol blue</td>
</tr>
<tr>
<td>6 mM MgCl₂</td>
<td>2.5 mM MgCl₂</td>
<td>0.03 % xylene cyanol FF</td>
</tr>
<tr>
<td>1.6 mM dNTPs</td>
<td>200 µM dNTPs</td>
<td>0.4 % orange G</td>
</tr>
<tr>
<td>0.8 µL Rnasin</td>
<td>2 pmol Primer forward</td>
<td></td>
</tr>
<tr>
<td>4 µL M-MLV RT</td>
<td>2 pmol Primer reverse</td>
<td></td>
</tr>
<tr>
<td>150 mM KCl (pH 8.3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*5X TBE (Tris-Borate-EDTA) Buffer (pH 8.3)*
0.45 M Trizma Base
0.45 M Boric Acid
10 mM EDTA

*1.5 % Agarose Gel in 1 X TBE*
1.5 % SeaKem LE Agarose (Biowhittaker Molecular Applications, UK) in 1 X TBE with 0.5 µg ml⁻¹ Ethidium Bromide
  - Mixture (without EtBr) was heated in the microwave for approximately 4 minutes or until agarose was completely dissolved. EtBr was added poured into a tray whilst still molten.

**ELISA**

<table>
<thead>
<tr>
<th><strong>Substrate Buffer</strong></th>
<th><strong>Reagent Diluent</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric acid 3.65 g</td>
<td>1 x TBS</td>
</tr>
<tr>
<td>Na₂HPO₄·7H₂O 8.94 g</td>
<td>0.05 % Tween20</td>
</tr>
<tr>
<td>Dissolve in 400 mls dH₂O, pH to 5.0 and make up to 500 mls.</td>
<td>0.1 % BSA</td>
</tr>
</tbody>
</table>
Glutathione Assay

0.1 M Phosphate Buffer with 5 mM EDTA (pH 7.5) (KPE)

Solution A
6.8 g KH$_2$PO$_4$ in 500 mls dH$_2$O

Solution B
8.5 g KH$_2$PO$_4$ or 11.4 g K$_2$HPO$_4$.3H$_2$O in 500 mls dH$_2$O

0.1 M Phosphate Buffer
16 mls of Solution A + 84 mls Solution B. pH adjusted to 7.5 and 0.327 g EDTA added.

Extraction Buffer – 0.1 % Triton/0.6 % SSA in KPE
20 µl Triton X-100 + 120 mg SSA* in 20 mls in 0.1 M Phosphate Buffer with 5 mM EDTA (pH 7.5) (KPE). Extraction buffer was prepared freshly for every experiment and kept on ice.

* Sulfosalicylic acid inhibits γ-glutamyl transferase activity.

Electrophoretic Mobility Shift Assay (EMSA)

Buffer A | Buffer C | 5x Binding Buffer
---|---|---
10mM HEPES | 50mM HEPES | 20 % Glycerol
10mM KCl | 50mM KCl | 5 mM MgCl$_2$.6H$_2$O
2mM MgCl$_2$ | 300mM NaCl | 2.5 mM EDTA
1mM dithiothreitol (DTT) | 0.1mM EDTA | 2.5 mM DTT
0.1mM EDTA | 1mM DTT, | 250 mM NaCl
0.4mM (PMSF) | 0.4mM PMSF | 50 mM Tris-HCl pH7.5
0.2mM NaF, | 10% glycerol | |
1µgml-1 leupeptin | 0.2mM NaF | |
0.2mM NaVO$_3$, | | |

6 % Non-Denaturing Polyacrylamide Gel
30 % acrylamide/bis-acrylamide solution (29:1) | 10mls
5X Tris-buffered EDTA | 10 mls
ddH$_2$O | 20.46 mls
10 % Ammonium Persulfate | 0.5 mls
TEMED | 0.04 mls

Gels were poured and allowed to polymerise for approximately 30 minutes
SDS-Polyacrylamide Gels

**10 % Separating Gel**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>4X Tris-HCl; pH 8.8</td>
<td>3.75 mls</td>
</tr>
<tr>
<td>Acrylamide/Bis (37.5:1)</td>
<td>3.75 mls</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>6.25 mls</td>
</tr>
<tr>
<td>10 % APS</td>
<td>0.1 mls</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.04 mls</td>
</tr>
</tbody>
</table>

**4 % Stacking Gel**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>4X Tris-HCl; pH 6.8</td>
<td>1.25 mls</td>
</tr>
<tr>
<td>Acrylamide/Bis (37.5:1)</td>
<td>0.65 mls</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>3.05 mls</td>
</tr>
<tr>
<td>10 % APS</td>
<td>0.5 mls</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.02 mls</td>
</tr>
</tbody>
</table>

Separating gels was poured and topped-up with water-saturated isobutanol and left to polymerize. Once set, the isobutanol was removed and the gels washed with ddH₂O and dried with Whatman paper. The 4 % Stacking gels was added on top of the separating gel and the combs inserted and allowed to set for approximately 30 minutes.

**Sample Loading Buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>1 ml</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>0.5 mls</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>2 mls</td>
</tr>
<tr>
<td>1 M Tris-HCl; pH 6.7</td>
<td>1.25 mls</td>
</tr>
<tr>
<td>0.05 % Bromophenol blue</td>
<td>3 mls</td>
</tr>
</tbody>
</table>

**RIPA Buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS-CMF</td>
<td>90 mls</td>
</tr>
<tr>
<td>NP-40</td>
<td>1 ml</td>
</tr>
<tr>
<td>Deoxcholic acid</td>
<td>0.5 g</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

**5X Running Buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trizma Base</td>
<td>15.1 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>72.0 g</td>
</tr>
<tr>
<td>SDS</td>
<td>5.0 g</td>
</tr>
</tbody>
</table>

Made up to 1 litre with ddH₂O

**10X Transfer Buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>144 g</td>
</tr>
<tr>
<td>Trizma Base</td>
<td>30 g</td>
</tr>
</tbody>
</table>

Make up to 1 litre with ddH₂O

**10X TBS**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trizma Base</td>
<td>21.2 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>30 g</td>
</tr>
</tbody>
</table>

Dissolve in 800 mls ddH₂O pH to 7.4 with HCl. Make up to 1 litre with ddH₂O.

