INTRACELLULAR THIOL TARGETS OF CHLORINATED OXIDANTS

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Abstract

The neutrophil oxidative burst during inflammation is a main source of oxidants \textit{in vivo}. As a result of neutrophil activation, the inflammatory oxidants produced include $\text{H}_2\text{O}_2$ and chlorinated oxidants (HOCl and chloramines). These oxidants have been linked to endothelial dysfunction, a key component of various inflammatory diseases such as cancer, atherosclerosis and obesity.

The relevance of chloramines to inflammatory disease is an area of great interest. Some chloramines have been shown to traverse membranes and are cytotoxic, reacting with a number of cell components, with particular preference for Cys and Met residues. Understanding which cellular targets are preferentially oxidised by chloramines could provide important clues as to how endothelial dysfunction might be mediated in the context of inflammation. The aim of this thesis was to explore the cellular thiol targets HOCl and cell-permeable chloramines (glycine chloramine, GlyCl; and monochloramine, $\text{NH}_2\text{Cl}$) in an endothelial cell model. Specifically, the focus was to investigate each oxidant’s effects on cells grown in culture, with treatment exposure kept to a minimum in order to specifically identify early protein thiol modifications which could be involved in the determination of cell fate and/or antioxidant protection.

Peroxiredoxins (Prxs, a family of thiol proteins known to detoxify cells against $\text{H}_2\text{O}_2$) in both endothelial cells and erythrocytes were shown to react with chloramines and HOCl, but accumulated in a different redox state than that seen with $\text{H}_2\text{O}_2$. Instead of becoming inactivated due to hyperoxidation, as observed with $\text{H}_2\text{O}_2$, Prxs were reversibly oxidised to disulfides and thus remained catalytically active following chloramine or HOCl treatment, suggesting that these enzymes may be important in detoxifying cells against chlorinated compounds. In addition, this study provided some evidence that Prx redox status could be involved in (or at least related to) cell viability in an endothelial model.

This study has also identified novel proteins which specifically underwent thiol redox modifications in cells briefly exposed to cell-permeable chlorinated oxidants. The two-dimensional SDS-PAGE approach used illustrated that many proteins underwent thiol oxidation
following treatment, and a subset of these proteins were selected and identified by mass spectrometry. Many of the proteins identified by this method are known to participate in antioxidant protection of cells and/or in apoptotic cell signalling, which makes them worthy of further exploration.

Finally, haem oxygenase-1 (HO-1), another important antioxidant enzyme with strong links to inflammatory disease, was shown to be highly induced in cells exposed to low doses of NH₂Cl. Surprisingly, nuclear translocation of nuclear factor erythroid-2-related factor 2, one of the major transcription factors known to induce HO-1 expression, could not be detected. The highly sensitive HO-1 expression in response to chloramine treatment, in addition to its known roles in protecting cells against inflammatory tissue injury, mean that HO-1 is likely involved in protecting cells against oxidative injury during inflammation.

This study has explored a number of thiol proteins which undergo redox modification in response to exposure to chlorinated oxidants. The identification and characterisation of these targets helps to understand the inflammatory condition and the roles specific to the various chlorinated oxidants. Further understanding of the processes involved in inflammatory disease will assist in directing future studies toward the development of therapeutics for preventing inflammatory tissue damage.
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Publications arising from this thesis


**Stacey, M.M.,** Vissers, M.C., Winterbourn, C.C. 2-cys peroxiredoxins are oxidised by inflammatory oxidants in human endothelial cells (manuscript in preparation).
CHAPTER 1. INTRODUCTION ................................................................. 1

1.1 ROS, Inflammation & Disease ..................................................... 1
   1.1.1 ROS and Oxidative stress .................................................. 1
   1.1.2 Oxidative stress during inflammation .................................. 2
      1.1.2.1 The neutrophil oxidative burst ..................................... 2
      1.1.2.2 Hydrogen Peroxide (\(\text{H}_2\text{O}_2\)) ................................. 3
      1.1.2.3 Hypochlorous acid (\(\text{HOCl}\)) .................................. 4
      1.1.2.4 Chloramines .............................................................. 6
   1.1.3 Erythrocytes and oxidative stress ...................................... 8
   1.1.4 Endothelial cells, inflammation and oxidative stress ............. 10
   1.1.5 Inflammation and disease .................................................. 10
      1.1.5.1 Atherosclerosis ....................................................... 10
      1.1.5.2 Type 2 diabetes ..................................................... 14

1.2 Thiol proteins ........................................................................... 15
   1.2.1 Redox chemistry of thiol residues ....................................... 16
   1.2.2 Thiol: redox switches and antioxidants ................................. 17
1.2.2.1 Glutathione ................................................................. 18

1.3 Peroxiredoxins (Prxs) ........................................................... 19
  1.3.1 2-Cys Prx: Structure and catalytic mechanism ........................ 20
  1.3.2 Reactivity of 2-Cys Prx .................................................... 21
    1.3.2.1 Reactivity with MPO-derived oxidants .......................... 21
  1.3.3 Peroxiredoxins and disease ............................................. 22

1.4 Haem oxygenase 1 ............................................................... 23
  1.4.1 Induction of haem oxygenase-1 (HO-1) .............................. 23
    1.4.1.1 Nuclear factor-erythroid-2-related factor 2 (Nrf2) .......... 23
    1.4.1.2 Targets of Nrf2 ...................................................... 24
  1.4.2 HO-1 enzymatic activity .................................................. 24
    1.4.2.1 HO-1 as an antioxidant .......................................... 24
  1.4.3 Role of HO-1 in endothelial cells ..................................... 25
    1.4.3.1 Role of HO-1 in models of vascular disease .................. 25
    1.4.3.2 Roles in atherosclerosis and associated disease pathologies 26
  1.4.4 Effect of MPO-derived oxidants on HO-1 expression ............. 26

1.5 Aims of the thesis ............................................................... 28

CHAPTER 2. METHODS .................................................................. 29

2.1 Materials ............................................................................. 29

2.2 Buffers ................................................................................... 31

2.3 Erythrocytes ......................................................................... 32
  2.3.1 Cell isolation ................................................................. 32

2.4 HUVECs ............................................................................... 32
  2.4.1 Cell isolation and culture .................................................. 32

2.5 Hepa 1c1c7 ........................................................................... 33
  2.5.1 Culture ............................................................................ 33
2.6 Cell Treatments ........................................................................................................ 34
  2.6.1 Chloramines and hypochlorous acid ..................................................................... 34
    2.6.1.1 Chloramines and TNB Assay ................................................................. 34
    2.6.1.2 Hypochlorous acid ................................................................................. 34
    2.6.1.3 Hypothiocyanous acid (HOSCN) ......................................................... 34
    2.6.1.4 Intact erythrocytes ................................................................................ 35
    2.6.1.5 Lysed erythrocytes ................................................................................ 35
    2.6.1.6 HUVECs and HEPA 1c1c7 cells ............................................................. 35
  2.6.2 Hydrogen peroxide .............................................................................................. 36
  2.6.3 Carbon monoxide ............................................................................................... 36
  2.6.4 Sodium azide ...................................................................................................... 36
  2.6.5 Auranofin .......................................................................................................... 36
  2.7 Consumption of HOCl or chloramines ................................................................. 36
    2.7.1 Oxidant consumption: Erythrocytes ............................................................ 37
    2.7.2 Oxidant consumption: HUVECs ................................................................. 37
  2.8 Consumption of H$_2$O$_2$ (FOX assay) ............................................................... 37
  2.9 Thioredoxin reductase (TrxR) activity assay ....................................................... 38
  2.10 Protein assay ......................................................................................................... 38
  2.11 Protein precipitation ............................................................................................. 38
  2.12 SDS-Polyacrylamide gel electrophoresis ............................................................ 39
  2.13 Immunoblot analysis ............................................................................................ 39
  2.14 Detection of Prx redox state ................................................................................ 40
  2.15 Coomassie R250 PVDF staining ......................................................................... 41
  2.16 Silver stain procedure .......................................................................................... 41
  2.17 Cell viability .......................................................................................................... 42
    2.17.1 Propidium iodide ....................................................................................... 42
    2.17.2 MTT assay ............................................................................................... 42
  2.18 Assessment of total protein thiols ....................................................................... 42
CHAPTER 3. EFFECT OF CHLORINATED OXIDANTS ON PRX2 OXIDATION IN ERYTHROCYTES ....................................................... 52

3.1 Introduction.............................................................................. 52

3.2 Experimental Approach.......................................................... 52

3.3 Results .................................................................................... 55
  3.3.1 Oxidant consumption by erythrocyte cell suspension................. 55
  3.3.2 Prx2 oxidation....................................................................... 56
  3.3.3 Comparison of Prx2 and GSH oxidation...................................... 58
  3.3.4 Glucose-dependent recycling of Prx2 and GSH............................ 58
  3.3.5 Time-dependent regeneration of reduced Prx2 and GSH............... 60
  3.3.6 Oxidation & loss of GSH from treated erythrocytes..................... 61
Chapter 3.7 Oxidation of Prx2 & GSH in haemolysate .................................................. 64

3.8 Testing for endogenous H₂O₂ production ......................................................... 65

3.4 Discussion ........................................................................................................... 66

3.5 Summary ............................................................................................................ 69

Chapter 4. EFFECT OF MPO-DERIVED OXIDANTS ON PRX OXIDATION IN ENDOTHELIAL CELLS .................................................................................. 70

4.1 Introduction ......................................................................................................... 70

4.2 Experimental Approach ..................................................................................... 71

4.3 Results ................................................................................................................ 75

4.3.1 Prxs in HUVECs ............................................................................................... 75

4.3.2 Effect of H₂O₂ on Prx redox state ................................................................. 75

4.3.3 Chloramine or HOCl-induced oxidation of Prxs ........................................... 79

4.3.4 Chloramine consumption ............................................................................... 79

4.3.5 Regeneration of reduced Prxs following dimerisation .................................. 81

4.3.6 Comparison of Prx oxidation with loss of reduced GSH ............................... 83

4.3.7 Oxidant-induced loss of viability ................................................................. 84

4.3.1 Relationship between Prx oxidation and viability ...................................... 86

4.4 Discussion ........................................................................................................... 90

Chapter 5. PROTEOMIC INVESTIGATION OF NOVEL TARGETS OXIDISED BY CHLORINATED OXIDANTS IN ENDOTHELIAL CELLS .......... 94

5.1 Introduction ......................................................................................................... 94

5.2 Experimental approach ..................................................................................... 94

5.3 Results ................................................................................................................ 96

5.3.1 Time-dependent oxidation of HUVEC thiol proteins by chloramines and HOCl .................................................. 96

5.3.2 Disulfide modifications induced by hypothiocyanous acid .......................... 98

5.3.3 Effect of prolonged incubation in HBSS alone ........................................... 99
5.3.4 Effect of cell-impermeable taurine-chloramine on disulfide formation in HUVECs............100
5.3.5 Dose-dependent oxidation of HUVEC thiol proteins by chloramines, HOCl and H₂O₂.....100
5.3.6 Visualisation of oxidised proteins by two-dimensional IAF labelling................................103
5.3.7 Identification of proteins undergoing disulfide modification........................................107
  5.3.7.1 GAPDH.....................................................................................................................109
  5.3.7.2 Peroxiredoxins (spots 3, 4, 5 and 17).........................................................................109
  5.3.7.3 Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A; spots 7, 8, 9, 10 & 11).....114
5.4 Discussion ......................................................................................................................115
  5.4.1 Peroxiredoxins spots (3, 4, 5 and 17)........................................................................116
  5.4.2 Cyclophilin A................................................................................................................117
  5.4.3 Nucleoside diphosphate kinase B (spots 9, 11)..............................................................117
  5.4.4 Cofilin 1.........................................................................................................................118
  5.4.5 Parkinson disease protein 7 (Spot 4)...............................................................................118
  5.4.6 Glutathione-S-Transferase P (Spot 17)...........................................................................119
  5.4.7 Glutathione peroxidase 1 (spots 4 & 17).........................................................................120
  5.4.8 Annexin A1/A2 (Spots 1 & 2)..........................................................................................120
  5.4.9 Small ubiquitin-related modifier 3 (or 4) (SUMO3/SUMO4) (Spot 18)..........................121
  5.4.10 Beta-2-microglobulin precursor (Spot 14).................................................................122
  5.4.11 Interpretation of these results......................................................................................122
    5.4.11.1 Limitations...............................................................................................................122
    5.4.11.2 Advantages.............................................................................................................123
  5.4.12 Chlorinated oxidants as thiol-targeting agents............................................................124
  5.4.13 Summary and future directions..................................................................................124

CHAPTER 6. INDUCTION OF HO-1 EXPRESSION BY MONOCHLORAMINE..126

6.1 Introduction...................................................................................................................126
6.2 Experimental Approach ........................................................................................................127

6.3 Results ..................................................................................................................................129

6.3.1 Nrf2 & HO-1 in HUVECs .................................................................................................129

6.3.1.1 HO-1 expression and nuclear levels of Nrf2 in HUVECs grown in 12% FBS..............129

6.3.1.2 HO-1 expression and Nrf2 localisation in serum-deprived HUVECs......................129

6.3.1.3 SFN-induced nuclear accumulation of Nrf2 and expression of HO-1 in serum-deprived HUVECs ..................................................................................................................131

6.3.1.4 Monochloramine-induced HO-1 expression in HUVECs ......................................133

6.3.2 Nrf2 localisation in Hepa 1c1c7 cells ................................................................................133

6.3.3 Effect of monochloramine on Nrf2 and HO-1 in Hepa 1c1c7 cells .........................134

6.3.4 Nuclear accumulation of Nrf2 in NH₄Cl-treated cells .............................................137

6.4 Discussion ............................................................................................................................139

CHAPTER 7. GENERAL DISCUSSION ......................................................................................144

7.1 Summary ..............................................................................................................................144

7.2 Inflammatory oxidants and Prxs .....................................................................................145

7.2.1 Conformational changes in Prxs differ depending on oxidant treatment ..................145

7.2.2 Potential role of Prx in apoptosis ..................................................................................147

7.3 Novel thiol targets of inflammatory oxidants .................................................................148

7.4 Chloramine-induced HO-1 Expression .............................................................................150

7.5 General Conclusions ..........................................................................................................151

References ..................................................................................................................................153

APPENDIX A .............................................................................................................................192
List of Figures

Figure 1.1 Oxidants produced as a result of NOX2 assembly in the activated neutrophil. .......... 5
Figure 1.2 Structure of the chloramines investigated in this thesis. ........................................ 7
Figure 1.3 Scheme of atherosclerotic plaque development. ..................................................... 13
Figure 1.4 Role of ROS in Diabetes-induced atherosclerosis. .................................................. 14
Figure 1.5 Redox modifications of thiol residues. ................................................................. 16
Figure 1.6 Redox control of glutathione. ................................................................................ 18
Figure 1.7 The catalytic cycle of typical 2-cys peroxiredoxins. ................................................. 21
Figure 1.8 Products of the enzymatic haem degradation by haem oxygenase. ......................... 25
Figure 1.9 Activation of HO-1 and the downstream effects on inflammation. ......................... 27
Figure 2.1 Bio-Rad 2-D SDS-PAGE Standards were separated and detected using silver staining. ........................................................................................................................................ 49
Figure 2.2 Spot excision from 2-dimensional gels. ................................................................... 50
Figure 3.1 Oxidation of TNB by thiol-targeting oxidants. ......................................................... 53
Figure 3.2 Oxidation of 3,3’,5,5’ tetramethylbenzidine (TMB). ............................................... 53
Figure 3.3 Method used to capture native Prx redox state in cells........................................... 54
Figure 3.4 GSH was detected by derivatisation with monobromobimane. ............................... 55
Figure 3.5 Concentration-dependent consumption of oxidants by erythrocytes during 20 min incubation ......................................................................................................................... 56
Figure 3.6 Prx2 oxidation in erythrocytes treated with the given concentrations of oxidant for 20 min. ........................................................................................................................................... 57
Figure 3.7 Western blotting with antibodies against the hyperoxidised forms of Prx.............. 57
Figure 3.8 Concentration-dependent oxidation of Prx2 and GSH after 20 min treatments of erythrocytes ................................................................................................................................. 59
Figure 3.9 Regeneration of GSH and Prx2 in erythrocytes treated with NH2Cl. ...................... 60
Figure 3.10 GSH and its oxidation products GSSG and GSA were detected by LC-MS/MS. .... 62
Figure 3.11 GSH retrieved from erythrocytes treated with chloramines or HOCl. ................. 63
Figure 3.12 Prx and GSH oxidation in GlyCl-treated haemolysate. ......................................... 65
Figure 3.13 Prx2 oxidation in cells pretreated to either enhance or reduce the effect of any endogenous H2O2. .................................................................................................................... 66
Figure 4.1 Method used to quantify the amount of hyperoxidised Prx in cells ....................... 71
Figure 4.2 TrxR Activity was monitored spectrophotometrically. ................................................. 72
Figure 4.3 In mitochondria of living cells, MTT is converted to a formazan product. ............. 73
Figure 4.4 Scheme for Protein Carbonyl ELISA. .................................................................. 74
Figure 4.5 Quantification of Prxs by Western blotting. ......................................................... 75
Figure 4.6 Prx redox state in H$_2$O$_2$-treated HUVECs. ....................................................... 76
Figure 4.7 TrxR activity and Prx dimerisation in Auranofin-treated HUVECs. ................. 77
Figure 4.8 Regeneration of reduced Prxs following H$_2$O$_2$-induced hyperoxidation. .... 78
Figure 4.9 Chloramine-induced oxidation of peroxiredoxins. ............................................. 80
Figure 4.10 Regeneration of reduced Prxs following chloramine-induced oxidation. ....... 82
Figure 4.11 TrxR activity in treated HUVECs. .................................................................... 83
Figure 4.12 Concentration of reduced GSH in cells treated with various oxidants. ......... 84
Figure 4.13 Dose-dependent loss of viability in HUVECs treated with different concentrations of oxidant. ............................................................................................................ 85
Figure 4.14 Regression analysis of Prx oxidation and cell death measured by uptake of propidium iodide. ................................................................................................................. 86
Figure 4.15 MTT measurement of HUVEC viability 24 hr after treatment. ......................... 87
Figure 4.16 Levels of protein thiols and protein carbonyls in HUVECs treated with exogenous oxidants. ................................................................................................................. 89
Figure 5.1 Protocol for the specific labelling of disulfide-modified proteins in cell lysate. .... 95
Figure 5.2 Detection of HUVEC thiol proteins which become oxidised during treatment. .... 97
Figure 5.3 Thiol oxidation in HOSCN-treated HUVECs. ....................................................... 98
Figure 5.4 Effect of incubation in HBSS on HUVEC thiol oxidation. .................................... 99
Figure 5.5 HUVECs treated with TauCl were monitored for disulfide-modified proteins. .... 101
Figure 5.6 Thiol proteins in HUVECs which underwent disulfide modification during 10 min exogenous oxidant treatment. ...................................................................................... 102
Figure 5.7 Two-dimensional electrophoresis with IAF labelling of oxidised thiols. ........... 106
Figure 5.8 Spots excised from large 2-dimensional SDS-PAGE gels. ................................. 109
Figure 5.9 IAF spots correspond to Prxs. .............................................................................. 110
Figure 5.10 Spots excised from GlyCl-treated gel and identified as CypA by LC-MS/MS. .. 114
Figure 5.11 Immunoblots for CypA with 2-dimensional and 1-dimensional IAF. ............ 115
Figure 6.1 In-plate nuclear extraction procedure employed for HUVEC and Hepa 1c1c7 cytosolic and nuclear isolation. ....................................................................................... 128
Figure 6.2 Nrf2 localisation and HO-1 expression in HUVECs. ........................................... 130
Figure 6.3 Effect of serum deprivation on HO-1 protein expression in HUVECs. ............. 131
Figure 6.4 Nuclear accumulation of Nrf2 and cytosolic HO-1 protein levels in serum-deprived HUVECs treated with sulforaphane.