**Strip Buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 % SDS</td>
<td>100 mls</td>
</tr>
<tr>
<td>Trizma Base</td>
<td>3.78 g</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>400 mls</td>
</tr>
</tbody>
</table>

pH to 6.7 with HCl

To strip blots add 400 µl β-mercaptoethanol to 50 mls strip buffer per blot. Heat at 60 °C for 45 minutes. Wash thoroughly (3 x 5 minutes) in 1 X TBS/0.05 % Tween-20 prior to blocking.
**MIP-2 ELISA**

**Coating Buffer R**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO₃</td>
<td>4.3 g</td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>5.3 g</td>
</tr>
</tbody>
</table>

Make up to 1 litre with distilled H₂O, pH 9.4

**Blocking Buffer**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.0 g</td>
</tr>
<tr>
<td>Na₂HPO₄·6H₂O (Na₂HPO₄)</td>
<td>1.42 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.2 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2 g</td>
</tr>
<tr>
<td>BSA (fraction V)</td>
<td>5.0 g</td>
</tr>
</tbody>
</table>

Make up to 1 litre with dH₂O, pH 7.4

**Standard Diluent/Assay Buffer**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.0 g</td>
</tr>
<tr>
<td>Na₂HPO₄·6H₂O (Na₂HPO₄)</td>
<td>1.42 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.2 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2 g</td>
</tr>
<tr>
<td>BSA (fraction V)</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Tween -20</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

Make up to 1 litre with dH₂O, pH 7.4
The data regarding the particle sizes of welding fume NIMROD c276 was obtained from the Institute of Occupational Medicine as part of a report into the London Underground (IOM, London Underground Report, 2003). Welding fumes were dispersed onto Nuclepore polycarbonate filters, which were then coated with several layers of carbon. Portions of the carbon-coated filters were then excised and mounted on 200 square mesh, 3.05mm copper Transmission Electron Microscope (TEM) grids. The copper grids were placed in a Jaffe washer, filled with chloroform and left overnight. The chloroform digested the polycarbonate filters and left the particles suspended in the carbon. Each copper grid was placed in the TEM and examined at low magnification for uniformity of deposit. The particle analysis was carried out using a Link AN10000 semi-automatic system with the TEM set at 5000X magnification on a slow scan speed. For each sample, approximately 1000 particles were analysed (each mean diameter being the result of ten individual measurements per particle). This approach measures actual rather than aerodynamic diameter, and will therefore underestimate the aerodynamic diameter of dense, e.g. iron-containing, particles. The work was carried out by Steve Clark at the IOM, Research Park North, Riccarton, Edinburgh.
Appendix IV  IL-8 Gene Expression in Alveolar Epithelial Cells

A549 cells were treated with the washed particulate fraction of welding fumes (63 μg ml⁻¹) for 6 hr, RNA was isolated and IL-8 mRNA quantified by RT-PCR. The figure shows a representative PCR gel for IL-8 and GAPDH. C: control; TNF: TNF-α; 1: NIMROD 182; 2: NIMROD c276; 3: COBSTEL 6.

Appendix V  IL-8 Standard Curve

Representative standard curve for R&D IL-8 ELISA. Concentrations of IL-8 range from 0-1000 pg/ml.
A549 cells were treated with whole, soluble or washed welding fume particles (63 µg ml⁻¹) for 24 hr and the supernatant was analysed for IL-10 protein using an R&D ELISA kit. The values are expressed as percent control (untreated cells 0 µg ml⁻¹). The histogram represents the mean of three separate experiments performed in triplicate and the bars shown ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared to control.
Publication
Soluble transition metals cause the pro-inflammatory effects of welding fumes in vitro

Jane D. McNeilly, a Mathew R. Heal, b Iain J. Beverland, c Alan Howe, d Mark D. Gibson, c Leon R. Hibbs, b William MacNee, a and Ken Donaldson a, * 

a Respiratory Medicine Unit, ELEGI/Colt Laboratories, University of Edinburgh, Edinburgh EH9 9AG, UK 
b Department of Chemistry, University of Edinburgh, UK 
c Department of Civil Engineering, University of Strathclyde, UK 
d Health and Safety Laboratory, Sheffield, UK 

Received 9 September 2003; accepted 24 November 2003

Abstract

Epidemiological studies have consistently reported a higher incidence of respiratory illnesses such as bronchitis, metal fume fever (MFF), and chronic pneumonitis among welders exposed to high concentrations of metal-enriched welding fumes. Here, we studied the molecular toxicology of three different metal-rich welding fumes: NIMROD 182, NIMROD c276, and COBSTEM 6. Fume toxicity in vitro was determined by exposing human type II alveolar epithelial cell line (A549) to whole welding fume, a soluble extract of fume or the “washed” particulate. All whole fumes were significantly toxic to A549 cells at doses >63 μg ml⁻¹ (TD 50; 50, 25, and 12 μg ml⁻¹, respectively). NIMROD c276 and COBSTEM 6 fumes increased levels of IL-8 mRNA and protein at 6 h and protein at 24 h, as did the soluble fraction alone, whereas metal chelation of the soluble fraction using chelax beads attenuated the effect. The soluble fraction of all three fumes caused a rapid depletion in intracellular glutathione following 2-h exposure with a rebound increase by 24 h. In addition, both nickel based fumes, NIMROD 182 and NIMROD c276, induced significant reactive oxygen species (ROS) production in A549 cells after 2 h as determined by DCFH fluorescence. ICP analysis confirmed that transition metal concentrations were similar in the whole and soluble fractions of each fume (dominated by Cr), but significantly less in both the washed particles and chelated fractions. These results support the hypothesis that the enhanced pro-inflammatory responses of welding fume particulates are mediated by soluble transition metal components via an oxidative stress mechanism.

© 2004 Elsevier Inc. All rights reserved.

Keywords: Welding fumes; Transition metals; Inflammation; Oxidative stress

Introduction

At present, it is estimated that more than one million workers are employed as welders worldwide (Sundin, 1998), with more than three million performing welding intermittently as part of their work duties (Sferlazza and Beckett, 1991). Manual metal arc welding (MMAW) and flux core arc welding (FCAW) are two commonly used procedures in the construction industry and in numerous other industrial processes. Welding involves the fusion of metals by high temperature generated via an electrical arc resulting in the formation of metal-enriched fumes. Welding fumes are a complex mixture of gases and small particulates of metal oxides formed by the vaporization and oxidation of metal during the welding process (Lockey et al., 1988; Yu et al., 2000). The nature of respirable fumes depends upon the type of welding and the composition of electrode, filler wire, and fluxes (Antonini et al., 1996; Lockey et al., 1988; Sferlazza and Beckett, 1991; Yu et al., 2000).

Numerous studies have reported welding fume particles to be well within the respirable size range <1 μm (reviewed by Antonini et al., 1998; Lockey et al., 1988; Yu et al., 2000). Thus, upon inhalation, particle deposition occurs in the lower respiratory tract, including the terminal bronchioles and alveoli, beyond the mucusciliary escalator (Sferlazza and Beckett, 1991; Yu et al., 2000).
The health of welders has been studied extensively (Doig and Duguid, 1951; Sferlazza and Beckett, 1991). A number of epidemiological studies have reported a higher incidence of respiratory illness such as bronchitis, airway irritation, metal fume fever (MFF), chemical pneumonitis, and also changes in lung function in welders (Antonini et al., 1996; Sferlazza and Beckett, 1991). Furthermore, the severity, duration, and frequency of acute upper and lower respiratory tract infections have been shown to be greater among welders compared to the general population (Howden et al., 1988).

The most frequently described respiratory illness among welders is metal fume fever (MFF), which is an acute, self-limiting, systemic, febrile illness caused by inhalation of high concentrations of metal oxides, primarily zinc oxide (Blanc et al., 1993; Sferlazza and Beckett, 1991). Symptoms include high fever and sweating, throat irritation, chest tightness, dry cough, and general malaise associated with a pulmonary inflammatory cellular response (Kuschner et al., 1995; Lockey et al., 1988), peaking 5–12 h postexposure and resolving within 24–48 h (Antonini et al., 1996; Sferlazza and Beckett, 1991). Although the pathogenesis of MFF is poorly understood, allergic and immunological mechanisms are most frequently postulated (Graeme and Pollack, 1998). Cytokine networking mediated by the release of pro-inflammatory cytokines TNF-α and IL-8 by pulmonary macrophage causes both local pulmonary inflammatory cellular response and systemic response (Blanc et al., 1993; Gordon, 1991; Kuschner et al., 1995). This theory is consistent with evidence showing that tolerance to metal fumes develops and symptoms are not observed on successive days of fume exposure (Blount, 1990; Graeme and Pollack, 1998; Nemery, 1990).

Reports of a dose-dependent increase in pro-inflammatory cytokines and neutrophilia in the human bronchoalveolar lavage (BAL) 20–22 h following zinc oxide inhalation provide further evidence supporting the role of a cytokine-mediated mechanism causing the symptoms of MFF (Blanc et al., 1993; Kuschner et al., 1995). Furthermore, TNF-α levels were significantly greater than 3 h postexposure, suggesting an initial role for TNF-α in the pathophysiology of MFF (Blanc et al., 1993; Kuschner et al., 1997). Similar findings were reported in rats following intratracheal installation of stainless steel welding fumes with increased levels of TNF-α and IL-β in BALF (Antonini et al., 1996). These cytokines, produced predominantly by alveolar macrophages, are involved in numerous inflammatory processes such as neutrophil recruitment and increased oxygen radical production (Driscoll et al., 1990, 1991; Goldring and Krane, 1986; Schmidt et al., 1982; Tsujimoto et al., 1986). These findings provide further evidence supporting the role of cytokines in the inflammatory response associated with exposure to certain environmental and occupational particles (Antonini et al., 1996).

The importance of metal content, metal bioavailability, and interactions among transition metals in mediating pulmonary inflammation and injury following exposure to many different particles has been demonstrated. Such particles include residual oil fly ash (ROFA), stone quarry particles (Hetland et al., 2001), Provo PM10 (Costa and Dreher, 1997), and ambient airborne particles (Costa and Dreher, 1997; Dreher et al., 1997; Gilmour et al., 1996). Furthermore, the pathogenicity associated with exposure to crocidolite-asbestos has been linked to the high iron content on fiber surfaces (Jiménez et al., 2000; Weitzman and Weitberg, 1985).

Mechanistic studies report that particle-associated transition metals can undergo redox cycling resulting in the production and release of reactive oxygen species (ROS). These reactive compounds can deplete antioxidants, cause cellular damage, lung injury, and inflammation (Carter et al., 1997; Dreher et al., 1997; Jiménez et al., 2000; Stohs and Bagchi, 1995; Toyokuni, 1996).

The aims of this study were to investigate the molecular toxicology of three compositionally different welding fumes by comparing their potential to activate lung epithelial cells to release the pro-inflammatory cytokine IL-8. We hypothesized that transition metals present in or on welding fume particles cause pulmonary inflammation and lung injury observed in welders. In addition, we postulated that the soluble metal components were the main contributing factors.

To investigate this we initially performed experiments to determine the toxicity of three different welding fumes on alveolar epithelial cells. To examine the possible mechanisms by which nontoxic concentrations of welding fume particles may cause lung inflammation, the ability of fumes to cause expression of the pro-inflammatory cytokine IL-8 in epithelial cells was assessed. A recent study by Antonini et al., 1999 identified the soluble components of fume from stainless steel MMAW to be most cytotoxic and have the greatest effect on macrophage function. Similar studies using other environmental particulates have shown that the soluble metal fraction plays an important role in the induction of lung injury (Dreher et al., 1997; Adamson et al., 1999). Therefore, to determine which components of welding fume particles were responsible for the pro-inflammatory response, alveolar epithelial cells were exposed to soluble, insoluble, or whole fume components at subtoxic concentrations. In addition, we investigated whether welding fumes exerted some of their effects via oxidative stress by assessing the production of intracellular ROS and levels of the important antioxidant glutathione.

Materials and methods

Materials. All reagents were obtained from Sigma-Aldrich, UK unless otherwise stated.