Figure 6.5 Immunofluorescence of HUVECs was investigated using antibodies against Nrf2 with Hoechst counterstaining.

Figure 6.6 HO-1 protein expression in HUVECs treated with chloramines.

Figure 6.7 Intracellular localisation of Nrf2 in Hepa 1c1c7 cells treated with sulforaphane or monochloramine.

Figure 6.8 Nrf2 accumulation and Haem oxygenase-1 expression in Hepa 1c1c7 cells.

Figure 6.9 Accumulation of Nrf2 and Haem oxygenase-1 in HUVECs.

Figure 6.10 Western blotting of nuclear and cytosolic fractions to investigate intracellular localisation of Nrf2 in Hepa 1c1c7 cells and HUVECs.

Figure 6.11 Simplified schematic of the transcriptional regulation of HO-1 expression.

Figure 7.1 Relationship between Prx redox state, conformation, and cellular function.
List of Tables

Table 1.1 Some chloramines, their properties and demonstrated effects on cells. .......................... 9
Table 1.2 Second-order rate constants for the reaction of H$_2$O$_2$ and chloramines with GSH and Prx2. ......................................................................................................................... 22
Table 2.1 Materials used in the thesis .................................................................................................................. 29
Table 2.2 Buffers used in the thesis ......................................................................................................................... 31
Table 2.3 Comparison of plate sizes in terms of cell number, protein content and treatment volumes used. ........................................................................................................................................... 35
Table 2.4 Primary and secondary antibodies used in the thesis .............................................................. 40
Table 2.5 Gradient for elution of GSH-MBB samples ......................................................................................... 44
Table 2.6 Settings for detection of glutathione analytes and standards .................................................. 45
Table 2.7 Conditions for isoelectric focusing of 11-cm and 17-cm IPG strips ........................................... 48
Table 3.1 Glutathione oxidation products in erythrocytes treated with chloramines or HOCl .......... 63
Table 4.1 Chloramine consumption by HUVECs is concentration-dependent ....................................... 80
Table 5.1 Proteins identified from Mascot search of amino acid sequences detected by MS/MS. .................................................................................................................................................. 111
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>1-D</td>
<td>One-dimensional</td>
</tr>
<tr>
<td>2-D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>AFN</td>
<td>Auranofin</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycosylation end product</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
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<td>AP-1</td>
<td>Activator protein 1</td>
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<td>ApoE</td>
<td>Apolipoprotein E</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CAM</td>
<td>Carbamidomethylation</td>
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<tr>
<td>cAMP</td>
<td>3',5'-cyclic monophosphate</td>
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<tr>
<td>CGD</td>
<td>Chronic granulomatous disease</td>
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<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<tr>
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<td>Hypochlorous acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>hsCRP</td>
<td>High sensitivity C-reactive protein</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>IAF</td>
<td>Iodoacetamidofluorescein</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Inter-Cellular Adhesion Molecule 1</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focusing</td>
</tr>
<tr>
<td>IL-</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IPG</td>
<td>Immobilized pH gradient</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>Keap1</td>
<td>Kelch-like ECH-associated protein 1</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>m/z</td>
<td>mass-to-charge ratio</td>
</tr>
<tr>
<td>M199</td>
<td>Medium 199</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MBB</td>
<td>monobromobimane</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MTT</td>
<td>Thiazolyl Blue Tetrazolium Bromide</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NADH</td>
<td>reduced nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethyl maleimide</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NH2Cl</td>
<td>Monochloramine</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NOX</td>
<td>NADPH oxidase</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonidet® P-40</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Nuclear factor erythroid-2-related factor 2</td>
</tr>
<tr>
<td>O2−</td>
<td>Superoxide</td>
</tr>
<tr>
<td>oxLDL</td>
<td>Oxidised low-density lipoprotein</td>
</tr>
</tbody>
</table>
PAGE Polyacrilamide gel electrophoresis
PBS Phosphate buffered saline
pI Isoelectric point
PI Propidium iodide
pK\textsubscript{a} Acid dissociation constant
PMA Phorbol 12-myristate 13-acetate
Prx Peroxiredoxin
PTEN Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase
PTP Protein tyrosine phosphatase
PTP-1B protein tyrosine phosphatase-1B
PVDF Polyvinylidene fluoride
RNS Reactive nitrogen species
ROS Reactive oxygen species
RT Room temperature
SDS Sodium Dodecyl Sulfate
SFN Sulforaphane
SH2 Src-homology 2
SHP2 SH2-containing protein tyrosine phosphatase
siRNA Small interfering RNA
SMC Smooth muscle cell
TauCl Taurine chloramine
TBS Tris buffered saline
TBST Tris buffered saline containing Tween® 20
TCA Trichloroacetic acid
TCEP Tris(2-carboxyethyl)phosphine hydrochloride
TMB 3,3′,5,5′-tetramethylbenzidine
TNB 5-thio-2-nitrobenzoic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris-(hydroxymethyl)-methylamine</td>
</tr>
<tr>
<td>Trx</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>TrxR</td>
<td>Thioredoxin reductase</td>
</tr>
<tr>
<td>UHPLC</td>
<td>Ultra high performance liquid chromatography</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>πGST</td>
<td>π-class glutathione-S-transferase</td>
</tr>
</tbody>
</table>
Chapter 1. Introduction

Understanding the tissue damage which results from inflammation is an area of great interest and importance. Many diseases such as atherosclerosis involve a heightened inflammatory response which actually contributes to the pathophysiological disease state. Increasingly, the damage to cells and tissues during inflammation has been associated with increased oxidative stress as a direct result of the body's inflammatory response. Oxidants which are intended by the body's immune system to defend against invading pathogens are readily able to react with the body's own cells during inflammation. This can lead to negative side effects and even contribute to the disease pathology in tissues during inflammation, especially when the inflammatory response becomes chronic.

This thesis is focused on the mechanisms of oxidative stress induced by hydrogen peroxide and chlorinated oxidants, which are generated as a result of neutrophil activation as part of the inflammatory response. In particular, the aim was to understand the major cellular targets (with particular interest in thiol targets) of these oxidants in an endothelial model. Identification of these targets will enable better understanding of the cellular systems which might protect from and/or contribute to endothelial damage during inflammation, and provide insight for future therapeutic development.

1.1 ROS, Inflammation & Disease

1.1.1 ROS and Oxidative stress

Reactive oxygen species (ROS) are constantly produced during normal physiological conditions and consist of a variety of oxidative molecules derived from O₂ (including hydrogen peroxide, H₂O₂, superoxide, 'O₂⁻, and the hydroxyl radical 'OH). ROS are capable of modifying macromolecules including lipids, proteins and DNA, which are susceptible to oxidative modification; this is often accompanied by either reversible or irreversible dysfunction of the target. Under normal homeostatic conditions, the level of ROS produced— as a result of normal aerobic respiration, for instance – is balanced by the activity of various endogenous antioxidant
Chapter 1: Introduction

systems. However, an imbalance in ROS production and scavenging in favour of ROS production results in a condition referred to as oxidative stress.

Increasingly, this cellular redox imbalance (or lack of redox homeostasis) has been associated with a number of pathological conditions and diseases. When the level of ROS increases, cellular defences against oxidative damage include a variety of enzymatic (e.g. catalases and glutathione peroxidases, GPxs) and non-enzymatic (e.g. glutathione, GSH, and ascorbate) strategies. These may scavenge ROS directly or simply repair/replace damaged molecules. The level of ROS produced has the potential to be beneficial or harmful to cells, depending on the degree of imbalance; despite the potential damaging effects of macromolecular oxidation, it has been repeatedly suggested that low levels of ROS produced may be responsible for activating signalling cascades which help cells respond to various physiological stresses (from xenobiotic or electrophilic stimuli, for example).

There are several main cellular sources of endogenously-produced ROS, including the mitochondrial electron transport chain (of which Complexes I and III are responsible for electron ‘leakage’, reducing O$_2$ to ‘O$_2$‘ at the mitochondrial membrane) and enzymatic sources such as NADPH oxidases (NOXs), which reduce O$_2$ to ‘O$_2$‘ at the cell membrane, with reducing equivalents from NADPH (reviewed in [2; 13; 14]). Phagocytic NOX2 is one of the most well-studied members of this widely-distributed family of flavoprotein peroxidase enzymes. The components of the NOX2 enzyme assemble at the phagosome membrane and this activated complex is responsible for the hallmark ‘oxidative burst’ of activated neutrophils (reviewed in [15]; further discussed in Section 1.1.2.1).

1.1.2 Oxidative stress during inflammation

1.1.2.1 The neutrophil oxidative burst

Neutrophil recruitment to a site of inflammation is one of the body’s first responses to pathogenic or other inflammatory stimuli. Neutrophils are the most abundant leukocytes in humans and provide an important defence in terms of bacterial phagocytosis and killing. Bacterial killing by neutrophils has been shown to include both oxidative and non-oxidative mechanisms [16; 17], and in recent years the oxidative killing has generated much scientific attention.
Chapter 1: Introduction

As mentioned in Section 1.1.1, the NOX2 complex assembles on the phagosome membrane of activated neutrophils, resulting in the characteristic oxidative (or respiratory) burst which is at least partly responsible for the neutrophil’s bactericidal capacity. This is illustrated in patients with chronic granulomatous disease (CGD), who have inactive NOX2 resulting in a failure to produce a respiratory burst. Neutrophils from patients with CGD are ineffective at clearing infection, and these patients consequently suffer severe chronic infections which are often life-threatening [18; 19]. Study of patients with this exceedingly rare disease has highlighted the importance of NADPH oxidase function in the inflammatory response.

Although important for the bactericidal activity of the neutrophil, NOX2-derived oxidants are also reactive with the body’s own cells. The collection of inflammatory oxidants generated as a result of the neutrophil oxidative burst is responsible for contributing to inflammatory tissue damage. In fact, many diseases (such as cancer, diabetes and atherosclerosis) and their associated pathologies have strong links to the heightened ROS production during chronic inflammation (discussed in Section 1.1.5). The specific effects of the various oxidants produced are still not fully understood, but depend on the particular oxidant’s local concentration, specific reactivity, targets, cell permeability and possible diffusion distances. These oxidants are discussed below.

1.1.2.2 Hydrogen Peroxide (H₂O₂)

In addition to the mitochondrial electron transport chain, a number of enzymes (including NOXs), are endogenous sources of superoxide radicals. Superoxide readily dismutates (with or without the assistance of superoxide dismutases) in cells to generate H₂O₂, a molecule which has important roles in cell signalling (reviewed in [20; 21]).

Unlike superoxide, H₂O₂ is readily membrane-permeable and although it is a 2-electron oxidant, is not particularly reactive with most biological molecules. H₂O₂ does appear to favourably react with thiol residues and in particular those which are in the anionic thiolate form (Cys-S⁻, refer to Section 1.2.1) at physiological pH (i.e. have a low pKₐ). Included among the thiol protein targets of H₂O₂ which have been identified are protein tyrosine phosphatases (PTPs) and Prxs [22].

Catalases (Equation 1.1), glutathione peroxidases (GPxs) (Equation 1.2) and Prxs (further discussed in Section 1.3) are the main cellular antioxidants responsible for scavenging...
intracellular H$_2$O$_2$, and appear to scavenge H$_2$O$_2$ at similar rates [9]. Unfortunately, attempts to understand precisely how H$_2$O$_2$ functions in signalling cascades have been hampered by a lack of sensitive and specific probes to accurately detect H$_2$O$_2$ within the cell environment; increasingly, however, H$_2$O$_2$ is considered to mediate cell signalling events by reacting locally with specific targets. Although methodologies to investigate these local reactions within the cell have yet to be perfected, there is a strong focus on attempting to understand where H$_2$O$_2$ is produced and what its specific targets are in proximity to the site(s) of production.

\[
2\text{H}_2\text{O}_2 \xrightarrow{\text{Catalase}} 2\text{H}_2\text{O}+\text{O}_2 \quad (1.1)
\]

\[
2\text{GSH}+\text{H}_2\text{O}_2 \xrightarrow{\text{GPN}} \text{GSSG}+2\text{H}_2\text{O} \quad (1.2)
\]

### 1.1.2.3 Hypochlorous acid (HOCl)

Myeloperoxidase (MPO) is a neutrophil enzyme which catalyses the reaction of H$_2$O$_2$ with Br$^-$, Cl$^-$ or SCN$^-$ to generate hypohalous acids (HOBr, HOCl or HOSCN, respectively) [13-17] (Equation 1.3 and Figure 1.1). Of these oxidants, HOCl has received the most attention and has been suggested to be responsible for much of the bactericidal effects of neutrophil activation [23].

\[
\text{X}^- + \text{H}_2\text{O}_2 \xrightarrow{\text{MPO}} \text{OX}^- + \text{H}_2\text{O} \quad (1.3)
\]

X=Cl, Br, I, SCN

Neutrophils are present in the blood at 2.4 × 10$^6$ cells/ml, and can accumulate to much higher numbers at inflammatory sites [24]; in vitro studies have shown that stimulated neutrophils (at normal physiological cell concentrations) are capable of steady generation of hypochlorous acid (HOCl) which amounts to 100 μM produced in under an hour [25]. It has been estimated that as much as 45% of the H$_2$O$_2$ consumed by Myeloperoxidase (MPO) is converted to HOCl [26].
At physiological pH, HOCl consists of roughly equal concentrations of HOCl and OCl\(^-\) (pKa ~ 7.5) \([27]\), but for simplicity this thesis refers to these compounds collectively as HOCl. Myeloperoxidase is the only mammalian peroxidase known to catalyse the reaction of H\(_2\)O\(_2\) with Cl\(^-\) to generate HOCl (Equation 1.3), and the formation of this compound is favoured due to high plasma concentrations of Cl\(^-\) \([26]\).

HOCl is therefore a major reactive compound produced by neutrophils. It is both cell-permeable and highly reactive with a range of substrates \([28-30]\), making it the neutrophil’s most effective bactericidal oxidant. While the high reactivity of HOCl makes it an ideal antimicrobial agent, this oxidant also reacts readily with targets in host cells, making it a potentially damaging oxidant \([31-33]\). This cell-permeable oxidant is capable of inducing cell death and growth arrest in a number of cell types \([34]\), as well as activating mitogen-activated protein kinase (MAPK) pathways\([35]\), inactivating glyceraldehyde-3-phosphate dehydrogenase (GAPDH) \([36]\) and inducing thiol oxidation \([37; 38]\) and apoptosis \([34]\), among other events. Because of its reactivity, HOCl is not

---

**Figure 1.1 Oxidants produced as a result of NOX2 assembly in the activated neutrophil.**

H\(_2\)O\(_2\), HOCl and chloramines are produced as a result of NOX2 assembly and the resultant generation of superoxide. (Figure has been adapted from [2])
generally expected to accumulate under physiological conditions; instead, it is believed to react with biological targets, and potentially result in the accumulation of secondary, less reactive (but nevertheless oxidising) species such as chloramines [39] (further discussed below).

1.1.2.4 Chloramines

Taurine, or 2-aminoethanesulfonic acid, is an organic acid derivative of cysteine, present in high concentrations in neutrophils. In fact, taurine is the most abundant free amino acid in neutrophils (measured at concentrations up to 50 mM), accounting for ~75% of all free acids in these cells [40; 41]. In stimulated neutrophils, taurine is released and reacts with HOCl to produce taurine chloramine (TauCl; Figure 1.2 A). It has been suggested that this reaction could detoxify against the harmful effects of released HOCl due to the relative unreactivity of the chloramine [41].

Chloramines other than TauCl are undoubtedly generated during inflammation [39] (reviewed in [42]); excess HOCl produced as a result of the oxidative burst may react directly with other amines (Equation 1.4). In this thesis, a strong focus was placed on the moderately permeably glycine chloramine [43] (GlyCl; Figure 1.2 B) and highly cell-permeable monochloramine [44] (NH$_2$Cl; Figure 1.2 C), generated from the reactions of HOCl with glycine and ammonium, respectively. These compounds have both been associated with a number of biochemical events in treated cells.