Cell culture. The type II human alveolar-like epithelial cell line A549 (European Collection for Animal Cell Culture)
was maintained in continuous culture in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamate, and 100 IU ml penicillin⁻¹-100 μg ml streptomycin⁻¹.

Cells were grown to confluency at 37 °C in a humidified atmosphere containing 5% CO₂, washed with Ca²⁺/Mg²⁺-free phosphate-buffered saline (PBS-CMF), harvested with trypsin-EDTA, and passaged.

The cells were seeded at a density of 0.2 × 10⁶, 1.1 × 10⁶, or 9.0 × 10⁶ ml⁻¹ in 6-, 24-, or 96-well culture plates, respectively, and grown until approximately 80% confluent. The resultant monolayers were washed with PBS-CMF and grown to confluency in media containing 2% FBS.

Welding fume particles. Samples of welding fume particles produced by two nickel-based stainless steel welding consumables (NIMROD 182, and NIMROD c276) and one cobalt-based stainless steel welding consumable (COBSTEL 6) were obtained from Health and Safety Laboratory, Sheffield, UK. These samples were generated under controlled conditions at The Welding Institute (TWI), Great Abington, Cambridge, UK, by carrying out mechanical welding inside a fume box and collecting the fume on paper filters. The resulting material was removed from the filters by brushing and stored in airtight glass containers until required.

Welding fume characterization. Welding fume particles were characterized as described by Brown et al. (2001). Briefly, fume particles were suspended at a stock concentration of 1 mg ml⁻¹ in deionized water. Five microliters of each suspension was applied to the surface of 200-mesh size carbon-coated electron microscope (EM) grids (Agar Scientific). Grids were placed on filter paper and dried at room temperature and subsequently examined by transmission electron microscopy (TEM).

Treatment of A549 cells with welding fumes. Welding fume particles were prepared at a stock concentration of 1 mg ml⁻¹ in media containing 2% FBS (2% DMEM), sonicated for 10 min, and vortexed briefly to ensure complete dispersion. For all experiments after the initial dose-response assessments, monolayers were treated with concentrations ranging from 2 to 63 μg ml⁻¹. Cells were exposed to either whole particle suspensions, soluble fraction, or washed particles only at a concentration of 63 μg ml⁻¹. Nontoxic concentrations of TNF-α (10 ng ml⁻¹) (R&D systems, Abingdon UK) and carbon black (Huber 900) (100 μg/ml) (Degussa, Frankfurt, Germany) were used as positive control and an inert particle, respectively.

Soluble components were isolated from whole particle suspensions at the relevant concentration by centrifugation at 13,000 rpm (5 min) to pellet any particulates. The resultant supernatant was transferred into a clean Eppendorf tube and the procedure was repeated four times to ensure that any particulate contaminant was removed before treatment. The concentration of the soluble fraction was taken to be the concentration of the soluble components derived from the whole welding fume particle suspension at the indicated concentration.

To determine whether insoluble fume particulates had any effect on A549 cells, particulates were pelleted by centrifugation and repeatedly washed and centrifuged in PBS-CMF. The resultant particles were resuspended in 2% DMEM and applied to monolayers for the time indicated.

Chelation of welding fumes. Whole particle suspensions and soluble fractions of welding fume particles (63 μg ml⁻¹) were suspended in 2% DMEM containing 50 mg ml⁻¹ chelax beads and mixed on a rotating wheel for 4 h at room temperature. After incubation, samples were centrifuged at 13,000 rpm (5 min) to pellet the chelax beads. The resultant suspensions were applied to monolayers and incubated at 37 °C for 24 h.

Cytotoxicity. Cytotoxicity was assessed by lactate dehydrogenase (LDH) release. Cells seeded in 96-well flat bottomed culture plates were grown to confluency in media containing 2% FBS and exposed to a range of fume particle concentrations (1–250 μg ml⁻¹) for the various times indicated. LDH release was measured according to manufacturers instructions (Roche Molecular Biochemicals) using pyruvic acid as a substrate. LDH activity was determined spectrophotometrically at 490 nm and expressed as percentage of total cellular LDH, which was measured in the cell lysates obtained by trypsin treatment with 0.1% Triton X-100.

Assessment of metabolic activity. The ability of A549 cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was used as an indicator of the metabolic competence of the cells and was performed as described by Mosmann (1983) with minor modifications. Cells were seeded at a density of 0.1 × 10⁶ cells ml⁻¹ in 24-well flat-bottomed plates and grown until approximately 80% confluency in media containing 10% FBS. Cells were washed with PBS-CMF and grown until confluent in 2% DMEM. Monolayers were treated with particle suspensions in concentrations of 1–250 μg ml⁻¹ for 4–48 h. Four hours before harvest, 100 μl MTT (0.5 mg ml⁻¹) was added and incubated for a further 4 h. Following incubation, isopropanol/0.1 M HCl (500 μl) was added to each well, mixed thoroughly, and centrifuged at 2000 rpm for 5 min to sediment any particles. Supernatants (200 μl) were transferred in triplicate to a flat-bottomed 96-well plate and the absorbance was determined spectrophotometrically on a microplate reader at 570 nm (reference wavelength: 630 nm). MTT reduction for each treatment was expressed as a percentage of control values.

IL-8 mRNA by RT-PCR. Following treatment, cells were washed with PBS-CMF and total cellular RNA was isolated using Trizol Reagent (GIBCO-BRL) according to manufacturers instructions and dissolved in 50 μl diethylpyrocarbonate (DEPC)-treated water. RNA (2 μg) was reverse-
transcribed using M-MLV RT (Promega) and the resultant cDNA was stored at −20 °C until required.

PCR was performed for the genes IL-8 and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using 5 and 3 µl cDNA, respectively. Oligonucleotide primers used for PCR reactions were obtained from (MWG-Biotech AG).

 Primer sequences were IL-8: sense 5'-ATT GAG AGT GGA CCA CAC TGC GCC-3', antisense 5'-CAC TGA TTC TTG GAT ACC ACA GAG-3'; GAPDH: sense 5'-CCA CCA TGG CAA ATT CCA TGG CA-3', antisense 5'-TCT AGA CGG CAG GTC AGG TCC ACC-3'.

 Conditions for PCR were IL-8: 31 thermal cycles of denaturation (94 °C for 1 min), annealing (60 °C for 1 min), extension (72 °C for 1 min), final extension for 5 min at 72 °C; GAPDH: 20 cycles of denaturation (72 °C for 90 s), annealing (94 °C for 30 s), extension (60 °C for 45 s), final extension for 10 min at 72 °C.

 The amplification products were electrophoresed through a 1.5% agarose gel containing ethidium bromide. The resultant bands were scanned and visualized using a UVP High Performance Ultraviolet Transilluminator and GRAB-IT software version 2.5. The intensity of the IL-8 (173 bp) and GAPDH bands (600 bp) was determined by densitometry using GelBase/GelBlot software and expressed as a ratio of the band intensity of IL-8/GAPDH.

 Enzyme-linked immunosorbent assay for IL-8. The IL-8 protein was determined using monoclonal and biotinylated antihuman IL-8 antibodies obtained from NIBSC (Potters-Bar, Herfordshire, UK) for initial dose-course experiments or R&D Systems (Oxon, UK) for all subsequent ELISA. Briefly, 96-well microplates (EIA/RIA, Costar, Cambridge) were coated with monoclonal IL-8 antibody (2 µg ml⁻¹, NIBSC; or 4 µg ml⁻¹, R&D). Cytokine levels of test supernatants were assessed according to manufacturer instructions utilizing a standard quantitative immunometric "sandwich" enzyme technique, with values being determined using a recombiant protein standard curve ranging from 5 to 800 pg ml⁻¹ IL-8. All samples were analyzed in triplicate and IL-8 protein levels were expressed as a percentage of the control.

 Total glutathione reduced (GSH) + oxidized (GSGG). Following treatment with either whole particle supernatant or soluble fractions only, for the time intervals indicated, cells were washed with PBS-CMF, harvested with trypsin-EDTA, and rewashed. Total intracellular glutathione was measured by the DTNB/GSSG reductase recycling method described by Tietze (1969) with slight modifications. Total GSH concentration was determined using linear regression of a GSH standard curve. The values were expressed as the amount of GSH in µmol per mg of protein as a percentage of the control.

 Intracellular reactive oxygen species. The level of intracellular ROS was determined by the change in fluorescence resulting from the oxidation of the fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich). Cells exposed to either whole particle suspensions or soluble fractions only were incubated with 40 µM of the fluorescent probe DCFH-DA for 30 min at 37 °C. Following incubation, cells were washed twice with PBS-CMF, harvested by trypsinization (0.25% Trypsin-EDTA), rewashed, and resuspended in PBS-CMF. The degree of fluorescence, corresponding to intracellular ROS, was determined using FACs Calibur flow cytometry (excitation λ 488 nm; emission 530 nm). The proportion of fluorescence cells was determined using Cell Quest software and expressed as a percentage control geo-mean fluorescence.

 Metal analysis. Samples of whole particle suspension, washed particle suspension, soluble fraction, and chelated soluble fraction were prepared, as above, from an initial welding fume concentration of 63 µg ml⁻¹, as applied in the biological assays. One milliliter of each sample was diluted to 5 ml in ultrapure water and the aqueous fraction analyzed by ICP-MS (VG Elemental PlasmaQuad 3) for the transition metals Co, Cr, Cu, Fe, Mn, Ni, Ti, V, and Zn selected for their potential to take part in redox cycling. Sample concentrations were quantified against 11-point calibration graphs constructed using a multi-element ICP standard.

 Statistical analysis. Individual experiments were conducted in triplicate unless otherwise stated. Data are expressed as means ± SEM (n = 3) and were analyzed on MINITAB version 10.5. Statistical significance was determined using one-way analysis of variance (ANOVA) with post hoc Tukey's pairwise comparisons. Significance is reported at *P < 0.05, **P < 0.01, ***P < 0.001 compared to control; $P < 0.05, $$$P < 0.01, $$$$P < 0.001 NIMROD 182 vs. NIMROD c275 or #P < 0.05, ##P < 0.01, ###P < 0.001 NIMROD 182 vs. COBSTEL 6.

 Results

 Welding fume characterization

 TEM images of welding fume particles are shown in Fig. 1. All welding fumes were heterogeneous in nature containing a high proportion of ultrafine (uf) particles with some larger particles. Images of carbon black and ultrafine carbon black (Figs. 1D and E) are shown for comparison.

 Cytotoxicity assessments

 To establish the potential toxicity of the three welding fumes NIMROD 182, NIMROD c276, and COBSTEL 6, alveolar epithelial cells were incubated with welding fumes (1–250 µg ml⁻¹) for 24 h and LDH release was measured in cell lysates. At concentrations greater than 63
Fig. 1. Transition electron micrographs showing particles. (A) NIMROD 182. (B) NIMROD c276. (C) COBSTEL 6. (D) Carbon Black. (E) Ultrafine Carbon Black. Magnification: ×35000.

1g ml⁻¹, all fumes caused significant toxicity (P < 0.005) (Fig. 2). Furthermore, following treatment, all three welding fumes caused a significant dose and time-dependent decrease in the metabolic activity of alveolar epithelial cells as measured by the reduction of MTT (Fig. 3). There was also a clear time-dependent depletion in MTT (data not shown).

NIMROD 182 was found to be less toxic than either NIMROD c276 or COBSTEL 6 when compared to control values at lower concentrations. However, at the higher doses tested (63, 125, 250 µg ml⁻¹), there was no significant difference between fumes. As the experimental aim was to investigate the molecular mechanisms of the pro-inflammatory effects of welding fumes rather than their direct toxicity, all subsequent experiments were performed using...

Fig. 2. Welding fume particles cause increased lactate dehydrogenase (LDH) release in alveolar epithelial cells. LDH release is expressed as percentage of total cellular LDH measured in the cell lysates obtained by treatment with 0.1% Triton X-100 following treatment with welding fumes (1–250 µg ml⁻¹) for 24 h. The graph represents the mean of three experiments conducted in triplicate and the bars represent ± SEM *P < 0.05, **P < 0.005, ***P < 0.001 compared to 100% cytotoxicity from 0.1% Triton X-100.