Mono-chloramines are readily generated by the non-enzymatic reaction of HOCl with free amines [23; 45](Equation 1.4):

\[
\text{HOCl + R-NH}_2 \rightarrow \text{R-NHCl + H}_2\text{O}
\] (1.4)

While chloramines are less reactive than HOCl, they do retain the capacity to oxidise biological targets. In contrast to HOCl, chloramines tend to be long-lived [39] and react more selectively with sulfhydryls and thioethers [10; 46; 47]; these traits have led to the speculation that chloramines could be involved in cell signalling cascades (in response to inflammation, for instance), and/or the control of redox couples ([48]; reviewed in [42]).
Monochloramine derivatives are also able to react with excess HOCl to generate dichloramines (Equation 1.5), which are more highly oxidising (and mutagenic) species than monochloramines \[49\]. However, for the purposes of this thesis, attention has been devoted solely to the study of mono-chloramines; these are herein referred to simply as ‘chloramines’.

\[
RNHCl + HCl \rightarrow RHCl_2 + H_2O
\]  

(1.5)

Although TauCl itself is reported to be cell impermeable in most cell types \[45\], TauCl treatment in full culture medium has been shown to mediate various effects on cells, including oxidative damage and increased cell signalling via various pathways. Many of these events are undoubtedly mediated by oxidants other than TauCl due to the generation of other oxidants from the reactions of TauCl with medium components. This could potentially include other chloramines generated by transchlorination reactions \[50\], whereby the ‘R’ group of one amine can be exchanged for another (Reaction 1.6), but the complexity of the medium reaction mixture makes it impossible to determine definitively which oxidant(s) is/are present, let alone what oxidant(s) is/are mediating which cellular event(s).

\[
R^1\cdot NHCl + R^2\cdot NH_2 \leftrightarrow R^1\cdot NH_2 + R^2\cdot NHCl
\]  

(1.6)
Chapter 1: Introduction

A minority of studies have investigated the effects of exogenous TauCl on cells treated in buffer lacking thiol scavengers and free amines, and some biological effects have been attributed to this chloramine specifically. This includes activation of extracellular signal-regulated kinase (ERK) via the epidermal growth factor (EGF) receptor [51] (Table 1.1).

Various chloramines have been shown to modulate a number of cellular processes through such mechanisms as the interference of MAPK pathways [51], reduction in levels of inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production [52; 53], inhibition of tumour necrosis factor-α (TNFα) [53; 54], activation/translocation of nuclear factor-erythroid2-related factor 2 (Nrf2) [55], and interference with nuclear factor kappa-B (NF-κB) signalling pathways [36; 56-59] (Table 1.1).

Though it has been clearly demonstrated that transchlorination from one N-monochloramine to another amine or amino group occurs readily [45; 50; 60; 61], this phenomenon is often unaddressed in the literature, making it difficult to determine the specific effects of the various chlorinated species. Many studies applying exogenous chloramine (or HOCl) treatment to cells have used full culture medium. This means that the exact compound (or combination of compounds) responsible for any observed changes is speculative (but could admittedly be of biological relevance in the context of inflammation). There is nevertheless a lack of information regarding the specific effects of exogenous chloramines on living cells and tissues.

1.1.3 Erythrocytes and oxidative stress

Once erythrocytes are mature, neither haem nor haemoglobin is newly synthesised [62]. Haemoglobin is highly susceptible to autoxidation in these O₂-carrying cells, generating methaemoglobin and superoxide radicals from the reduction of O₂ [63; 64]; this has been shown to contribute much of the endogenously-generated H₂O₂ in these cells [65]. Therefore, to inhibit this oxidative event over the course of the erythrocyte’s 120-day life, a reducing cell environment must be maintained to inhibit oxidation of its proteins, and this is accomplished by high cellular levels of antioxidant enzymes, such as peroxiredoxin 2 (Prx 2).
### Table 1.1 Some chloramines, their properties and demonstrated effects on cells.

<table>
<thead>
<tr>
<th>N-Compound</th>
<th>N-Cl derivative</th>
<th>Charge</th>
<th>Cell permeability</th>
<th>Toxicity</th>
<th>Known cellular effects&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taurine</td>
<td>Taurine chloramine (Also: taurine mono-chloramine, TauCl)</td>
<td>Anionic</td>
<td>Very low</td>
<td>Low</td>
<td>↑ Generation of pro-inflammatory cytokines (NO, prostaglandins, TNF-α, etc.) ↑ Expression of COX-2 ↓ activity of NF-κB and AP-1 ↑ HO-1 activity ↑ Nrf2 activation ↑ ERK activation (via EGF receptor)</td>
<td>[29; 44; 45; 52; 53; 55; 59; 66-72]</td>
</tr>
<tr>
<td>Glycine</td>
<td>Glycine chloramine (Also: glycine mono-chloramine, GlyCl)</td>
<td>Anionic</td>
<td>Slowly permeable</td>
<td>Medium</td>
<td>↓ Caspase activation &amp; phosphatidyl-serine exposure (↓ apoptosis) ↓ GAPDH activity ↓ NF-κB activation (via Met ox’n of iκBα) ↓ ERK activation</td>
<td>[36; 43; 50; 73]</td>
</tr>
<tr>
<td>Ammonium (NH₄⁺)</td>
<td>Mono-chloramine (Also: ammonia chloramine, NH₂Cl)</td>
<td>Uncharged</td>
<td>High (rapid)</td>
<td>High</td>
<td>↑ Apoptosis (JNK-dependent); ↑ caspase activation ↓ TNF-α-induced adhesion molecule (e.g. ICAM-1) expression ↑ NF-κB activation Arrest of cell cycle ↑ Endothelial permeability</td>
<td>[43-45; 56; 66; 74; 75]</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cellular effects were investigated in a number of studies, some of which treated cells in full media, which cannot control for the generation of other chloramines or unknown oxidative compounds.

Low et al [65] demonstrated that haemoglobin autoxidation contributed to endogenous oxidation of Prx2, the third most abundant protein in the erythrocyte. Mice lacking this key antioxidant enzyme have been shown to develop haemolytic anaemia [76], which illustrates the negative repercussions of oxidative stress in the erythrocyte.
In addition, oxidative injury during inflammation could be very relevant in erythrocytes due to their co-existence in the bloodstream with activated neutrophils. Their exposure to locally high concentrations of oxidants produced by the phagocytes could put them at risk of oxidative damage and diminished function.

1.1.4 Endothelial cells, inflammation and oxidative stress

The endothelium is a particularly vulnerable tissue when it comes to oxidative injury from inflammation. In fact, endothelial cells have for a long time been known to play an important role in the inflammatory response, involving cross-talk between endothelial and immune cells (for example, via cytokines – including interleukins – and their receptors; reviewed in [77]). In spite of this, endothelial dysfunction is known to occur in various disease states in which inflammation appears to contribute to the disease pathologies, as seen in patients with cardiovascular diseases (reviewed in [78] and [79]).

Increased inflammation (measured as an increase in high sensitivity C-reactive protein (hsCRP), a widely-used biomarker for inflammation) has been positively associated with disease outcome and morbidity/mortality in clinical studies of various inflammatory diseases (reviewed in [80]). Increasingly, the persistent production of oxidants by low-grade inflammation appears to contribute to the negative pathologies associated with inflammatory diseases, including endothelial dysfunction and damage. This likely occurs due to a level of oxidant production which overwhelms the cells’ endogenous antioxidant systems.

1.1.5 Inflammation and disease

Increasingly, evidence suggests that chronic low-grade inflammation can lead to and exacerbate various disease pathologies. Diseases such as atherosclerosis [81; 82], diabetes [83], arthritis [84], obesity [85] and cancer [86] have been linked to inflammation and the associated generation of ROS. The roles of inflammation in atherosclerosis and diabetes – two diseases with strong inflammatory components – are discussed briefly below.

1.1.5.1 Atherosclerosis

Many adverse cardiovascular events are a result of an underlying vascular pathology known as atherosclerosis; these include coronary artery disease, stroke, and peripheral arterial disease.
Atherosclerosis is a chronic inflammatory disease characterised by the formation of an atheroma – a fibrous plaque which forms in the intima of a major artery – which can eventually lead to complete occlusion of the artery, depriving organs of blood and oxygen, and thereby cause one of the above-mentioned adverse events.

Briefly, atherosclerosis involves activation of the endothelium, followed by endothelial adhesion of recruited leukocytes in response to cytokines expressed on the endothelial membrane. Subsequently, leukocytes penetrate into the subendothelium; accrual of low density lipoproteins (LDL) in this space leads to the accumulation of oxidised LDL (oxLDL) and foam cell formation when macrophages take up this oxidised molecule. The formation of a plaque rich in lipids from these foam cells is followed by migration and proliferation of smooth muscle cells, which form a fibrous cap over the plaque (Figure 1.3). The result is a hardened artery, often with a decreased lumen and continued expression of inflammatory molecules which leads to further recruitment and infiltration of immunoinflammatory cells.

The role of inflammation in atherosclerosis is both highly reported and accepted in the scientific community. Inflammation has been described as “fundamental” [87] and even “central” [82] to all stages of the disease, which involves an ongoing inflammatory response. (Many other reviews have also been published on this concept [88-93]). In addition, the endothelium itself is directly involved in the disease [82]; early stages of atherosclerosis are understood to be initiated by endothelial activation and production of pro-inflammatory cytokines, chemokines and adhesion molecules. A number of key events which contribute to the disease pathology appear to be mediated by the membrane expression of these molecules, the roles of which have been elucidated using mouse models of experimental atherosclerosis (reviewed in [82]). Studies have employed these mouse models (apolipoprotein E (apoE) or LDL receptor knockout mice) crossed with mice deficient in cytokines or cytokine receptors to generate double mutants; this has enabled investigators to pinpoint the roles of inflammatory cytokines in various stages of atherosclerotic disease. It has become clear that endothelial cell cytokine expression is important for a number of events including: alterations in endothelial cell-cell adhesion allowing the transmigration of leukocytes to the subendothelium [94]; expression of adhesion molecules and chemokines which are important for adhesion of leukocytes to the endothelium [95]; and modulation of both coagulation and fibrinolysis [96-99], among other events [82], all of which seem to play a role in disease initiation and/or progression.
While the role of inflammation in atherosclerosis is now widely recognised, the roles of specific oxidants which would be generated as a result of the chronic inflammatory condition are less well defined. Nevertheless, it has been shown that oxidation of LDL by HOCl, for instance, is associated with the formation of the pro-atherogenic foam cell clusters [100; 101]. In addition, high-density lipoprotein (HDL) can become modified by HOCl in such a way that it fails to protect against the formation of an atherosclerotic plaque [42].

More broadly, atherosclerosis has been positively associated with a number of biomarkers for oxidative stress, including F₂-isoprostanes [102-104], 4-hydroxy-2-nonenal [101; 105; 106], and 3-chlorotyrosine [107; 108] (reviewed in [109]). The detection of these oxidation products (especially the latter) in human atherosclerotic lesions has led to speculation that indeed, MPO-derived oxidants (Section 1.1.2.3) could be responsible for oxidising LDL, generating the atherogenic molecule [108].

NADPH oxidase activity has also been implicated in atherosclerosis [110] (reviewed in [111]). Increased expression of NADPH oxidase subunits has been reported in human atherosclerotic arteries [112], and knockout mouse studies have shown that mutations in an NADPH oxidase subunit in mouse models of experimental atherosclerosis have a protective, anti-atherogenic effect on vascular cells [113; 114].
Nitric oxide also plays a key role in maintaining vascular function. Endothelial-derived NO promotes vasodilatation, prevents both leukocyte adhesion and oxidation of LDL, and inhibits proliferation of vascular smooth muscle cells, all of which protect against atherosclerosis. Thus, a decrease in NO (for example via dysfunction of endothelial NO synthase, eNOS) is strongly linked with endothelial dysfunction, vascular disease pathologies and atherosclerosis (reviewed in [111; 115]. Increased ROS are suspected to play an important role in leading to NO depletion during inflammation. The eNOS enzyme can become uncoupled by ROS (thereby depleting

**Figure 1.3 Scheme of atherosclerotic plaque development.**
Activation of the endothelium leads to the expression of chemokines and adhesion molecules which bind and result in transmigration of leukocytes from the bloodstream to the subendothelium. Accumulation of oxidised low-density lipoprotein (LDL) and leukocytes in the subendothelium leads to formation of foam cells and a lipid-rich plaque. Migration and proliferation of smooth muscle cells (SMCs) lead to the formation of a fibrous cap.
production of NO), and in addition superoxide (produced by xanthine oxidase or NADPH oxidase, for example) may directly react with NO, forming the cytotoxic compound peroxynitrite, which can also induce eNOS uncoupling (reviewed in [115]).

1.1.5.2 Type 2 diabetes

Aside from being an independent risk factor for the development of atherosclerosis, diabetes itself is a condition of heightened ROS production and inflammation. Unlike Type 1 diabetes – an autoimmune disease in which the islet antigens are targeted and destroyed by the immune system – Type 2 diabetes is a condition characterised by chronic hyperglycaemia, leading to the

Figure 1.4 Role of ROS in Diabetes-induced atherosclerosis.

Under diabetic condition, hyperglycaemic induction of ROS production occurs via glycation reactions and the mitochondrial electron transport chain. In addition, the products of glycation reactions (advanced glycosylation end products, AGEs) induce ROS formation via the NADPH oxidases. An inflammatory response is initiated as a combination of the resultant endothelial dysfunction, production of growth factors, induction of signalling pathways and endothelial membrane expression of adhesion molecules (Figure has been adapted from [7]). ↑ Denotes an increase in a process while ↓ shows processes which decrease.
deterioration of islet β-cells which leads to a drop in insulin production. Regardless of disease type, however, a patient with diabetes suffers chronic hyperglycaemia, and this hyperglycaemia has been shown to induce a condition of oxidative stress leading to inflammation [116] (reviewed in [7; 117]).

As with atherosclerosis, increased levels of biomarkers of oxidative stress have been associated with diabetes, including F_{2}-isoprostanes [102-104]. In addition, increased levels of protein-S-glutathionylation [118], protein carbonylation (reviewed in [119]) and glutathione oxidation [120] (also reviewed in [121] and [122]) have been detected in humans and animal models with this condition.

The negative physiological effects of chronic hyperglycaemia in diabetes have now been strongly associated with increased generation of ROS. High concentrations of glucose inundate a number of metabolic pathways (including the involvement of glycation reactions and/or the mitochondrial electron transport chain), and each of these pathways results in higher levels of ROS production (reviewed in [7; 117; 123]). In addition, glycation reactions lead to the formation of advanced glycosylation end products (AGEs), which are known to activate NADPH oxidases (as is insulin). The net result of this oxidant production is not only suspected to result in further damage to pancreatic β cells (thereby worsening the condition), but also to induce the production of pro-inflammatory cytokines in vascular endothelial cells. The activation of pro-inflammatory pathways is likely to explain the link between diabetes and the associated increased risk of developing atherosclerosis in these patients (reviewed in [7]).

1.2 Thiol proteins

Two common reversibly oxidisable residues exist in proteins: methionine (Met, containing a thioether [RSR'] group) and cysteine (Cys, containing a thiol [SH] group). In addition, the less common selenocysteine residue can undergo reversible oxidation. Of these oxidisable residues, Cys has been most well-studied in the literature, and also forms the basis of this thesis. Therefore, this section will focus on Cys residues in protein and their involvement in redox homeostasis.
Ten to 20% of all Cys residues are estimated to be susceptible to oxidative modification, consisting of between $2.1 \times 10^5$ and $4.2 \times 10^5$ cysteines in the human proteome [124]. This susceptibility depends largely on the $pK_a$ of the thiol at physiological pH; most intracellular thiol residues have a $pK_a$ of $\sim 8.5$, making them highly protonated and poorly reactive under normal physiological conditions [2; 124; 125]. Conversely, readily oxidisable Cys residues generally have a low $pK_a$; these residues exist mainly in the thiolate (deprotonated, Cys-S) form and are more highly reactive in the physiological setting.

1.2.1 Redox chemistry of thiol residues

In the most reduced form, Cys residues are in the sulfhydryl (SH) state. Reversible two-electron oxidation of thiol groups generates the sulfenate (-SO) oxidation states, which can either stabilise (by forming a sulfenic acid) or react with nearby thiols to generate disulfides (-SS-).

*Figure 1.5 Redox modifications of thiol residues.*

In its most reduced sulfhydryl form, a thiol may be oxidised by $\text{H}_2\text{O}_2$ to generate a sulfenic acid, which may be resolved by the reversible formation of a disulfide or a cyclic sulfenamide. Alternatively, further oxidation of the sulfenic acid generates the (generally) irreversible sulfinic and sulfonic acids; hyperoxidation of the disulfide can generate the thiosulfinate or thiosulfonate residues. Thiols may also be redox modified by NO (to generate the nitrosothiol), or undergo glutathionylation by oxidation by glutathione disulfide. Figure is from [6].
Other reversible oxidative modifications of these residues include S-glutathionylation and S-nitrosylation, while the sulfinate (\(-\text{SO}_2\)) and sulfonate (\(-\text{SO}_3\)) forms are generally considered to be irreversibly oxidised. (There are, however, exceptions to this as seen with the reduction of peroxiredoxin-\(-\text{SO}_2\) by sulfiredoxin (refer to Section 1.3)). The possible transitions between the reduced and oxidised forms of protein thiols are outlined in Figure 1.5.

### 1.2.2 Thiols: redox switches and antioxidants

It has become evident that a wide variety of proteins undergo redox modification as a means to control cellular function, protein localisation and/or activity (reviewed in [6; 124; 126]). Due to the various types of modifications which can occur, as well as the potential for redox modification of multiple residues within a single protein, a complex range of thiol-dependent regulatory changes are possible. Functionally, proteins containing redox-sensitive thiols are responsible (and essential) for a wide range of cellular processes, from signal transduction (e.g. protein tyrosine phosphatase-1B, (PTP-1B), SH2-containing PTP (SHP2) and PTEN) [127], to receptor activation (e.g. N-methyl-D-aspartate (NMDA) and EGF receptors)[128; 129], and transcription factor activity (DNA binding and gene expression; e.g. NF\(\kappa\)B, Activator protein 1 (AP1), tumour suppressor p53, SP-1) [130-133].

Enzymes containing active site thiols which are susceptible to reversible oxidative modification have become recognised as ‘redox switches’; proteins may undergo changes in function akin to an “on-off” mechanism which switches depending on the oxidation state of their active site thiol residue (reviewed in [6; 134]). Classes of enzymes which depend on cysteines in their active sites include caspases, kinases, phosphatases and proteases [124], and many of these have been shown to be under this type of redox control. For instance, the activities of PTP-1B [127], GAPDH [135], caspase-3 [136] and Prxs [137] (refer also to Section 1.3) have been shown to be regulated by reversible oxidation of their active site cysteine residues.