Fig. 3. Welding fume particles decrease the metabolic activity of alveolar epithelial cells. Metabolic activity was determined by the reduction of MTT following treatment with welding fumes (1–250 µg ml⁻¹) for 24 h and is expressed as percent of the control value (0 µg ml⁻¹). The graph represents the mean of four experiments conducted in triplicate. The bars represent ± SEM *P < 0.05, **P < 0.005, ***P < 0.001 compared to control.
Table 1

A. Toxic dose of welding fumes determined by LDH release

<table>
<thead>
<tr>
<th>Sample</th>
<th>TD 10</th>
<th>TD 25</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIMROD 182</td>
<td>37.5</td>
<td>117.5</td>
</tr>
<tr>
<td>NIMROD c276</td>
<td>35</td>
<td>70</td>
</tr>
<tr>
<td>COBSTEL 6</td>
<td>42.5</td>
<td>108.75</td>
</tr>
</tbody>
</table>

B. Toxic dose determined by reduction of metabolic activity of epithelial cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>TD 25</th>
<th>TD 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIMROD 182</td>
<td>11.2</td>
<td>42</td>
</tr>
<tr>
<td>NIMROD c276</td>
<td>6.25</td>
<td>25</td>
</tr>
<tr>
<td>COBSTEL 6</td>
<td>4</td>
<td>11.875</td>
</tr>
</tbody>
</table>

nontoxic concentrations (2–63 μg ml⁻¹). Acellular experiments showed no interference with either assays from welding fume particles alone (data not shown).

The toxic dose causing 10%, 25%, or 50% toxicity was calculated. TDX of each welding fume was calculated using results obtained from the above assays (Tables 1A and B).

**IL-8 protein production**

Enzyme-linked immunosorbance assay (ELISA) was used to assess IL-8 protein production from A549 cells exposed to welding fumes. A549 cells were treated with welding fumes at concentrations between 2 and 63 μg ml⁻¹ for 6 and 24 h. At higher concentrations (31 and 63 μg ml⁻¹), both NIMROD c276 and COBSTEL 6 caused a significant increase in IL-8 production at both time points (P < 0.01, P < 0.001) (Figs. 4A and B). Following 6-h exposure, NIMROD c276 induced the highest cytokine response in A549 cells. At both time points, NIMROD 182 was the least potent fume for IL-8 release. Following 6-h exposure to NIMROD 182 (63 μg ml⁻¹), a 4-fold increase in IL-8 release was measured in comparison to the 13-fold and 8-fold increase induced by NIMROD c276 and COBSTEL 6, respectively (Fig. 4A).

We hypothesized that the soluble metal components of welding fumes play a key role in the pro-inflammatory effects. To address this issue, alveolar epithelial cells were treated with either the soluble components or washed...
Table 2: Time course of release of soluble fractions of welding fume particles

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>0</th>
<th>0.16</th>
<th>0.5</th>
<th>1</th>
<th>4</th>
<th>8</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMROD 182</td>
<td>50.24 ± 5.2</td>
<td>258.44 ± 15.9***</td>
<td>221.67 ± 5.4***</td>
<td>191.80 ± 10.3***</td>
<td>216.62 ± 15.9***</td>
<td>227.65 ± 23.1***</td>
<td>210.64 ± 12.5***</td>
</tr>
<tr>
<td>IMROD c276</td>
<td>50.24 ± 5.2</td>
<td>291.53 ± 7.4***</td>
<td>265.34 ± 6.5***</td>
<td>285.56 ± 7.1***</td>
<td>310.84 ± 4.9***</td>
<td>294.75 ± 7.1***</td>
<td>337.95 ± 5.6***</td>
</tr>
<tr>
<td>OBSTEL 6</td>
<td>50.24 ± 5.2</td>
<td>231.78 ± 6.1***</td>
<td>188.12 ± 14.0***</td>
<td>215.24 ± 12.1***</td>
<td>212.48 ± 20.1***</td>
<td>195.02 ± 8.3***</td>
<td>200.07 ± 6.7***</td>
</tr>
</tbody>
</table>

Welding fume particles (63 μg ml⁻¹) were agitated over a 24-h period and supernatant was collected after 0.16, 0.5, 1, 2, 4, 8, and 24 h. Soluble fractions were isolated as explained in Materials and methods. A549 cells were then treated with the soluble fractions for 24 h and the supernatant was analyzed for IL-protein. The values are expressed as picograms per ml ± SEM.

**P < 0.001 compared to control (0 pg/ml) at 0 h.

Articles and IL-8 release was measured following 24-h exposure. The soluble fraction from all three fumes enhanced IL-8 expression at the highest concentrations (31 and 63 μg ml⁻¹). The levels of IL-8 measured were equivalent to those observed following exposure to whole fume suspensions (Figs. 4C and B). No increase in IL-8 production was shown after 24-h treatment with washed fume particles (Fig. 4D). These findings suggest that the soluble components are responsible for all of the proinflammatory effects of welding fumes.

To investigate the time-scale over which soluble components exert their inflammosenic effects, the particles were suspended in supernatant over a 24-h period. At various time points, the supernatant was collected, centrifuged to remove any particles, and alveolar epithelial cells were treated with them for 24 h and IL-8 release was measured. Supernatant obtained following 10-min incubation with all three fumes significantly enhanced IL-8 expression in A549 cells (Table 2). At all other time points, IL-8 production was similar to that measured at the initial time point.

Figure 5. Effect of welding fume particles on IL-8 gene expression. A549 cells were treated with welding fume particles 2–63 μg ml⁻¹ for 6 h, RNA was isolated, and IL-8 mRNA was quantified by RT-PCR. (A) Representative PCR gel for IL-8 and GAPDH for dose-response of A549 cells treated with welding fumes. (B) Representative PCR gel for IL-8 and GAPDH for cells exposed to either whole or soluble fraction of welding fumes. (C) Histogram represents n = 4 conducted on pooled triplicate samples ± SEM. Values expressed as the ratio of IL-8 mRNA/GAPDH expressed as percentage control (0 μg ml⁻¹).
showing that all of this soluble activity was released in the first few minutes.