Some antioxidant systems exploit the reactivity of oxidants with thiol residues. To protect cells against unwanted oxidative insult (to DNA, for example), these antioxidant molecules mop up intracellular ROS by undergoing reversible oxidation of one or more thiol residues through direct reaction with the target oxidant(s). One of the most well-known examples of this involves the tripeptide glutathione (GSH, discussed further in Section 1.2.2.1), which becomes oxidised by ROS and is reduced mainly via the glutathione reductase system (GR; Figure 1.6). Other
examples include the ubiquitous enzymes which make up the Prx enzyme family (Section 1.3) and the thioredoxins, as well as cysteine proteases [138; 139].

Although H$_2$O$_2$ is the primary oxidant which has been studied in the context of oxidative thiol modifications, it has become evident that other oxidants may modulate cellular processes via similar reactions. MPO-derived oxidants (refer to Section 1.1.2) including HOCl and chloramines, for instance, have been shown to reversibly oxidise thiol residues [11; 140-142].

1.2.2.1 Glutathione

One of the most well-known cellular thiol antioxidants is reduced glutathione, a tripeptide which overcomes the limitations of its high pKa (~8.8) by its high intracellular concentration (known to

![Figure 1.6 Redox control of glutathione.](image)

Control of GSH:GSSG ratio is under the enzymatic control of various enzymes. Depletion of cellular GSH by conjugation to electrophilic substrates (E) is accomplished by glutathione-S-transferases (GST). Glutathione peroxidases (GPx) catalyze the reduction of hydrogen peroxide with reducing equivalents from GSH, while the reduction of the oxidation product glutathione disulfide (GSSG) by glutathione reductase (GR) employs reducing equivalents from NADPH. Thiol proteins (P) can become S-glutathionylated; reduction of these is accomplished enzymatically by glutaredoxin (Grx). Inset shows the structure of reduced GSH.
occur in millimolar concentrations inside cells [143-145]). GSH and its primary oxidation product glutathione disulfide (GSSG) are maintained in non-equilibrium under physiological conditions [124], and a decrease in the ratio of GSH:GSSG is often measured and interpreted as an indication of increased oxidative stress.

Although GSH reacts slowly with $H_2O_2$ ($k = 0.9 \text{ M}^{-1}\text{s}^{-1}$) [144], its reactivities with MPO-derived oxidants such as HOCl and chloramines are substantially higher ($3 \times 10^7$ and 100-700 $\text{M}^{-1}\text{s}^{-1}$, respectively) [10; 11]. This makes GSH a likely candidate for protecting cells against the endogenous generation of oxidants during inflammation.

In addition to reacting directly with oxidants, GSH acts in co-operation with a system of antioxidant enzymes as shown in Figure 1.6. Reduced GSH reacts with oxidants such as $H_2O_2$ to generate the disulfide, GSSG, in a reaction catalysed by glutathione peroxidase (GPx); the reduction of GSSG to GSH is catalysed by GR. In addition, GSSG can participate in redox regulation of other proteins by S-glutathionylation of susceptible thiol residues, and this reaction is reversible by glutaredoxin (Grx). Finally, GSH levels may be depleted inside cells in the presence of electrophilic agents, which may become glutathionylated and subsequently exported; this process involves the activity of glutathione-S-transferase.

Therefore, GSH has important roles in cells, both as an antioxidant to directly scavenge oxidants and through its participation in redox-regulatory pathways.

### 1.3 Peroxiredoxins (Prxs)

The Prx family has attracted recent attention in terms of the potential antioxidant and redox signalling functions of these thiol enzymes. In humans, the family of Prxs consists of 6 members (Prxs 1 through 6) [22; 146-148]; these proteins are ubiquitous and highly expressed, even under normal physiological conditions. Along with a conserved molecular structure which increases their rate of oxidation, Prxs rely on a conserved low-pKa thiol residue at the active site to catalyse the reduction of peroxides (including hydrogen peroxide, organic hydroperoxides and peroxynitrite). Whereas $H_2O_2$ reacts with GSH with a rate constant of only $0.9 \text{ M}^{-1}\text{s}^{-1}$ [144], the rate constant for the reaction of $H_2O_2$ with Prx2 has been estimated at $1.3 \times 10^7 \text{ M}^{-1}\text{sec}^{-1}$ [9].
Chapter 1: Introduction

1.3.1 2-Cys Prxs: Structure and catalytic mechanism

Peroxiredoxins are classified into 3 types, depending on the number of conserved cysteines and mode of catalytic activity: typical 2-cysteine; atypical 2-cysteine; and 1-cysteine. All three types involve a common mechanism in which a conserved cysteine residue becomes oxidised by attacking the substrate, generating the cysteine sulfenic acid (Cys-SOH). The resolution of the Cys-SOH, however, differs between the three classes. Typical 2-Cys Prxs are obligate homodimers; they undergo resolution of the peroxidatic Cys-SOH (generally near residue 50) of one monomer by the C-terminal Cys (near residue 170) of an adjacent monomer, forming an intermolecular disulfide. Atypical 2-Cys Prxs undergo this same condensation reaction but the disulfide forms between the peroxidatic and resolving Cys of the same subunit; these are therefore functionally monomeric. On the other hand, in 1-Cys Prxs the Cys-SOH forms a disulfide with a small molecule thiol (such as GSH) or other protein thiol (the catalytic mechanisms of these isoforms are reviewed in [22; 149]. For the purposes of this thesis, this section will focus on the typical 2-Cys Prxs (1-4), which are the focus of Chapters 3 and 4.

Although Prxs 1-4 share a catalytic mechanism but differ in their intracellular localisations. Prxs 1 and 2 are primarily cytosolic (Prx1 has been shown to undergo nuclear translocation [150]), while Prx3 is confined to the mitochondria and Prx4 is localised to several organelles: the endoplasmic reticulum, lysosomes, and in proximity to mitochondria [151-153]. The catalytic mechanism of typical 2-Cys Prxs in the reduction of peroxides involves attack of the substrate by the active-site cysteine (the peroxidatic Cys), oxidising the residue to a sulfenic acid (-SOH). This sulfenic acid residue is resolved by attack from a second cysteine residue (the resolving Cys), on an adjacent monomer; this results in the formation of a disulfide-linked homodimer [22] (Figure 1.7). The thioredoxin system in most cells is capable of rapidly recycling the disulfide-linked Prxs back to the reduced form, making this system an efficient peroxide detoxification system. Alternatively, in the presence of high peroxide concentrations, the peroxidatic sulfenic acid may become hyperoxidised to a sulfinic or sulfonic acid before resolution by the resolving cysteine. This hyperoxidation of Prxs appears to occur only in situations where Prxs are actively cycling, as erythrocytes with low TrxR activity show very little propensity for Prx2 hyperoxidation [65]. This inactivation was long thought irreversible before it was determined that sulfiredoxin is capable of slowly reducing the hyperoxidised form to the active, reduced thiol form of the enzyme [154; 155], though hyperoxidation to the sulfonic acid is generally considered irreversible [156-158].
1.3.2 Reactivity of 2-Cys Prx

Prx oxidation status has been proposed as a potential sensor or indicator of in vivo oxidative stress, and the sensitivity of Prxs to oxidation by H$_2$O$_2$ in a variety of cell types has been investigated [65; 148; 159-161]. Human erythrocyte Prx2 reacts extremely rapidly with H$_2$O$_2$, with a rate constant of $\sim$10$^7$ M$^{-1}$s$^{-1}$, similar to the rates for catalases and glutathione peroxidases [9], and far greater than that for the reaction of GSH with H$_2$O$_2$ (Table 1.2).

1.3.2.1 Reactivity with MPO-derived oxidants

Generally, thiols react more quickly with chloramines (10$^2$ to 10$^3$ times faster) than with peroxides [9; 47; 48; 144]. Because of their low pKa thiol active sites and high reactivities with H$_2$O$_2$, Prxs are likely candidates for reaction with cell-permeable MPO-derived oxidants such as

![Figure 1.7 The catalytic cycle of typical 2-cys peroxiredoxins.](image)

Peroxiredoxins undergo oxidation of the peroxidatic cysteine to a sulfenic acid, which results in the formation of disulfide-linked homodimers. The dimer is rapidly reduced by the thioredoxin system. In the presence of high amounts of H$_2$O$_2$, however, the peroxidatic cysteine becomes hyperoxidised to the sulfinic or sulfonic acid form; sulfiredoxin slowly reduces the sulfinic form back to the catalytically active reduced (thiol) form.
HOCl and chloramines (refer to Section 1.1.2) [47; 48]. However, Prxs are unusual thiol proteins in that they are far more selective for peroxides than for chloramines (Table 1.2).

Table 1.2 Second-order rate constants for the reaction of H$_2$O$_2$ and chloramines with GSH and Prx2.

<table>
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<th>GSH</th>
<th>Prx2</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O$_2$</td>
<td>1$_{[3]}$</td>
<td>1.3×10$^7$$_{[9]}$</td>
</tr>
<tr>
<td>NH$_2$Cl</td>
<td>1×10$_3$</td>
<td>1.5×10$^4$</td>
</tr>
<tr>
<td>GlyCl</td>
<td>2.3×10$<em>2$$</em>{[10]}$</td>
<td>8</td>
</tr>
<tr>
<td>TauCl</td>
<td>1.2×10$<em>2$$</em>{[10]}$</td>
<td>3</td>
</tr>
<tr>
<td>HOCl</td>
<td>3×10$<em>7$$</em>{[11]}$</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Table is from [12], as are values lacking references. N.D. = value not determined.

Recently, it was shown that erythrocyte Prx2 is oxidised by exogenous addition of chloramines, and that purified Prx2 reacts with chlorinated oxidants [12]. However, very little investigation has explored the ability of the various Prxs to react with non-peroxyl groups or other oxidants; nor whether Prxs might behave differently inside living cells than expected from the rate constants.

1.3.3 Peroxiredoxins and disease

The 2-Cys Prxs (Prxs 1-4 in humans) have been shown to be oxidised in cells under a variety of stresses (including oxidative stress and shear stress), and are overexpressed and/or detected in the oxidised form in various disease states. Many of these diseases are at least partially characterised by a heightened inflammatory response. For example, Prx1 is up-regulated in lungs as a results of hyperoxia-induced oxidative stress [162-167], and both Prxs 1 and 2 are up-regulated in mouse testis following ionising radiation [168]. Up-regulation of 2-Cys Prxs has also been observed in relation to cancer [165; 169; 170], atherosclerosis [171; 172], Alzheimer’s disease [173] and Parkinson’s disease [174] (reviewed in [3]).

Although it is not yet clear exactly what the processes are which lead to Prx overexpression, overexpression of these enzymes appears to have a protective antioxidant effect. One of the
transcription factors known to induce Prx1 expression (as well as several other key antioxidant enzymes) is Nrf2, which is discussed below (Section 1.4.1.1).

Models of Prx deficiency have shown that Prxs have non-redundant functions in vivo, and have highlighted various disease pathologies which have resulted in these models. For instance, mice deficient in Prx1 [175; 176] and Prx2 [76] have been shown to develop haemolytic anaemia; the Prx1−/− mice were reported to have decreased lifespan and also develop various malignant tumours while Prx2 deficient mice suffer enlarge spleens (splenomegaly). Increased sensitivity to oxidative stress has also been demonstrated in mouse models of Prx3 [177; 178] and Prx6 [179-181] deficiencies.

1.4 Haem oxygenase 1

1.4.1 Induction of haem oxygenase-1 (HO-1)

HO-1 (otherwise known as inducible haem oxygenase) is considered one of the main genes under Nrf2 transcriptional control, and is an enzyme with strong links to inflammatory disease [182-184]. Unlike HO-2, which is non-inducible and constitutively expressed, HO-1 is inducible by an increasingly wide range of stimuli (reviewed in [185]). Various signal transduction pathways and transcription factors have been implicated in the upregulation of HO-1, including members of the MAPK signalling cascades [186; 187] and regulatory element binding of various proteins including early growth response-1 (EGR-1) [188], NF-κB [187], and AP-1[189; 190](reviewed in [3]). Studies have uncovered a multitude of ways in which HO-1 appears to be regulated under varying cellular stresses and in different cell types, highlighting the complexity of the HO-1 response (refer to Chapter 6 for further discussion of HO-1 expression). Nrf2 is nevertheless the most well-studied transcription factor involved in HO-1 expression (reviewed in [3; 191]), and is discussed below.

1.4.1.1 Nuclear factor-erythroid-2-related factor 2 (Nrf2)

Nrf2 is a basic leucine zipper transcription factor which is usually bound to its repressor, Kelch-like ECH-associated protein 1 (Keap1), which targets it for ubiquitination and proteasomal degradation. Upon stimulation (usually involving oxidant- or electrophile-mediated modification of specific cysteine residues on Keap1), Nrf2 is released, allowing its translocation to the nucleus, where it can bind to the antioxidant responsive element or electrophile responsive element (ARE
or EpRE, respectively; cis-acting elements) in the regulatory region of various genes encoding phase II detoxification enzymes and antioxidant proteins (reviewed in [3; 8]). Thus, Nrf2 has a protective effect in cells by regulating endogenous defences, and has been shown to have an important role in both intracellular redox signalling and protection against carcinogens (reviewed in [192-194]).

1.4.1.2 Targets of Nrf2

Many phase II drug-detoxification enzymes and antioxidant enzymes are expressed constitutively at low levels to protect cells against oxidative damage. Upon cellular stimulation with a variety of compounds (such as oxidants, electrophiles, or phenolic compounds), Nrf2 is activated and binds to the ARE of various genes, inducing transcription and cellular accumulation of the enzymes. Nrf2 has been shown to have a role in both the constitutive and inducible expression of these protective antioxidant enzymes, which include Prxs, haem oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase, glutathione-S-transferase, glutathione peroxidase and thioredoxin (reviewed in [83]).

1.4.2 HO-1 enzymatic activity

HO-1 is a microsomal enzyme which catalyses the breakdown of free haem into biliverdin, iron \((\text{Fe}^{2+})\) and carbon monoxide (CO) (Figure 1.8). This enzyme spans the ER membrane [195] and its induction and activity have been shown to have important anti-oxidant, anti-apoptotic, and anti-inflammatory activities both \textit{in vitro} and \textit{in vivo} (discussed below).

1.4.2.1 HO-1 as an antioxidant

HO-1 induction in various human cell types has been shown to defend against oxidative stress induced by a wide range of stimuli, and in a range of pathological conditions. Cells exposed to various stimuli including haemin [196], cigarette smoke [197; 198], lipopolysaccharide [199], TauCl [55; 72] and nitric oxide (reviewed in [185]), have been determined to have heightened levels of HO-1 expression.

Not only is HO-1-dependent haem degradation likely to be important for mediating the cytoprotective effects of this enzyme, but its products biliverdin/bilirubin and CO (Figure 1.8) also have important roles in maintaining cell homeostasis. Low levels of bilirubin (which can
result from low HO-1 activity) have been linked to an increased risk for coronary artery disease [200-203]. CO also has various cytoprotective roles, presumably through inhibition of NADPH oxidase assembly [55] and/or indirect modulation of MAPK signalling cascades. Although the precise mechanisms are still unknown, CO has been shown to decrease the MAPK-dependent expression of various pro-inflammatory cytokines (including interleukin-6 (IL-6) and TNF-α), and to inhibit apoptosis, proliferation and thrombosis (reviewed in [185]).

1.4.3 Role of HO-1 in endothelial cells

1.4.3.1 Role of HO-1 in models of vascular disease

Studies using mice have been important in understanding the role of HO-1 in the context of disease, as well as the control of HO-1 expression under these conditions. Of interest, a number

![Diagram of enzymatic haem degradation by haem oxygenase.](image)

**Figure 1.8** Products of the enzymatic haem degradation by haem oxygenase.
of mutant models were developed which have highlighted an important role for HO-1 in the prevention of atherosclerosis (reviewed in [204]). Mice were protected against experimental atherosclerosis by haemin-induced HO-1 expression [205] or adenoviral vector-delivered HO-1 [206; 207]. In addition, various studies have shown an inverse relationship between HO-1 expression and the thickening of the intima (a negative result of inflammation in damaged blood vessels) following vascular injury [208-211]. Two cases of human HO-1 deficiency have been documented to date [212-214], and these have illustrated both a strong similarity to deficient mouse phenotypes of the deficiency (including iron deposition in liver and kidney, anaemia, growth retardation and severe inflammation), and the critical role of this enzyme in maintaining homeostasis.

1.4.3.2 Roles in atherosclerosis and associated disease pathologies
HO-1 activity in endothelial cells has been strongly linked to vascular protection and the prevention of cardiovascular disease [215]. Recently, Current Drug Targets published a series of reviews which summarised the role of HO-1 enzyme in a host of inflammatory conditions, including lung disease [216], liver inflammation [217], and cancer [192]. HO-1 also has strong links to diabetes; specific polymorphisms in the promoter region of the HMOX1 gene (which encodes HO-1) are correlated with a higher incidence of diabetes [218].

The induction of HO-1 under these conditions of inflammation is considered a protective event, since modulation of its expression has protective effects on cells and animals when increased and these effects are reversed if HO-1 protein or activity levels are decreased or inhibited. For this reason, HO-1 has become an enzyme of great interest as a potential drug target for inflammatory diseases. In macrophages, these anti-inflammatory effects are thought to be mediated by the co-ordinated suppression of pro-inflammatory cytokines and induction of anti-inflammatory cytokines (reviewed in [3]; Figure 1.9).

1.4.4 Effect of MPO-derived oxidants on HO-1 expression
The induction of HO-1 by MPO-derived oxidants has received some attention. Both TauCl and HOCI have been reported to induce an Nrf2-dependent increase in HO-1 mRNA and protein levels [55; 72; 219; 220]. Many questions remain unanswered regarding the activation of this system by chlorinated oxidants, as the studies mentioned above employed prolonged oxidant treatments in cell culture medium, with high doses of oxidant. Concentrations of TauCl of at
least 500 μM were applied, while HOCl was generally applied at concentrations in excess of 100 μM (though Wei et al. [221] did report effects at 24 h with doses of HOCl down to 10 μM), and treatments were sustained for at least 1 h, and up to 24 h. Therefore, although there has been some indication that the Nrf2-HO-1 system is activated in response to inflammatory oxidants, the conclusions which can be drawn from these studies are limited. Further study of this system in the context of inflammation is required.

**Figure 1.9 Activation of HO-1 and the downstream effects on inflammation.**
Pro-oxidant and pro-inflammatory pathways activate signalling cascades (e.g. MAPK pathways) which in turn initiate the transcription and activation of HO-1. The products of HO-1-induced haem degradation inhibit chronic inflammation both by inducing anti-inflammatory cytokines and inhibiting proinflammatory cytokines. (Figure is adapted from [3])
1.5 **Aims of the thesis**

It is widely accepted that inflammation plays a key role in mediating some of the disease pathologies which characterise conditions like atherosclerosis. Heightened levels of inflammation are associated with increased levels of ROS, as a result of MPO activity in activated neutrophils. Although there is some evidence for the involvement of chloramines in various cell signalling cascades, the specific intracellular targets of MPO-derived oxidants (while assumed to be thiol-based) are still largely unknown. Understanding how oxidants such as HOCl and chloramines act in cells to induce signalling cascades, apoptosis or antioxidant responses will be key to advancing our knowledge of inflammation toward potential therapies to protect against these oxidants’ negative effects. To help elucidate these areas, this thesis employs primarily an endothelial model system relevant to the inflammatory condition.

Because HOCl and chloramines are known to readily react with thiol residues, thiol proteins are a particular focus of this thesis. Peroxiredoxins and GSH are abundant antioxidant systems responsible for protecting cells against oxidative stress, and redox status of these enzymes is explored in cells treated with chlorinated oxidants. In addition, a proteomic analysis is used to uncover novel thiol targets of these oxidants, which could be relevant in antioxidant and/or signalling functions under conditions of inflammation.

HO-1 is another antioxidant system with strong links to inflammation, but limited studies have explored its activation in the context of inflammatory oxidants. HO-1 activation is also investigated using the endothelial model here in response to chloramine-induced oxidative stress.

Thus, the general aims of this thesis are to:

1) Explore the redox effects of HOCl and chloramines on endogenous thiol antioxidants (Peroxiredoxins and GSH);
2) Identify specific proteins which undergo reversible thiol oxidation upon treatment with HOCl and chloramines; and
3) Investigate the effect of exogenous addition of chloramine on Nrf2 activation and HO-1 transcription.
Chapter 2. Methods

2.1 Materials

A list of materials used in the thesis is presented in Table 2.1.

Table 2.1 Materials used in the thesis

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<tr>
<td>Medium 199</td>
<td>Gibco-BRL (Invitrogen)</td>
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<td>Minimum Essential Medium (MEM, 10X)</td>
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</tr>
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| Cell treatment reagents                          |                                   |                        |
| Hypochlorous acid (Janola)                       | Sara Lee                          | Auckland, NZ           |
| Sulforaphane                                      | LKT Laboratories Inc.             | St Paul, MN, USA       |
| Hydrogen peroxide                                 | LabServ supplied by BioLab        | Victoria, Australia    |
| Auranoquin                                        | ICN Biomedicals Inc.              | Costa Mesa, CA, USA    |

| GSH Assays                                        |                                   |                        |
| Reduced glutathione (GSH)                         | Sigma-Aldrich                     | St Louis, MO, USA      |
| Oxidised glutathione (GSSG)                       | Sigma-Aldrich                     | St Louis, MO, USA      |
| Glutathione sulfonamide (GSA)                     | Sigma-Aldrich                     | St Louis, MO, USA      |
| Monobromobimane (MBB)                             | Calbiochem                        | La Jolla, CA, USA      |

| TrxR Assays                                       |                                   |                        |
| NADPH                                            | Sigma-Aldrich                     | St Louis, MO, USA      |
| 5,5′Dithiobis(2-nitrobenzoic acid)                | Sigma-Aldrich                     | St Louis, MO, USA      |
| Bovine Serum Albumin (BSA)                        | Gibco-BRL (Invitrogen)            | Auckland, NZ           |

| Peroxiredoxin assays                              |                                   |                        |
| Bovine catalase                                   | Sigma-Aldrich                     | St Louis, MO, USA      |
| N-ethylmaleimide                                  | Sigma-Aldrich                     | St Louis, MO, USA      |

<p>| Antibodies                                        |                                   |                        |
| Polyclonal rabbit anti-peroxiredoxin 1            | AbCam Ltd.                        | Cambridge, UK          |
| Polyclonal rabbit anti-peroxiredoxin 2            | Sigma-Aldrich                     | St Louis, MO, USA      |
| Polyclonal rabbit anti-peroxiredoxin 3            | AbFrontier Co. Ltd.               | Seoul, Korea           |
| Polyclonal rabbit anti-peroxiredoxin (SO2/3)      | AbFrontier Co. Ltd.               | Seoul, Korea           |
| Polyclonal Goat anti-Nrf2                         | Santa Cruz Biotechnology, Inc.    | Santa Cruz, CA, USA    |</p>
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**Cell viability assays**

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**Immunofluorescence**

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**Chapter 2: Materials and Methods**

Polyclonal Rabbit anti-haem oxygenase 1 (Hsp32)

Monoclonal Mouse anti-β-actin

Polyclonal Rabbit anti-β-tubulin III

Polyclonal Mouse Anti-Parp1

Polyclonal rabbit anti-cyclophilin A

Goat anti-rabbit peroxidase conjugate

Goat anti-mouse peroxidase conjugate

Rabbit anti-goat peroxidase conjugate

Cell viability assays

Propidium Iodide

Thiazolyl Blue Tetrazolium Bromide (MTT)

Immunofluorescence

Fluoromount-G™

Hoechst 33342

Electrophoresis

Tris

40% acrylamide/bis solution 37.5:1

Benchmark™ pre-stained protein ladder

Hybond-P™ polyvinylidene fluoride (PVDF) membrane

ECL™ Plus western blotting detection system

Tween® 20

Bio-Lyte® 3-10 ampholytes

Readystrip pH 5-8 IPG strips

Mineral oil

Electrode wicks

DNA grade agarose

β-mercaptoethanol

Bromophenol blue

Sodium Dodecyl Sulfate (SDS)

PlusOne™ Tris

PlusOne™ Urea

PlusOne™ Thioureia

PlusOne™ Sodium Dodecyl Sulfate (SDS)

PlusOne™ Bromophenol blue

PlusOne™ Glycerol

PlusOne™ Dithiothreitol (DTT)

Miscellaneous

Dc Protein assay kit

Trichloroacetic acid (TCA)

Micro Bio-Spin 6 columns

DL-Dithiothreitol (DTT)

Iodoacetamide

Iodoacetamidofluorescein (IAF)

CHAPS

Chloroform

Dimethyl sulfoxide, Hybri-Max™

CompleteTM protease inhibitors

PhosSTOP™ phosphatase inhibitors

Xylenol orange
Chapter 2: Materials and Methods

### Sodium azide
Fisons, Loughborough, Leics, UK

### 3,3′,5,5′-tetramethylbenzidine (TMB)
Sigma-Aldrich, St Louis, MO, USA

### Tris(2-carboxyethyl)phosphine hydrochloride (TCEP)
Sigma-Aldrich, St Louis, MO, USA

### Acetic Acid
LabServ supplied by BioLab, Victoria, Australia

### Phorbol 12-myristate 13-acetate
Sigma-Aldrich, St Louis, MO, USA

All other chemicals were from BDH Laboratory Supplies (Poole, England), MERCK Ltd. (Palmerston North, NZ) and Sigma-Aldrich (St Louis, MO, USA)

#### 2.2 Buffers

A list of the buffers and solutions used in this thesis is presented in Table 2.2.

### Table 2.2 Buffers used in the thesis

<table>
<thead>
<tr>
<th>Buffer Name</th>
<th>Abbreviation</th>
<th>Recipe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate-buffered saline</td>
<td>PBS</td>
<td>140 mM NaCl, 13 mM KCl in 10 mM sodium phosphate buffer, pH 7.4</td>
</tr>
<tr>
<td>Hank’s balanced salt solution</td>
<td>HBSS</td>
<td>140 mM NaCl, 13 mM KCl, 0.5 mM MgCl2, 1 mM CaCl2 and 5.5 mM glucose in 10 mM sodium phosphate buffer, pH 7.4</td>
</tr>
<tr>
<td>FOX reagent</td>
<td></td>
<td>100 µM xylanol orange, 250 µM ferric ammonium sulfate, 100 mM sorbitol, 25 mM sulfuric acid</td>
</tr>
<tr>
<td>Extract buffer</td>
<td></td>
<td>40 mM HEPES, pH 7.4, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, Complete™ protease inhibitors* and 1% Triton X-100 [w/v]</td>
</tr>
<tr>
<td>SDS sample buffer</td>
<td></td>
<td>65.8 mM Tris-HCl, pH 6.8, 10.5% glycerol [v/v], 2.1% SDS [w/v], 0.053% bromophenol blue [v/v], with or without 5% β-mercaptoethanol [v/v] respectively</td>
</tr>
<tr>
<td>Tris-buffered saline</td>
<td>TBS</td>
<td>20 mM Tris-HCl, 140 mM NaCl, pH 7.6</td>
</tr>
<tr>
<td>Tris-buffered saline with Tween-20</td>
<td>TBST</td>
<td>TBS containing 0.05% Tween-20 [w/v]</td>
</tr>
<tr>
<td>Coomassie R250 Solution</td>
<td></td>
<td>0.25% Coomassie Brilliant Blue R-250 [w/v], 45% ethanol [v/v], 45% water [v/v], 10% acetic acid [v/v]</td>
</tr>
<tr>
<td>Coomassie destain solution</td>
<td></td>
<td>45.5% ethanol, 45.5% water, 9% acetic acid</td>
</tr>
<tr>
<td>Silver Stain Solution 1</td>
<td></td>
<td>0.2 g/l Na₂S₂O₃</td>
</tr>
<tr>
<td>Silver Stain Solution 2</td>
<td></td>
<td>2 g/l AgNO₃, 750 µl/l formaldehyde</td>
</tr>
<tr>
<td>Silver Stain Solution 3</td>
<td></td>
<td>60 g/l Na₂CO₃, 20 ml/l Silver Stain Solution 1, 500 µl/l formaldehyde</td>
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<tr>
<td>Silver Stain Solution 4</td>
<td></td>
<td>20 g/l EDTA</td>
</tr>
<tr>
<td>Cell detachment buffer</td>
<td></td>
<td>150 mM NaCl and 1 mM EDTA in 40 mM Tris-HCl, pH 7.6</td>
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<tr>
<td>Buffer A</td>
<td></td>
<td>10 mM KCl, 0.1 mM EDTA, 1 mM DTT in 40 mM Tris-HCl, pH 7.6, Complete™ protease inhibitors* and 0.5 % NP-40 [v/v]</td>
</tr>
<tr>
<td>Buffer B</td>
<td></td>
<td>(1 mM EDTA, 1 mM DTT, 400 mM NaCl, 1.5 mM MgCl₂ and 25% [v/v] glycerol in 20 mM Tris-HCl), 1.6 mg/ml Complete™ protease inhibitors* and PhosStop™ phosphatase inhibitors*</td>
</tr>
<tr>
<td>Sample rehydration buffer</td>
<td></td>
<td>7 M urea, 2 M thiourea, 10 mM DTT, 1% CHAPS [w/v], 0.2% Bio-Lyte 3-10 Ampholytes [v/v], 0.05% glycerol [v/v] and a few crystals of bromophenol blue</td>
</tr>
<tr>
<td>Equilibration buffer</td>
<td></td>
<td>6 M urea, 20 % glycerol [v/v], 2% SDS [w/v] and a few crystals of bromophenol blue in 0.375 M Tris-HCl, pH 8.8</td>
</tr>
</tbody>
</table>
Buffer Name | Recipe
--- | ---
Agarose solution | 0.5 % agarose [w/v], 24.8 mM Tris, 192 mM glycine, 0.1% SDS, and a few crystals of bromophenol blue

*Complete™ protease inhibitors and PhosSTOP™ phosphatase inhibitors were freshly added to buffers immediately before use, and employed as per the manufacturer's instructions (1× final concentration).

### 2.3 Erythrocytes

#### 2.3.1 Cell isolation

Human blood from healthy volunteers was collected, with informed consent, into heparinised tubes. Plasma was removed and erythrocytes were washed three times with at least 1.5 volumes of ice-cold PBS. With each wash removal, the buffy coat and/or top layer was also aspirated. Cells were suspended in either PBS alone or PBS with 5 mM glucose, and the cell concentration of packed erythrocytes was estimated using a haemocytometer. Erythrocyte suspensions were maintained on ice during preparation.

### 2.4 HUVECs

#### 2.4.1 Cell isolation and culture

Human Umbilical Vein Endothelial Cells (HUVECs) were isolated according to the methods of Jaffe et al [164]. Umbilical cords were obtained with informed parental consent; ethics were approved by the Upper South A Regional Ethics Committee (ethics ref CTY/02/12/209). Processing of cords for isolation of HUVECs occurred within 24 h of birth, with cords maintained at 4°C in PBS.

Cords were cannulated and the veins rinsed with sterile saline solution to rid of any blood and/or clots; cords were carefully inspected for any mechanical damage, which could result in detachment of smooth muscle cells (and subsequent contamination of HUVEC cultures). Sterile collagenase solution (100 U/ml in 10 mM phosphate buffered saline, pH 7.4) was used to disrupt the endothelium by filling the cord vein with the solution and incubating in a 37°C water bath for 12 min. Endothelial cell disruption was completed by gentle kneading of the cord before vigorous flushing the vein with the collagenase solution through several rounds of filling and draining of the cord. Finally, the solution containing detached cells was removed and added to sterile complete M199 medium (containing 12% heat-inactivated Cosmic Calf serum [v/v], 100 μg/ml heparin, 40 μg/ml endothelial cell growth supplement, 100 U/ml penicillin and 100
μg/ml streptomycin). Cells were pelleted by centrifugation at 1000×g for 4 min; the supernatant was removed and cells were gently resuspended in fresh, complete M199. The cell suspension was transferred to a 75 cm² flask pre-coated with 0.1% gelatine [w/v]; 0.5% Fungizone™ [v/v] was added to the flask and viable cells were allowed to adhere overnight. HUVEC were maintained over several days by washing with PBS and addition of M199 and Fungizone™.

Following harvest, HUVECs were stored long-term in liquid nitrogen for later use. Endothelial monolayer was disrupted by addition of 4 ml TrypLE® Express trypsin substitute to washed subconfluent (~80% confluent) cells in each 75 cm² flask for 5 min. Complete M199 was added to suspended cells before collection by centrifugation at 1000×g for 4 min. Cells from one 75 cm² flask were resuspended in 1 ml ice-cold Freezing Medium (MEM [10X stock used for 1X final concentration] containing 5% DMSO [v/v], 75% heat-inactivated Cosmic Calf serum [v/v], 200 U/ml penicillin and 200 μg/ml streptomycin, and 0.2% NaHCO₃ [w/v]) and transferred to a single sterile cryotube, on ice. For cryopreservation, cryotubes were transferred to a Nalgene® Mr. Frosty freezing container containing isopropanol for cooling to -80ºC at a rate of 1ºC min⁻¹. Cells were subsequently stored in liquid nitrogen until further use.

Subculture of confluent cells was accomplished by addition of TrypLE® Express trypsin substitute for 5 min. M199 was added to detached cells before centrifugation at 1000×g for 4 min and cells were resuspended in fresh complete M199. Cells were treated at confluence (~5 – 6 × 10⁴ cells/cm²) by the 5th passage after thawing.

### 2.5 Hepa 1c1c7

#### 2.5.1 Culture

The Hepa 1c1c7 cell line (ATCC® number CRL-2026™) is a murine hepatoma epithelial cell originally harvested from C57L mouse liver. Hepa 1c1c7 cells were maintained in a humidified atmosphere with 5% CO₂ at 37ºC. Cells were grown directly on tissue culture dishes, in Minimum Essential Medium alpha (MEM-α) with renewal every 2 days. Subculture of subconfluent cells was performed by disruption of the monolayer using TrypLE® Express. Cells were passaged or treated at ~75% confluence.
2.6 **Cell Treatments**

2.6.1 **Chloramines and hypochlorous acid**

2.6.1.1 *Chloramines and TNB Assay*

Glycine chloramine (GlyCl), taurine chloramine (TauCl), and monochloramine (NH$_2$Cl) were freshly prepared on the day of use by mixing a 10:1 molar excess of amine solution with HOCl (added drop-wise with vortexing), thereby ensuring no dichloramine production [222]. Chloramine concentration was determined by reaction with 5-thio-2-nitrobenzoic acid (TNB). After a 15 min incubation in the dark, the change in absorbance was monitored at 412 nm ($\lambda_{412}=14,100$ M$^{-1}$ cm$^{-1}$)[166]. Chloramine solutions were kept on ice and generally prepared at ~5 mM stock and used within 1 h. For treatment, the solutions were diluted in PBS or HBSS pre-warmed to 37ºC immediately before being applied to cells.

2.6.1.2 *Hypochlorous acid*

The concentration of HOCl in stock solution was determined spectrophotometrically after dilution in NaOH, final pH≥10 ($\varepsilon_{292}=350$ M$^{-1}$cm$^{-1}$). HOCl treatments were made up to the desired concentration by dilution in PBS (for treatment of erythrocytes) or HBSS (for treatment of HUVECs) immediately before applying to cells. Immediate, vigorous mixing ensured that the cells in the sample received a homogenous dose of the oxidant. The pK$_a$ of HOCl is 7.5 [27]; therefore, at the pH used in experiments HOCl and OCl$^-$ would be present in approximately equal concentrations. For the sake of brevity, the oxidant is herein referred to as HOCl. Alternatively, HOCl concentration was determined by the TNB assay, as above.