**IL-8 gene expression**

RT-PCR analysis was used to evaluate changes in steady-state gene expression of IL-8 in A549 cells exposed to either whole welding fumes or soluble fraction alone (63 µg ml\(^{-1}\)) for 6 or 24 h. All three fumes significantly increased IL-8 mRNA at the 6 h time point (Fig. 5C). More importantly, there was no difference between the whole particle suspensions; that is, particles plus any soluble components, compared to the soluble fraction alone. By 24 h, mRNA expression had returned to basal levels (data not shown), suggesting that welding fumes elicited their inflammogenic response within the first few hours of exposure.

**Chelation of soluble welding fumes**

To assess the contribution of soluble transition metal components in inducing cytokine release, welding fumes were treated with the transition metal chelator, chelex beads (50 mg ml\(^{-1}\)) before addition to epithelial cells. We expected that transition metals released or mobilized from the fumes would be chelated by the beads and removed from the solution following centrifugation and removal of the beads. A significant reduction in IL-8 protein release was observed following treatment of supernatants of all three welding fumes with chelex beads when compared to untreated supernatant (Fig. 6). These findings provide further evidence for the role of soluble transition metals in the induction of the pro-inflammatory effects of welding fumes.

**Intracellular glutathione concentrations**

To investigate the involvement of ROS in the inflammatory and toxic responses following particle exposure, levels of the intracellular antioxidant glutathione were measured as an indicator of oxidative stress. Treatment of alveolar epithelial cells with all three welding fumes (63 µg ml\(^{-1}\)) for 2 h caused a significant depletion in intracellular glutathione concentrations (\(P < 0.05\)) (Fig. 7). However,
Welding fume particles induce intracellular ROS production in alveolar epithelial cells. A549 cells were treated with either the whole welding fume particles (w) or soluble fractions (s) for 2 h and then incubated with the probe DCFH-DA and levels of intracellular ROS were determined by flow cytometry. Values are expressed as percent control (0 pg ml⁻¹) geometric mean fluorescence. Each graph represents the mean of six experiments conducted in triplicate and the bars represent the SEM, *P < 0.05, **P < 0.01 compared to control. #P < 0.05, NIMROD 182 (w) vs. COBSTEL 6 (w). §P < 0.05, NIMROD 182 (s) vs. COBSTEL 6 (s). §§P < 0.01 NIMROD 182 (s) vs. COBSTEL 6 (s).

When exposure was extended over a 24-h period, glutathione concentrations increased above control levels, suggesting a rebound effect.

To examine the mechanisms by which soluble components exert their effects, we compared total glutathione concentrations following 2-h exposure to either whole particle suspension or soluble components. Both whole particle suspensions and soluble components of each fume caused a similar degree of depletion in total glutathione compared to control (Fig. 8). These data further confirm that the soluble components exert their effect via oxidative stress.

**Intracellular ROS levels**

To further examine the possible mechanism by which welding fumes may cause an inflammatory response, we measured intracellular ROS levels in alveolar epithelial cells using the fluorescent probe DCFH-DA and flow cytometry. Both the soluble fraction and whole particle suspension of the nickel-based fumes, NIMROD 182 and NIMROD c276, caused a significant increase in intracellular ROS in epithelial cells following 2-h exposure (P < 0.05, P < 0.005) (Fig. 9). As both the soluble fraction and whole particle suspension caused a similar degree of oxidative stress, this further supports our hypothesis that the soluble components of welding fumes play a fundamental role in initiating the pro-inflammatory response.

However, the cobalt-based fumes, COBSTEL 6, appeared to have no effect on the oxidative status of epithelial cells. This result is somewhat surprising as COBSTEL 6 was shown to significantly deplete glutathione at the same time point (Fig. 8), which would suggest a role for ROS.
Metals analysis

The total concentration of all measured metals present in solution in each of the whole and washed particle samples, and soluble chelox-treated fractions is shown in Fig. 10 for each welding fume. Fig. 10 clearly shows that the concentrations of metals were similar in the whole particle and soluble fractions, and were considerably greater than the concentration of metals in the washed particle fraction. The washed particle fraction is synonymous with the particle core and is shown to differ with different welding fume particles (Fig. 10).

The chelox-treated samples also have low concentrations of transition metals. This pattern mimics very closely the observations of the inflammation in the bioassays and provides strong evidence that the soluble transition metal content of the welding fumes mediates the inflammatory responses.

For all three fumes, the soluble metal content was dominated by Cr (>80%) (of the metals measured). The COBSTEL 6 fume also contained significant amounts of soluble Co. The remaining soluble metal content was composed principally of Mn, Ni, and Fe, with some Ti and V in the NIMROD c276 fumes (Fig. 10).

Discussion

The purpose of this study was to investigate the specific role of particle-associated transition metals in welding fumes in initiating pro-inflammatory effects in alveolar epithelial cells in vitro, to determine the importance of soluble metal in the welding fumes' ability to cause inflammation.

All of the fumes were cytotoxic at high doses (>63 μg ml⁻¹); however, following inhalation exposure, the deposited mass is likely to be very low due to effective clearance mechanisms such as the mucociliary escalator and alveolar macrophages, consequently, cell stimulation is the most probable mechanism. Therefore, to investigate the role of cell stimulation, we only utilized nonlethal concentrations for studies to stimulate IL-8 release.

We demonstrated that exposure of alveolar epithelial cells to the welding fumes, NIMROD c276 and COBSTEL 6, induced a significant increase in the production of IL-8 with a more pronounced effect at 6 than 24 h. However, NIMROD 182 was the least potent fume, inducing only a 4-fold increase above control at 6 h compared to 10-fold and 8-fold increases following exposure to NIMROD c276 and COBSTEL 6, respectively. We suggested that the difference in welding fume particle potency may be due to the lower total metal concentration present in this fume compared to both NIMROD c276 and COBSTEL 6. Previous studies have shown that different fluxes and shielding gases used during the welding process may affect the oxidation state of the fumes generated (Eagar et al., 1997). Consequently, the pulmonary responses of welding fumes vary according to the materials and processes used (Antonini et al., 1996). As the welding fumes examined in this study were obtained from compositionally different fluxes, and different welding processes were employed, the composition of the resultant fumes varied slightly.