2.6.1.3 *Hypothiocyanous acid (HOSCN)*

Enzymatic production of HOSCN was performed at 4º C using the method described [223]. Sodium thiocyanate (NaSCN, 7.5 mM) was prepared in 10 mM potassium phosphate buffer (pH 6.6). To the NaSCN, 2 µM lactoperoxidase was added, followed by four additions of 10 µl/ml H$_2$O$_2$ (from 75 mM stock solution), each addition separated by a 1 min interval. HOSCN concentration was determined by the TNB assay, as above.
2.6.1.4 Intact erythrocytes
For treatment of intact erythrocytes, equal volumes of suspended erythrocytes (in either PBS alone or PBS with 5 mM glucose, as indicated) and oxidant (chloramine or HOCl, diluted in the same buffer as the erythrocytes) were mixed for a 0.25% final cell suspension (2.5 × 10^7 cells/ml). Erythrocytes were added with immediate mixing by pipette to ensure an equal distribution of the oxidant and incubated at 37°C with occasional gentle mixing to maintain the cells in suspension. To quench any remaining oxidant and thereby interrupt the treatment, 5 mM methionine was added at the end of the time course.

2.6.1.5 Lysed erythrocytes
For treatment of haemolysate, a 10% cell suspension of erythrocytes was prepared in PBS. Cells were lysed by freezing at −80°C and thawing. Lysate (25 μl, representing the same amount of cellular material as was used in whole-cell experiments) was added to the desired amount of GlyCl (in PBS) and immediately mixed. Treated lysate was incubated at 37°C for 20 min before quenching by addition of 5 mM methionine. For samples destined for Western blot analysis of Prx2, PBS in these preparations contained 1 ng/ml catalase to inhibit spontaneous oxidation of the peroxiredoxin upon cell lysis.

2.6.1.6 HUVECs and HEPA 1c1c7 cells
HUVECs and HEPA 1c1c7 cells were grown in various plate sizes, including: 24-well (2 cm^2), 6-well (9.6 cm^2) and 100 × 20mm culture dishes. Treatment volumes and protein yield for the various plate sizes are shown in Table 2.3 (below).

<table>
<thead>
<tr>
<th>Dish size</th>
<th>Surface area (cm^2)</th>
<th>Approximate number of cells</th>
<th>Total protein (μg) (mean ± SEM, n=6)</th>
<th>Treatment vol (ml)</th>
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</thead>
<tbody>
<tr>
<td>24-well plate</td>
<td>2.0</td>
<td>(HUVEC) 1.2 × 10^5, (HEPA) 2.8 × 10^5</td>
<td>39.6 ± 4.8</td>
<td>1.0</td>
</tr>
<tr>
<td>6-well plate</td>
<td>9.6</td>
<td>(HUVEC) 5.8 × 10^5, (HEPA) 13.4 × 10^5</td>
<td>203 ± 8, 267 ± 19</td>
<td>1.0*</td>
</tr>
<tr>
<td>100-mm culture dish</td>
<td>78.5</td>
<td>(HUVEC) 47.1 × 10^5, (HEPA) 110 × 10^5</td>
<td>976 ± 210, 1880 ± 560</td>
<td>4.0</td>
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</table>

All values shown are per well for 24- or 6-well plates or per plate for 100-mm dishes.
*Except where specified; 2.0-2.5 ml M199 were used for some treatments.
*n=1 measurement of protein for this plate size.
2.6.2 Hydrogen peroxide

The concentration of $\text{H}_2\text{O}_2$ in stock solutions was quantified by measuring $A_{240}$ ($\varepsilon_{240} = 43.6 \text{ M}^{-1}\text{ cm}^{-1}$). $\text{H}_2\text{O}_2$ was diluted into HBSS or M199 immediately before addition to cells. No differences were observed in either the concentration of $\text{H}_2\text{O}_2$ in HBSS or M199 alone, nor in the effect of cells treated in either buffer (i.e. Prx oxidation, morphology as observed visually). Where possible, HUVECs were treated with $\text{H}_2\text{O}_2$ diluted in M199 except where directly compared with the effect of a chloramine or HOCl treatment. Cells cultured in 24-well plates were treated in a total volume of 1 ml whereas those cultured in 6-well plates were treated in 2.5 ml.

2.6.3 Carbon monoxide

Inhibition of haemoglobin autoxidation in erythrocytes was achieved by pretreatment with carbon monoxide; this involved gentle bubbling of a 0.25% erythrocyte cell suspension with the gas until a colour change from red to pink was observed. An approximate 7-nm shift of the oxyhaemoglobin peak ($\sim 577$ nm) was verified spectrophotometrically to confirm the conversion of oxyhaemoglobin to carboxyhaemoglobin ($\sim 570$ nm).

2.6.4 Sodium azide

Inhibition of erythrocyte catalase was achieved by incubation of a 0.25% erythrocyte cell suspension with 5 mM sodium azide for 5 min. Spectrophotometric analysis of $\text{H}_2\text{O}_2$ ($\lambda = 230$ nm) [224] confirmed that this incubation achieved an approximate 70% decrease in the rate of $\text{H}_2\text{O}_2$ consumption.

2.6.5 Auranofin

A stock solution of auranofin was prepared at 4 mM in DMSO and stored at -20°C. Auranofin was diluted to a working concentration in M199 or HBSS and applied to washed HUVECs.

2.7 Consumption of HOCl or chloramines

Consumption of chlorinated oxidants by erythrocytes or HUVECs was assessed using a spectrophotometric assay that measures the iodide-catalysed oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) [225]. TMB was dissolved in dimethylformamide for a stock concentration of 20 mM. TMB reagent consisted of a final concentration of 2 mM TMB and
100 μM NaI in 0.1 M Sodium Acetate buffer, pH 5.4 (10% final concentration of dimethylformamide [v/v]).

For this assay, chloramine and HOCl concentrations were first measured by the TNB assay (refer to section 2.6.1.1). Chloramine or HOCl alone was prepared in serial dilution for a standard curve, and applied to cells at appropriate concentrations for treatment. At appropriate time points an aliquot was removed from each standard and treatment for immediate mixing in 96-well plate with 50 μl TMB solution. For treated erythrocytes, this involved rapidly pelleting cells by centrifugation and removing a portion of the supernatant for analysis, whereas an aliquot of the treatment (supernatant) was simply removed from each well of treated HUVECs. Total volume for analysis (in the 96-well plates) was 200 μl, with some dilution of samples to achieve absorbance readings in the linear range. Absorbance was read at 650 nm and concentrations calculated based on the standard curve.

2.7.1 Oxidant consumption: Erythrocytes
The concentration of each oxidant was measured after 20 min incubation at 37°C for each of the treatment concentrations investigated. Cells were quickly pelleted and supernatant was sampled for reaction with TMB. Data were obtained from at least two separate experiments.

2.7.2 Oxidant consumption: HUVECs
The concentration of each oxidant was measured after 10- and 30 min incubations at 37°C. Because cells remained adhered to the plate, a sample of the treatment volume was removed from each well and immediately reacted with TMB. Data were obtained from at least 3 separate experiments.

2.8 Consumption of H$_2$O$_2$ (FOX assay)
The rate of H$_2$O$_2$ consumption by HUVECs was measured using the ferrous oxidation of xylenol orange 1 (FOX) assay [226]. After treatment of erythrocyte suspensions with H$_2$O$_2$ for 10 or 30 min at 37°C, a 20- or 50-μl sample (depending on the original concentration of H$_2$O$_2$) was removed from the cell supernatant and added with immediate vortexing to the FOX reagent (Table 2.2) with a final volume of 1.0 ml. The mixture was incubated at room temperature (RT) for at least 40 (but not more than 80) min and absorbance at 560 nm recorded by dispensing
each sample into wells of a 96-well plate and reading each sample in triplicate. Sample treatments were in duplicate and data were obtained from at 2 separate experiments. Standards were also sampled at each time point to detect any loss of $H_2O_2$ in medium or buffer alone.

2.9 Thioredoxin reductase (TrxR) activity assay

Following treatment, confluent HUVECs were lysed in Lysis Buffer (Table 2.2). Insoluble material was pelleted by centrifugation and protein content of the soluble fraction was assessed. TrxR activity was measured by monitoring the NADPH-dependent reduction of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) to TNB [227]. Lysates (20 μg total protein, in duplicate) were added to 96-well plates and mixed with 200 μl of a 5 mM solution of DTNB in 100 mM phosphate buffer (potassium phosphate, pH 7.0, 10 mM EDTA); each well was brought to a final volume of 245 μl with phosphate buffer and the absorbance was read at 412 nm using a SoftMax Pro spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Baseline curve was established from an initial 5 min, and the reaction was initiated by addition of NADPH (final concentration 200 μM). The increase in absorbance at 412 nm was followed for 5 min and the average change in absorbance (slope) – minus baseline – was used to calculate TrxR activity.

2.10 Protein assay

The Bio-Rad DC protein assay kit was used to estimate protein concentration in prepared cell lysates. Samples (between 2 and 5 μl, depending on the particular experiment) and BSA standards were transferred in duplicate to wells of a 96-well plate and mixed with 25 μl kit reagent A′ and 200 μl of kit reagent B. After 15 min incubation at RT, the absorbance was measured at 750 nm using a SoftMax Pro spectrophotometer. Protein concentrations were calculated using the BSA standard curve.

2.11 Protein precipitation

Where protein precipitation from cell extracts was required, a chloroform-methanol method was employed [228]. The cell lysate, methanol, chloroform and water were mixed (with solvents and water added consecutively with thorough mixing by vortex after each addition) at a ratio of 1:4:1:3, respectively. The mixture was centrifuged at 10,000 × g, resulting in proteins layered at the liquid interface. The aqueous top layer was removed, and 4 volumes of methanol were added, followed by vortexing. The precipitated proteins were pelleted by centrifugation at 10,000 × g, and the supernatant was removed as much as possible without disturbance of the pellet. The
pellet was thoroughly air-dried at 37°C before addition of solubilising buffer (this was generally SDS sample buffer or 2D-PAGE rehydration buffer, depending on the application).

2.12 SDS-Polyacrylamide gel electrophoresis

The Laemmli electrophoresis system [229] was employed using the Bio-Rad Mini-Protean 3 cell apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Samples were prepared by dilution in reducing or non-reducing SDS-Sample Buffer (Table 2.2) and proteins (generally 15-25 μg per lane) were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). A 4% acrylamide [w/v] stacking gel and a 12% resolving gel were used unless otherwise specified.

2.13 Immunoblot analysis

The Bio-Rad Mini Trans-Blot apparatus (Bio-Rad Laboratories, Hercules, CA, USA) was used to electrophoretically transfer proteins resolved by SDS-PAGE to polyvinylidene difluoride (PVDF) membrane. Following electrotransfer, non-specific antibody binding sites were blocked by incubation of the membrane with TBST (Table 2.2) and 5% non-fat dried milk [w/v] (also in TBST). Alternatively, the PVDF membrane was allowed to dry thoroughly, which blocked non-specific binding due to the hydrophobic nature of the membrane. Membranes with proteins from erythrocyte samples were blocked with 15 mM sodium azide and 2% H2O2 included in the 5% milk blocking buffer to prevent reaction of chemiluminescent reagents with haemoglobin through inhibition of its pseudoperoxidase activity [230; 231].

Incubation with the relevant antibody (Table 2.4) was performed overnight at 4°C in TBST containing 2% non-fat dried milk, unless otherwise stated. Horseradish peroxidase-conjugated secondary antibodies (Table 2.4) were diluted in TBST containing 2% non-fat dried milk and the membranes were incubated for 1 h at RT. After incubation with antibodies, membranes were thoroughly washed with TBST. Membranes were incubated with enhanced chemiluminescence (ECL) reagents for 2-3 min and visualised with a ChemiDoc XRS gel documentation system (Bio-Rad Laboratories, Hercules, CA, USA) for detection of bands. Bands were quantified by densitometric analysis using Quantity One analysis software version 4.6.1 (Bio-Rad Laboratories).
Table 2.4 Primary and secondary antibodies used in the thesis

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Application</th>
<th>Concentration of primary antibody</th>
<th>Secondary antibody</th>
<th>Concentration of secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal rabbit anti-peroxiredoxin 1</td>
<td>WB</td>
<td>1:10,000</td>
<td>Goat anti-rabbit peroxidase conjugate</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Polyclonal rabbit anti-peroxiredoxin 2</td>
<td>WB</td>
<td>1:10,000</td>
<td>Goat anti-rabbit peroxidase conjugate</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Polyclonal rabbit anti-peroxiredoxin 3</td>
<td>WB</td>
<td>1:10,000</td>
<td>Goat anti-rabbit peroxidase conjugate</td>
<td>1:10,000</td>
</tr>
<tr>
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<td>1:2,500</td>
<td>Goat anti-rabbit peroxidase conjugate</td>
<td>1:10,000</td>
</tr>
<tr>
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<td>WB</td>
<td>1:1,000</td>
<td>Rabbit anti-goat peroxidase conjugate</td>
<td>1:10,000</td>
</tr>
<tr>
<td></td>
<td>IF</td>
<td>1:100</td>
<td>Rabbit anti-goat Alexa-488 conjugate</td>
<td>1:150</td>
</tr>
<tr>
<td>Polyclonal rabbit anti-Haem oxygenase-1</td>
<td>WB</td>
<td>1:10,000</td>
<td>Goat anti-rabbit peroxidase conjugate</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Monoclonal mouse anti-β-actin</td>
<td>WB</td>
<td>1:10,000</td>
<td>Goat anti-mouse peroxidase conjugate</td>
<td>1:3,000</td>
</tr>
<tr>
<td>Polyclonal rabbit anti-β-tubulin III</td>
<td>WB</td>
<td>1:10,000</td>
<td>Goat anti-rabbit peroxidase conjugate</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Polyclonal mouse anti-Parp1</td>
<td>WB</td>
<td>1:10,000</td>
<td>Goat anti-mouse peroxidase conjugate</td>
<td>1:3,000</td>
</tr>
<tr>
<td>Polyclonal rabbit anti-Cyclophilin A</td>
<td>WB</td>
<td>1:10,000</td>
<td>Goat anti-rabbit peroxidise conjugate</td>
<td>1:10,000</td>
</tr>
</tbody>
</table>

2.14 Detection of Prx redox state

To monitor the transition from monomeric to dimeric Prx, cells were lysed in the presence of 10 μg/ml catalase to scavenge small amounts of peroxide in Extract Buffer (Table 2.2), and 100 mM NEM to alkylate the cysteine residues. To monitor the transition to hyperoxidised Prx (sulfenic, Prx-SO\(_2\)H, or sulfonic, Prx-SO\(_3\)H, acid), cells were lysed in HEPES extract buffer lacking NEM and catalase; under these conditions the Prxs become oxidised to the dimeric form by trace amounts of peroxide present in the buffer, enabling the amount of hyperoxidised protein to be quantified as the sole species running in the monomer position on non-reducing SDS-PAGE [161].

Protein content was assessed (Section 2.10) and proteins were denatured by addition of SDS-Sample Buffer; in some cases protein was precipitated (Section 2.11) before resolubilisation in SDS sample Buffer (Table 2.1).
All samples were heated to 100°C for 5 min before loading lysate containing equal amounts of protein (generally between 15 and 25 μg) onto each lane of a non-reducing, 12% (unless otherwise indicated) SDS-PAGE gel (Section 2.12). Protein bands were transferred by electroblotting to PVDF membrane (Section 2.13) and immunoblotted (Section 2.13) with antibodies against the specific isoforms of Prx.

To ensure that detection of the Prxs were in the linear range for detection and quantification, concentration curves of reduced pure Prx (1, 2 or 3) were analysed alongside aliquots of reduced control cell lysate of known total protein concentration. Subsequent analyses were performed using sufficient total protein (10-20 μg) for Prx detection in the linear range.

### 2.15 Coomassie R250 PVDF staining

To visualise complete protein complement on PVDF, membranes were stained with Coomassie R-250 solution (Table 2.2) for 5 min with constant rocking. Background staining was removed by washing membranes several times with Coomassie destain solution (Table 2.2). Membranes were rinsed with water and dried at RT. Protein was visualised using a Fluor-S® multi-imager (Bio-Rad Laboratories, Hercules, CA, USA).

### 2.16 Silver stain procedure

For visualisation of proteins on SDS-PAGE gels, fixing was performed for 30 min in a solution of 50% ethanol [v/v] and 10% acetic acid [v/v]. Gels were then washed for 15 min in a 10-fold dilution of the fixing solution, followed by three 5 min washes in MilliQ water. Gels were incubated for 2 min in Silver Stain Solution 1 (Table 2.1), and then subjected to three 30 sec washes in MilliQ water before 30 min incubation in Silver Stain Solution 2 (Table 2.2). Two 20 sec washes with MilliQ water were performed, and then Silver Stain Solution 3 was added to gels (Table 2.2). Once the colour had developed to the desired intensity, gels were immediately washed in MilliQ water and the reaction stopped by addition of Silver Stain Solution 4 (Table 2.1). Stained gels were imaged using a ChemiDoc™XRS system (Bio-Rad Laboratories, Hercules, CA).
2.17 **Cell viability**

2.17.1 **Propidium iodide**

Plasma membrane integrity in HUVECs was monitored using propidium iodide (PI) staining. Following a 10 min treatment, any remaining oxidant was quenched by addition of methionine (10-fold excess in concentration over initial treatment concentration). Cells were washed with PBS and fresh M199 was replaced for 24 h. The adherent cell layer was disrupted by trypsinisation and pooled with any non-adhering cells. Cells were pelleted by centrifugation and resuspended in M199 containing 2 μg/ml PI. Cell fluorescence was measured using a FC500 MPL Flow Cytometry system (Beckman Coulter Inc.). PI-positive cells (non-viable) were expressed as a percentage of the total cells analysed (5,000 cells). Cells were incubated in the dark for 10 min prior to analysis.

2.17.2 **MTT assay**

Cell viability was monitored spectrophotometrically by the ability of HUVEC mitochondrial reductases in living cells to convert thiazolyl blue tetrazolium bromide (yellow) to purple formazan. Following treatment, cells plated in 6-well culture dishes were incubated 24 h in fresh M199. MTT solution was prepared at 5 mg/ml in PBS and filter-sterilised; 150 μl were added to each well and incubated 3 h. Following incubation, some cells became detached from the plate; these suspended cells were pelleted, and to remaining adherent cells 1 ml of acidic isopropanol (0.04 M HCl in isopropanol) was added to each well to solubilise the converted dye. Plates were incubated in the dark with gentle agitation for 10 min. Pelleted cells were added to the dye solution and fully solubilised by pipetting. Insoluble cell material was pelleted by centrifugation (10,000 × g) and the absorbance of the converted dye was measured at 570 nm with background subtraction at 650 nm. Measurements were made using a Hitachi U-3900 spectrophotometer.

2.18 **Assessment of total protein thiols**

Total protein thiols were assessed using monobromobimane (MBB) derivatisation with spectrofluorometric detection [232]. Following treatment (in duplicate), washed HUVECs grown in 6-well plates were derivatised using 1.2 mM MBB (in 1 ml PBS per well, pH 8). Cells were incubated in the dark 20 min at 37°C. Following addition of 50 μl saturated trichloroacetic acid (TCA) per well to precipitate proteins, contents of the wells were removed from the plate by gentle pipetting, and insoluble matter was pelleted by centrifugation. Pellets were washed with 5% TCA ([v/v], from saturated TCA solution) three times, and then resuspended in 1% SDS
[w/v] to a final volume of 1.6 ml. Any remaining insoluble material was pelleted and the supernatant was analysed using a Hitachi fluorescence spectrophotometer (excitation 390 nm; emission 480 nm).

2.19 Protein carbonyl assay

Protein carbonyls were assessed by the enzyme-linked immunosorbent assay (ELISA) previously used in our laboratory [233]. HUVECs grown in 6-well plates were gently detached with a rubber policeman in cell detachment buffer (Table 2.2). Cells were pelleted, and the cell pellet subjected to three freeze-thaw cycles (alternating between dry ice and 37ºC heat block). The cell lysate was speed vacuumed to dryness and frozen at -80ºC until further analysis. The ELISA was performed using the BioCell Protein Carbonyl ELISA kit (Papatoetoe, New Zealand) according to the manufacturer’s instructions (low protein procedure).

2.20 Measurement of glutathione

2.20.1 High performance liquid chromatography (HPLC) method

Intracellular glutathione (GSH) concentration was determined by MBB derivatisation and HPLC analysis with fluorescence detection [232]. Details for the methods employed for analysis of erythrocytes and HUVECs are further described below.

2.20.1.1 Erythrocytes

Following oxidant treatment, erythrocytes (2.5 × 10⁷ cells) were pelleted by centrifugation. Erythrocyte pellet was resuspended in 10 mM phosphate buffer (pH = 8.0 with potassium hydroxide) and MBB (40 mM in acetonitrile) was added for a final concentration of 1 mM and volume of 400 μl; cells were incubated in the dark for 20 min at 37ºC. Proteins were precipitated by addition of 5% TCA [v/v]. Insoluble material was pelleted by centrifugation (10,000 × g for 5 min) and resulting supernatants (100 μl per sample) were analysed by HPLC with fluorescence detection (excitation wavelength 394 nm; emission wavelength 480 nm) using a C18 column (Brownlee Spheri-5 ODS, 5 μm, 100 × 4.6 mm). GSH was eluted using a stepwise gradient (Table 2.5). The concentration of GSH in samples was calculated using a standard curve generated from GSH solutions of known concentration (2-20 μM).
2.20.1.2 HUVECs

Confluent HUVECs plated in 24-well plates were used. Following oxidant treatment, cells (~120,000 per well) were derivatised and precipitated directly in the culture plates according to the methods described above, but with a final volume of 200 μl per well. Analysis was as described for erythrocytes.

Table 2.5 Gradient for elution of GSH-MBB samples

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow rate (ml•min⁻¹)</th>
<th>% Solvent A</th>
<th>% Solvent B</th>
<th>% Solvent C</th>
<th>Gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>1.5</td>
<td>95</td>
<td>5</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>10</td>
<td>1.5</td>
<td>90</td>
<td>10</td>
<td>0</td>
<td>Continuous (linear)</td>
</tr>
<tr>
<td>12</td>
<td>1.5</td>
<td>90</td>
<td>10</td>
<td>0</td>
<td>Continuous (linear)</td>
</tr>
<tr>
<td>17</td>
<td>1.5</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>Step</td>
</tr>
<tr>
<td>22</td>
<td>1.5</td>
<td>95</td>
<td>5</td>
<td>0</td>
<td>Step</td>
</tr>
</tbody>
</table>

Solvent A = water containing 0.25% acetic acid
Solvent B = acetonitrile containing 0.25% acetic acid
Solvent C = 75% acetonitrile with 25% water

2.20.2 LC-MS/MS Method

The concentrations of GSH and its oxidation products (glutathione disulfide, GSSG, and glutathione sulfonamide, GSA) in the intracellular and extracellular fractions following erythrocyte treatment with chloramines and HOCl were detected by LC-MS/MS as described [234].

Following treatment, erythrocytes were pelleted and prepared essentially as described [235]. NEM was added to the supernatant (extracellular fraction) at a final concentration of 2 mM; internal standards of GSA (100 pmoles), GSSG (100 pmoles) and NEM-alkylated GSH (200 pmoles) generated from isotopically labelled glycine (¹³C, ¹⁵N) were also added to each sample. The erythrocyte pellet was resuspended in 200 μl of 100 mM NEM and incubated at 37°C for 20 min; isotopic standards were added as above, and protein was precipitated by the addition of 800 μl ice-cold ethanol. Following 20 min incubation on ice, the precipitate was pelleted and the supernatant removed for analysis. Both cytosolic and extracellular fractions were dried under vacuum and resuspended in 200 μl PBS; any undissolved material was pelleted prior to injection of the resulting supernatant (50 μl) for analysis.
Chapter 2: Materials and Methods

Standard curves were generated by serial dilution of a known concentration of each glutathione species: GSH, GSSG and GSA. Each standard also included internal standards of each species in amounts equal to those shown above for samples.

Liquid chromatographic separation was performed using a Thermo Hypercarb column (100 mm×2.1 mm) held at 40°C. Mobile phases were 0.5% [v/v] formic acid as Solvent A and 1:1 acetonitrile:isopropanol (0.1% formic acid) as Solvent B. The flow rate was 0.2 ml/min. GSH, GSSG and GSA were eluted using a linear gradient of 0–30% solvent B over 15 min, and the column was flushed with 100% solvent B for 5 min before re-equilibration to initial conditions.

<table>
<thead>
<tr>
<th>Species</th>
<th>m/z Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSA</td>
<td>338→263</td>
</tr>
<tr>
<td>GSSG</td>
<td>613→484</td>
</tr>
<tr>
<td>GSH-NEM</td>
<td>433→304</td>
</tr>
<tr>
<td>Isotopically labelled GSA</td>
<td>341→263</td>
</tr>
<tr>
<td>Isotopically labelled GSSG</td>
<td>619→490</td>
</tr>
<tr>
<td>Isotopically labelled GSH-NEM</td>
<td>436→307</td>
</tr>
</tbody>
</table>

Mass spectrometric detection of the species was performed using a LCQ DECA XP$^\text{plus}$ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) with the parameters described by Harwood et al [234]. The target analytes and internal standards were detected by the m/z range shown in Table 2.6. Peak areas were normalised by dividing the area of the compound of interest by the area of the internal standard. Concentration of each species was calculated based on the standard curves. Mass spectrometry was performed by Dr. Rufus Turner (University of Otago, Christchurch, NZ).

### 2.21 Preparation of nuclear and cytosolic fractions from oxidant-treated HUVECs

Nuclear protein extraction was based on the methods described by Hsieh et al [236]. HUVECs grown in 100-mm tissue culture dishes were gently lysed in 1 ml ice-cold Buffer A (Table 2.2). Cell lysis and non-disruption of the nuclei were confirmed visually by microscopy. After 5 min incubation on ice, the lysate containing the non-nuclear fraction (organelles, cytosol and membranes) was removed from the plate, leaving only the attached nuclei on the plate. Nuclei
were lysed in 300 µl Buffer B (Table 2.2). Nuclei were removed by gentle scraping using a rubber policeman. Both fractions were maintained at 4ºC for 30 min with periodic vortexing. Insoluble material was pelleted by centrifugation (14,000 × g, 30 min, 4ºC) and the soluble fraction used for immunoblot analysis (Section 2.13). Where protein concentration was less than 1 µg/µl (determined as per Section 2.10), proteins were precipitated using the chloroform-methanol extraction procedure (Section 2.11) before dilution in reducing SDS sample buffer. Proteins were resolved by reducing SDS-PAGE (Section 2.12) and transferred to PVDF (Section 2.13); blots were probed with antibodies against the proteins of interest, including antibodies against Parp1 and β-tubulin to confirm nuclear and cytosolic isolation, respectively.

2.22 Immunofluorescent detection of Nrf2 localisation

2.22.1 Preparation of coverslips

Prior to cell subculture for the purposes of immunofluorescence, 13-mm glass coverslips were prepared with a glutaraldehyde-fixed gelatin coating [237]. Coverslips were submerged in 0.5% gelatin solution [w/v] (in water) for 1 h with constant rocking. Coverslips were then submerged in 2% glutaraldehyde [v/v] for 20 min, washed twice with PBS, then submerged in 70% ethanol for 1 h, still with constant rocking. Finally, coverslips were transferred to sterile 24-well plates, washed twice with sterile PBS and incubated 1 h with PBS alone. PBS was replaced prior to storage under sterile conditions for any coverslips not immediately used.

2.22.2 Immunofluorescence analysis

Cells (HUVECs or Hepa 1c1c7 cells) were subcultured onto glass coverslips (prepared as in Section 2.22.1, above) 24-48 h prior to treatment. At the end of the treatment period, cells were thoroughly washed using PBS and fixed by addition of 4% paraformaldehyde [w/v] (10 min) and permeabilised 5 min incubation in 0.5% Triton X-100 [v/v] (in PBS). Cells were blocked in 10% BSA [w/v] for 1 h. Coverslips containing fixed, permeabilised cells were washed in PBS and incubated overnight at 4ºC with antibodies against Nrf2, in 3% BSA. Cells were again washed with PBS before incubation with Alexa 488-conjugated secondary antibodies (in 3% BSA) for 1 h in the dark at RT. Secondary incubation was followed by Hoechst staining for 5 min (10 µg/ml Hoechst 33342, in PBS). Coverslips were washed and dipped in MilliQ water before mounting onto glass slides using Fluoromount-G™. Fluorescence imaging was performed using a Zeiss microscope.
Control slides included unstained cells, cells incubated with primary antibody only or alexa-488-conjugated secondary antibody alone. No fluorescence was visualised on the DAPI channel in cells lacking Alexa-488-conjugated secondary antibodies. Exposure time was consistent between experiments and was based on cells labelled with secondary antibody alone (no primary antibody); exposure time was set such that little to no fluorescence was detected in these control cells.

### 2.23 Identification of intracellular thiol protein targets of chlorinated oxidants

For separation of proteins by 2-dimensional electrophoresis, HUVECs were grown in 100-mm tissue culture dishes. Cells were treated in a 4 ml volume of HBSS, and following oxidant quenching and gentle washing of the cells, plates were scraped using a rubber policeman into 1 ml cell detachment buffer (Table 2.2) containing 100 mM NEM and pelleted by centrifugation. Cells were lysed by incubation with 190 μl of 100 mM NEM dissolved in Extract Buffer (Table 2.2) containing 1% CHAPS [w/v], with occasional vigorous agitation. Insoluble matter was pelleted and the soluble fraction was desalted using Bio-Gel P-6 DG desalting gel columns (Bio-Rad Laboratories) pre-equilibrated with extract buffer lacking NEM. Protein content was assessed using the Bio-Rad D assay (Section 2.10) and DTT was added (5 mM final concentration) to reduce disulfide bonds. Samples were incubated 30 min at 37°C, after which 200 μM 5-iodoacetamidofluorescein (IAF, freshly dissolved in DMSO as a 10 mM stock) was added and samples were incubated for an additional 30 min. Samples were protected from light from this point onward. Proteins were precipitated (Section 2.11) and the pellet was resolubilised in sample rehydration buffer (Table 2.1). All reagents in buffer (except Ampholytes and CHAPS) were PlusOne™ reagents (GE Healthcare, Buckinghamshire, UK). Protein concentration of reconstituted samples was 75 μg/100 μl (based on total protein calculated before the precipitation step). Each sample (200 μl) was resolved by two-dimensional electrophoresis (Section 2.24) using 11-cm, pH 5-8 IPG strips followed by second dimension separation on Criterion™ Tris-HCl 8-16% gradient gels (Section 2.24).
For spot excision and identification, 300 μl of pooled sample (equal amounts from four separate sample preparations) were rehydrated onto 17-cm, pH 5-8 IPG strips and separated by 2-D Electrophoresis (Section 2.24).

### 2.24 Two-dimensional electrophoresis

Each sample was loaded into a channel of a rehydration/equilibration tray. IPG strips were placed in direct contact with the samples and strips were overlaid with mineral oil to prevent dehydration. IPG strips were allowed to rehydrate overnight at RT to ensure complete sample absorption. Following sample absorption, strips were transferred to focusing tray channels with moistened electrode wicks placed between the strips and tray electrodes. Strips were overlaid with mineral oil and focused using the Bio-Rad Protean® IEF Cell (Bio-Rad Laboratories, Hercules, CA, USA). Isoelectric focusing was performed according to the voltage conditions set out in Table 2.7.

<table>
<thead>
<tr>
<th>Step</th>
<th>Voltage (Volts)</th>
<th>Time (Hours)</th>
<th>Voltage (Volts)</th>
<th>Time (Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>300</td>
<td>1</td>
<td>300</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1,000</td>
<td>1</td>
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<td>1</td>
</tr>
<tr>
<td>3</td>
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</tr>
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</tr>
<tr>
<td>5</td>
<td>500</td>
<td>HOLD</td>
<td>500</td>
<td>HOLD</td>
</tr>
</tbody>
</table>

Focused strips were prepared for the second dimension by incubation in equilibration buffer (Table 2.2). All reagents in equilibration buffer were PlusOne™ reagents (GE Healthcare). Strips were allowed to equilibrate on a rocker with equilibration buffer for 15 min; this was repeated a second time with fresh equilibration buffer in clean tray channels. 11-cm IPG strips were laid atop Criterion™ Tris-HCl 8-16% gradient gels, and 17-cm IPG strips were laid atop Tris-HCl 8-16% gradient gels cast using the Bio-Rad Protean® II XL Vertical Electrophoresis Cell. Strips were overlaid with melted agarose solution (Table 2.2). 10 μl of Precision Plus Prestained protein standards (Bio-Rad Laboratories, Hercules, CA, USA) were included on the gels.

Where Criterion™ gels were used, proteins were separated electrophoretically at a constant voltage of 150 V for approximately 100 min (or just until the dye front began to diffuse from the
Chapter 2: Materials and Methods

On large-format gels, proteins were separated at a constant amperage of 12 mA per gel for approximately 1000 min. Proteins were detected by fluorescence (488 nm) using a BioRad Molecular Imager® FX (Bio-Rad Laboratories, Hercules, CA, USA) on the ‘medium’ sample intensity setting.

Bio-Rad 2-D SDS-PAGE Standards were separated in an identical way for molecular weight (MW) and pI ranges on the gels (Figure 2.1). The standard gel image was superimposed with sample images such that pI and MW values could be assigned to spots using PDQuest™ software. Gel images were analysed using PDQuest™ software. Digital images (3 replicate images from each of 5 treatments, including the control) were used for comparison; automated detection and spot-matching were carefully scrutinized, and in many cases corrected by visual inspection and manual spot-matching. The software was used for automated statistical comparison of spots, enabling the identification of those which differed significantly compared to the control gels; for this, the replicate group comparison mode was used, along with Gaussian spot quantitation.
the spots identified as undergoing significant changes in intensity, many were manually excluded due to either low intensity, inconsistent appearance between replicate gels, or due to their appearance in streaky areas of the gel (which would result in difficult excision of the spots). Finally, a shortlist of spots was generated for further investigation.

Protein spots of interest were excised from unstained, large-format gels, and the correct excision of spots was confirmed by fluorescence imaging as described in Figure 2.2. Excised spots were sent to the Centre for Protein Research (University of Otago, Dunedin, New Zealand), where they were subjected to automated in-gel tryptic digestion using a robotic workstation (DigestPro MSi, Intavis AG, Cologne, Germany) [238]. Eluted peptides were concentrated by speed-vacuum and the tryptic peptides analysed by LC/MS-MS.

![Spot excision from 2-dimensional gels](image)

**Figure 2.2 Spot excision from 2-dimensional gels.**
Fluorescence imaging was used both in the identification of spots for excision, and to confirm the correct (and complete) excision of these spots. Spots of interest were identified following fluorescence detection of 2D SDS-PAGE gels (white arrows). Excision in the wrong area of the gel (black arrows) and incomplete excision (red arrows) were identified and any errors were corrected by re-alignment of the gel and images, and further excision. Blue arrows indicate confirmation that the intended spot was correctly excised from the gel.

### 2.25 LC-MS/MS of tryptic peptides
Resolubilisation of samples from SDS-PAGE gels was performed using a solution of 5% acetonitrile [v/v], 0.2% formic acid [v/v] in water. Solubilised samples were then injected onto
an Ultimate 3000 nano-flow UHPLC-System (Dionex Co, CA) which was in-line coupled to the nanospray source of a LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Scientific, San Jose, CA). Peptides were separated on an in-house packed emitter-tip column by a gradient developed from 5% acetonitrile, 0.2% formic acid to 80% acetonitrile, 0.2% formic acid in water over 15 min at a flow rate of 400 nl/min. Column packing consisted of 75 um ID PicoTip fused silica tubing (New Objectives, Woburn, MA) with C-18 material on a length of 8-9 cm.

### 2.26 Instrument settings for the LTQ-Orbitrap:

The Orbitrap mass analyser was used to perform a full MS of the range m/z 300 and 2000, with a resolution setting of 60,000 at m/z 400 and an automated gain control target of 4e5. Preview mode for FTMS master scan was enabled to generate precursor mass lists, from which the strongest 4 signals were selected for CID (collision induced dissociation)-MS/MS in the LTQ ion trap. Normalised collision energy was 35% using an AGC target of 2e5 and one microscan. Dynamic exclusion was enabled with 2 repeat counts during 30 sec and an exclusion period of 180 sec. Exclusion mass width was set to 0.01.

#### 2.26.1.1 Data Analysis

Protein identities from MS/MS data were obtained using the Human reference protein sequence database (downloaded in June 2010) with the Mascot search engine (www.matrixscience.com). Full tryptic peptides were searched with settings for matching of full tryptic peptides with a maximum of 3 missed cleavage sites; variable modification included carboxyamidomethyl cysteine, oxidised methionine, and pyroglutamate. The precursor mass tolerance threshold was 10 ppm and the maximum fragment mass error was 0.8 Da.