It is widely accepted that transition metals such as iron, copper, and chromium undergo redox cycling, while nickel and cadmium cause the production of ROS and consequent GSH depletion (Stohs and Bagchi, 1995). Recent studies have demonstrated that the pulmonary toxicity of complex metal-containing particulates can be associated with the soluble forms of transition metals and the dose (Adamson et al., 2000; Carter et al., 1997; Dreher et al., 1997; Hetland et al., 2001; Kennedy et al., 1998; Kodavanti et al., 1997). Indeed, particles containing easily solubilized metals appear to cause a more rapid onset and severity of acute pulmonary injury (Dreher et al., 1997). Furthermore, a study by Antonini et al. (1998) demonstrated that the solubility of welding fumes influenced the viability and ROS production in lung macrophages in vitro. There is also considerable evidence supporting the role of ultrafine (uf) particles (<0.1-μm diameter) in mediating such responses. Previous work has demonstrated that uf particles induce a greater inflammatory response and free radical activity than fine counterparts of the same material, and this enhanced reactivity could be attributed to the larger surface area (Brown et al., 2001; Ferin et al., 1992; Li et al., 1999). Several studies have reported that fumes generated by welding are <0.5-μm diameter and are therefore likely to be deposited in the bronchiolar and alveolar regions of the lungs (Akselsson et al., 1976; Howden et al., 1988). As all three welding fumes were composed predominantly of (uf) particles (data not shown), any soluble metals present on particle surfaces may generate more free radical activity and thus induce a greater inflammatory response. In this study, we attempted to elucidate the specific components of welding fumes responsible for inducing inflammation by exposing epithelial cells to the soluble fraction and the washed particle fractions of each fume. Our results demonstrated following treatment with the washed particles, IL-8 release remained at basal levels. However, by 6 h, the soluble components of all three fumes significantly increased IL-8 mRNA expression and subsequent protein release by 24 h. More importantly, these results mimicked those observed following exposure to the whole welding fumes. This supports our hypothesis that the soluble components of all three welding fumes studied were entirely responsible for the increased pro-inflammatory responses observed in epithelial cells, and in this instance particle size or surface area did not contribute to the inflammatory response as has been proposed for a range of low toxicity particles (Duffin et al., 2001).

Having clearly established that the soluble fractions play a fundamental role in initiating welding fume toxicity, we...
further examined the involvement of transition metals in cytokine release by treating the soluble fractions of welding fumes with the chelator, chelex-100. Chelex-100 works on the principle that any cation present in solution is chelated into the surface of the beads and thus, any released or solubilized transition metals are removed from the soluble action (Gao et al., 2002). Although there is evidence that metal amounts of nonmetal chemical constituents and organic materials such as DNA and amino acids can be moved by chelation, this only occurs in alkaline condition (Jiraffa et al., 2000; Hemmasi and Bayer, 1975; Molinelli et al., 2002). As treatments were all slightly acidic (data not shown), chelation treatment should only remove soluble transition metals. Following pretreatment with chelex-100, L-8 expression was completely attenuated, returning to asal levels (Fig. 6). The observation that IL-8 production was attenuated by chelax treatment of the samples is entirely consistent with the data in Fig. 10, which show that chelax-treated samples contain very significantly less soluble transition metal than the whole particle and soluble fractions that were not chelax-treated. Figs. 6 and 10 together provide clear evidence for the role of soluble transition metals in the induction of inflammatory gene expression by welding fumes. These data corroborate earlier evidence for the role of soluble transition metals in the induction of inflammatory gene expression by welding fumes. The observation that all three fumes readily released intracellular antioxidants such as glutathione may also result in ROS production. Therefore, we attempted to establish a link between GSH depletion and intracellular ROS production following exposure to the soluble components of welding fumes and thus determine whether ROS was responsible for the differences in inflammagonicity between fumes. Alveolar epithelial cells exposed for 2 h to the soluble fractions of both nickel-based fumes, NIMROD 182 and NIMROD c276, caused a significant increase in intracellular ROS production. The rise in ROS production may occur due to direct redox cycling of transition metals or as an indirect effect of GSH depletion.

Although COBSTEL 6 contains numerous transition metals known to undergo redox cycling, such as Fe, Mn, and Cr, the production of ROS as measured using the fluorescent probe DCFH-DA did not appear to play a role in the molecular toxicity of COBSTEL 6, as no difference in DCF fluorescence was observed. This result is somewhat surprising as we previously reported that soluble components of COBSTEL 6 caused depletion of GSH to the same extent as both NIMROD 182 and NIMROD c276, suggesting the involvement of intracellular ROS. Although previous studies have identified metal-induced ROS production using the DCFH-DA fluorescent probe, these compounds on specific metal compounds, such as Ni, Cr, and Co (Huang et al., 1993; Martin et al., 1998; Salnikow et al., 2000). We speculate that the lack of ROS detected in our system may be because the chemical properties of welding fumes are highly complex, and the interactions between different metals may synergize, attenuate, or block free radical formation detectable by this system. Furthermore, it is difficult to oxidize cobalt to Co$^{3+}$ from its normal Co$^{2+}$ oxidation state, in contrast to the more facile oxidation of other transition metals. Consequently, the cobalt present in COBSTEL 6 may not be as effective a redox cyycler for the generation of specific ROS detected by DCFH-DA. This observation is consistent with those of Salnikow, who saw no detectable ROS in A549 cells treated with CoCl$_2$ at a concentration of 100 $\mu$M (50-fold higher than in our fume).
Conclusion

In conclusion, these studies demonstrate that the soluble fractions of welding fumes play a fundamental role in mediating pro-inflammatory responses in alveolar epithelial cells as shown by increased expression of IL-8. Further examination highlighted that the soluble metal component was entirely responsible for this effect. In addition, enhanced levels of ROS and concomitant depletion of the antioxidant GSH suggest an important role for metal-particulate mediated oxidative stress, although more detailed evaluation of the signaling mechanisms involved is required. These data support the hypotheses that an enhanced inflammatory response, mediated by transition metals and oxidative stress in the lungs following inhalation of welding fume particles, is a potential mechanism for MFF and potentially other adverse health effects of welding fume exposure.

Acknowledgments

JMcN is the recipient of a Colt Foundation Fellowship in Occupational/Environmental Health.

References