### 2.27 Statistics

Data are expressed as means ± standard error of the mean (SEM) for at least 3 separate experiments, unless otherwise indicated. Graphs were plotted using either Office Excel version 2007 (Microsoft, Redmond, WA, USA) or SigmaPlot version 11.0 (SPSS Inc., Chicago, IL, USA). Statistical analyses were performed using the SigmaStat software package, version 3.10 (Systat Software, San Jose, CA, USA). Differences between groups of data were assessed employing a significance level (α) of 0.05.
Chapter 3. Effect of chlorinated oxidants on Prx2 oxidation in erythrocytes

Results from this chapter have been included in:


3.1 Introduction

In erythrocytes, peroxiredoxin 2 (Prx2) is the third most abundant protein and plays an important role in protecting against oxidative damage. Erythrocytes are exposed to considerable oxidative stress; high levels of haemoglobin lead to spontaneous oxidation of this protein, accompanied by the production of superoxide and other reactive oxidants. Furthermore, these anucleate cells cannot replace irreversibly oxidised proteins. Prx2 is highly reactive with hydrogen peroxide (H$_2$O$_2$) and plays a nonredundant role in protecting the erythrocyte against its oxidative effects [65].

In addition to endogenous oxidative stress, erythrocytes are likely exposed to neutrophil-derived oxidants, including HOCl and chloramines, at sites of inflammation. These oxidants react readily with thiols, especially low-$pK_a$ thiol residues. Note that this work was performed before the kinetics had been measured for the reactivity of chloramines with Prx2; therefore, this chapter aimed to explore the hypothesis that erythrocyte Prx2, due to the low-$pK_a$ cysteine in its active site, might be sensitive to oxidation in cells treated with chloramines or HOCl.

3.2 Experimental Approach

The reactivity of HOCl and chloramines (glycine chloramine and monochloramine) with Prx2 was investigated in intact erythrocytes. To assess its reactivity, the sensitivity of Prx2 was compared to that of GSH. The recycling of Prx2 was examined in the presence of glucose to maintain metabolic activity and redox cycling through NADPH and GSH. Furthermore, it was investigated whether or not H$_2$O$_2$ is produced endogenously as a result of chloramine exposure.
Chloramines were generated by reacting amine solution (taurine, glycine or ammonium chloride, in PBS) at a 10-fold excess with HOCl (Section 2.6.1). Chloramine and HOCl concentrations were determined by reaction with TNB (Section 2.6.1.1; Figure 3.1). One mole of chloramine reacts with 2 moles of TNB to produce one mole of DTNB. This method involves measuring the loss in absorbance at 412 nm spectrophotometrically, which corresponds to the loss of TNB, a yellow solution ($\varepsilon_{412}=14,100 \text{ M}^{-1}\text{s}^{-1}$), and corresponding gain in DTNB, a colourless solution [166; 222; 225; 239].

![Figure 3.1 Oxidation of TNB by thiol-targeting oxidants.](image)

Oxidants such as chloramines oxidise TNB. For every mole of oxidant, 2 moles of TNB are converted to one mole of DTNB, and this reaction is measured as a decrease in absorbance at 412 nm.

To determine chloramine concentration in the supernatant of treated cells a spectrophotometric assay measuring the oxidation of 3,3',5,5' tetramethylbenzidine (TMB, Figure 3.2) was used [225]. This assay uses iodide which is known to catalyse the oxidation of chloramines (RNHCl; Equations 1 - 4; [240]); TMB is oxidised by hypoiiodous acid (HOI) to a blue compound with maximal absorbance at 650 nm [225; 241]. Actual concentrations of each oxidant were determined by a standard curve generated using known concentrations of that oxidant.

![Figure 3.2 Oxidation of 3,3',5,5' tetramethylbenzidine (TMB).](image)

In the presence of iodine, chloramines oxidise to form HOI. HOI causes TMB to undergo a 2-electron oxidation, producing a blue colour which is detected spectrophotometrically.
Chapter 3: Effects of chlorinated oxidants on Prx2 oxidation in erythrocytes

(25x443) Absolute quantification of the standard curves in this instance therefore relied on accurate quantification of the chloramine solutions.

Peroxiredoxin redox status was examined by redox western blotting (Section 2.14) [65]. This method exploits the fact that Prxs which have been oxidised (to the sulfenic acid) readily form disulfide-linked dimers (refer to Section 1.3). Initially, free thiol residues are blocked with N-ethylmaleimide (NEM), preventing disulfide formation with the alkylated residues. Cell lysates were then separated under denaturing non-reducing conditions by SDS-PAGE and the oxidised (dimeric) and reduced (monomeric) forms are distinguishable by immunoblotting (Figure 3.3).

Intracellular levels of reduced GSH were measured by derivatisation with MBB (Figure 3.4). Fluorescent adducts were separated by HPLC with fluorescence detection (Section 2.20.1).

Intra- and extra-cellular concentrations of GSH, GSA and GSSG were quantified by LC/MS/MS (Section 2.20.2) [234]. Peaks corresponding to each species were separated chromatographically and identified based on the largest fragment ion. These peaks were normalised to internal standards and compared to a standard curve for quantification.
Chapter 3: Effects of chlorinated oxidants on Prx2 oxidation in erythrocytes

3.3 Results

3.3.1 Oxidant consumption by erythrocyte cell suspension

The change in extracellular concentration of each oxidant after 20 min incubation with suspended erythrocytes was monitored. Consumption of GlyCl was slow and concentration-dependent, with an approximate 20% decrease with each of the concentrations examined (Figure 3.5A). Almost all of the NH₂Cl and HOCl were consumed during the 20 min incubation with erythrocytes (Figure 3.5B & C). There was no significant change in TauCl concentration (different from control by less than 1%, on average; Figure 3.5D) at any of the exposures, indicating that little to no TauCl entered the cells. This is consistent with other reports that this chloramine has very low membrane permeability [45; 51; 242]. The oxyhaemoglobin spectrum in a lysed cell sample remained unchanged following treatment with all oxidants, and only occasionally the highest concentration of either NH₂Cl or HOCl caused a small amount of cell lysis over the treatment period, evident by a faint colouration of the supernatant.

Figure 3.4 GSH was detected by derivatisation with monobromobimane. MBB reacts with sulphhydrils including GSH, producing fluorescent adducts which can be separated chromatographically (image is from [1]).
3.3.2 Prx2 oxidation

Under normal physiological conditions proteins tend to be in a reduced state due to the reducing cell environment. Accordingly, untreated erythrocytes from healthy patients have Prx2 present primarily in the reduced form, with a small amount of dimer sometimes evident (<5%; refer to control lanes in Figure 3.6). Under situations of oxidative stress, Prx2 undergoes oxidation to a dimer which can be distinguished from the monomer by non-reducing SDS-PAGE. This reaction is reversible under reducing conditions, where Prx2 appears as a single band (not shown). The extent of Prx2 oxidation in erythrocyte suspensions treated with GlyCl, TauCl, NH$_2$Cl or HOCl varied depending on the oxidant (Figure 3.6). Analysis of replicate Western blots by densitometry indicated that complete oxidation of Prx2 required approximately 500 μM
GlyCl (Figure 3.6A), but only 2 μM NH$_2$Cl (Figure 3.6B) or 5 μM HOCl (Figure 3.6C). Analysis of blots probed with an antibody specific for the hyperoxidised Prxs (sulfinic and sulfonic acid forms) showed no detectable hyperoxidised species in samples treated with the highest concentrations of each oxidant (Figure 3.7). TauCl caused no observable oxidation of Prx2 in intact cells (Figure 3.6D); this is consistent with the inability of TauCl to enter the cells.

Figure 3.6 Prx2 oxidation in erythrocytes treated with the given concentrations of oxidant for 20 min.
Extracts were prepared in the presence of NEM, separated by non-reducing SDS-PAGE and immunoblotted with antibodies for Prx2; a shift from the monomeric (M) to the dimeric (D) form corresponds to Prx2 oxidation to an interchain disulfide.

Figure 3.7 Western blotting with antibodies against the hyperoxidised forms of Prx.
Erythrocyte samples treated with chloramines or HOCl were separated by reducing SDS-PAGE alongside untreated erythrocyte lysate (Control) and an endothelial cell positive control (HUVECs treated with 1 mM H$_2$O$_2$). N.S.: non-specific band. Of the Prx-SO$_2$/3 bands, the uppermost bands co-migrated with Prxs 3 and 1, but the lower band did not appear to co-migrate with any Prxs detected using available antibodies.
It is worth nothing that when proteins were separated by non-reducing SDS-PAGE the Prx2 dimer appeared as a doublet; it is suspected that the slower-migrating band consists of a Prx2 dimer linked by a single disulfide bond, whereas the band which migrates faster corresponds to the more “thoroughly” oxidized, 2-disulfide-linked dimer. The progression from the higher to lower band with increasing oxidant concentration as seen in several of the immunoblots in Figure 3.6 is consistent with this.

3.3.3 Comparison of Prx2 and GSH oxidation

Prx2 and GSH were oxidised to a similar extent when erythrocytes were treated with GlyCl (Figure 3.8A). However, complete oxidation of Prx2 was achieved with lower concentrations of NH$_2$Cl (Figure 3.8B) or HOCl (Figure 3.8C) than were required to fully oxidise GSH. For NH$_2$Cl, significantly less Prx2 remained in the monomeric form with both the 2 and 10 μM treatments (p < 0.05) and there was almost a 10-fold difference in the concentrations required for complete oxidation. Likewise, 5 μM HOCl was sufficient to induce complete oxidation of Prx2, whereas some GSH remained in the reduced state following treatment with as much as 10 μM HOCl; the percentages of reduced Prx2 compared to GSH differed significantly with the 5 μM HOCl treatment (p < 0.05).

3.3.4 Glucose-dependent recycling of Prx2 and GSH

Erythrocyte thiols were less sensitive to the chloramines and HOCl when exogenous glucose was added. Two to three times more GlyCl was required to achieve the same level of Prx2 oxidation as seen in the absence of glucose (Figure 3.8:D versus A), and a similar level of protection was seen with GSH. Oxidation of both GSH and Prx2 was significantly less in the presence of glucose at all GlyCl concentrations (Figure 3.8 D) above 50 μM. With glucose present, there was significantly less oxidation of Prx2 than GSH at GlyCl concentrations up to 200 μM.

Although GSH values were significantly higher at all NH$_2$Cl concentrations when glucose was present, Prx2 was significantly less oxidised with only the 2 μM treatment (compare Figure 3.8 B & E). Treatment of erythrocytes with up to 10 μM HOCl caused significantly less oxidation of Prx2 and GSH in the presence than absence of glucose (Figure 3.8: F versus C). At the lower HOCl concentrations in the presence of glucose, Prx2 and GSH oxidation were similar, but at higher concentrations Prx2 was more readily oxidised (p < 0.05 with 30 μM HOCl).
Figure 3.8 Concentration-dependent oxidation of Prx2 and GSH after 20 min treatments of erythrocytes.

Amounts of reduced Prx2 and GSH in erythrocytes were calculated as percent controls that had been incubated without oxidant. Prx2 oxidation was calculated by densitometry of western blots as shown in Figure 3.6. A, B and C represent data obtained from treatments performed in the absence of 5 mM glucose, while D, E, and F represent results obtained when glucose was included. Darker bars, relative quantity of reduced Prx2 (in the monomeric form); light bars, relative quantity of reduced GSH. All values represent means ± SD from at least 3 separate experiments. Typically, control cells contained 85-100% monomer; values have been calculated relative to control for each separate experiment. Statistically significant differences were calculated either relative to control for the given treatment, or between the two measures for the same treatment; these were assessed by one-sample or independent-samples t-tests (* indicates p < .05; ** indicates p < .005).
These results demonstrate that for NH$_2$Cl and HOCl, glucose gave greater recycling of GSH than Prx2. Compared to cells treated in the absence of glucose, approximately 10 times more of either oxidant was required for 50% GSH oxidation when glucose was present, but only 2 to 3 times more for 50% oxidation of Prx2.

### 3.3.5 Time-dependent regeneration of reduced Prx2 and GSH

To establish whether Prx2 and GSH could be regenerated over time, erythrocytes were treated with NH$_2$Cl in the presence of glucose; samples were taken over a 2 h time period and analysed by HPLC and immunoblotting for reduced GSH and Prx2, respectively. As demonstrated in Figure 3.5, NH$_2$Cl was consumed by the cells within 20 min. At that time point 5 and 10 μM NH$_2$Cl caused significant GSH oxidation, with no subsequent regeneration evident (Figure 3.9A).
Within 20 min, erythrocytes treated with as little as 2 µM NH₂Cl showed significant oxidation of Prx2 compared to control cells (less than 50% control levels of monomer); 5 and 10 µM treatments induced complete oxidation at the same time point (Figure 3.9B). Regeneration of Prx2 was evident with 2 µM NH₂Cl, with the relative quantity of monomer back to within normal range by 40 min. With 5 µM NH₂Cl, only 50% of the reduced form was regenerated, whereas with 10 µM the level of reduced Prx2 at the 2 h time point remained less than 10% that of control cells.

3.3.6 Oxidation & loss of GSH from treated erythrocytes

It has been shown that in erythrocytes exposed to HOCl, most of the GSH loss was due to GSSG formation and reversed on adding glucose [38]. However, it has previously been observed that other cells treated with HOCl lose GSH without a corresponding gain in GSSG or sulfonamide [243-247]. To examine the fate of the GSH lost in the presence of glucose, the oxidation products GSSG and glutathione sulfonamide (an irreversible product of the reaction with HOCl) were measured by LC-MS/MS (Figure 3.10). For cells treated with GlyCl (200 or 500 µM), NH₂Cl (5 or 10 µM) and HOCl (10 or 20 µM), the net loss of intracellular GSH was between 28 and 109 pmoles per 10⁶ cells. No more than 11% (with 500 µM GlyCl) and as little as one percent (with 5 µM NH₂Cl) of the loss of GSH could be accounted for by intracellular GSSG, and only small amounts of glutathione sulfonamide were detected (< 0.3 pmoles). Very little of any glutathione species was detected extracellularly (less than 2 pmoles of GSH in control samples), and in fact there was a net loss of glutathione species not only intracellularly but also in the extracellular fraction. Altogether, more than 89% of the GSH lost was not accounted for by these products (Table 3.1).

A recent publication [243] reported the identification of a novel product (5-hydroxybutyrolactam) formed by the oxidation of GSSG by HOCl. This M-45 product should be readily detectable by the LC-MS/MS employed [234]; however, no peaks corresponding to the mass of this butyrolactam species were detected.
Figure 3.10 GSH and its oxidation products GSSG and GSA were detected by LC-MS/MS. Cytosolic (A, B and C) and extracellular (D, E, and F) GSH species were analysed separately. The GSH pool in control cells consisted almost exclusively of the reduced species. Significant loss of GSH was observed with all treatments. Relatively low amounts of GSSG were detected, which represented less than 11% of the GSH lost in treated cells, and even less GSA was detected. Extracellular GSH species accounted for only a very small percentage of the total GSH, but the changes to extracellular and intracellular pools of GSH by the various treatments showed similar trends. Data represent means from 3 separate experiments ±SEM.
Chapter 3: Effects of chlorinated oxidants on Prx2 oxidation in erythrocytes

To explore whether protein glutathionylation could account for the loss of GSH, treated cells were subjected to lysis, protein precipitation and tris(2-carboxyethyl) phosphine hydrochloride (TCEP) reduction to release the GSH from mixed disulfides. In control cells 4.5 nmoles of GSH were typically detected (180 pmol/10^6 cells), with an additional 10 pmol/10^6 cells released following TCEP reduction (Figure 3.11). Depending on the oxidant used, treated erythrocytes typically showed a 35-50% reduction in GSH, of which only some was reducible by TCEP. Less

Table 3.1 Glutathione oxidation products in erythrocytes treated with chloramines or HOCl

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% GSSG</th>
<th>% GSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 μM GlyCl</td>
<td>8.6 ± 2.5</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>500 μM GlyCl</td>
<td>10.6 ± 1.5</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>5 μM NH₂Cl</td>
<td>1.8 ± 0.9</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>10 μM NH₂Cl</td>
<td>4.5 ± 1.2</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>10 μM HOCl</td>
<td>6.5 ± 2.9</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>20 μM HOCl</td>
<td>7.1 ± 2.8</td>
<td>0.3 ± 0.1</td>
</tr>
</tbody>
</table>

Values (means ± SD) are expressed as a percentage of the GSH lost (compared to a control treatment) following 20 min treatment of intact cells (in PBS, 5 mM glucose) which could be accounted for by each of the oxidation products, measured by LC-MS/MS. Control values (GSSG = 0.04%, GSA = 0.001% of total GSH in control cells) were subtracted from measurements prior to calculation.

Figure 3.11 GSH retrieved from erythrocytes treated with chloramines or HOCl. Treated whole cells were lysed following derivatisation with MBB. Light bars represent the amount of GSH which was in the reduced state following treatment, while dark bars represent the proportion of GSH which was present in mixed disulfides, released by TCEP reduction. Data are means for 3 experiments, ±SEM.
than 20% of the loss of GSH following chloramine treatment was accounted for as mixed disulfides, and less than 30% in HOCl-treated cells. Thus, the majority of the GSH lost was not accounted for by any of these oxidation products.

### 3.3.7 Oxidation of Prx2 & GSH in haemolysate

According to the calculated rate constants [12] (Table 1.2), GSH should be approximately 10 times less sensitive than Prx2 to oxidation by NH₂Cl, and 30 times more sensitive to oxidation by GlyCl. The findings with NH₂Cl and intact erythrocytes (Figure 3.8 B) are in broad agreement with this, but the similar degree of oxidation of cellular Prx2 and GSH seen with GlyCl (Figure 3.8A) is an obvious inconsistency. To elucidate whether the different susceptibilities of Prx2 and GSH to oxidation reflected an intact cell system, lysed erythrocytes were treated with GlyCl (Figure 3.12). In the lysate, GSH was far more susceptible than Prx2; whereas 2 nmoles GlyCl induced a 50% loss of GSH, 45 nmoles were required to induce a similar loss of Prx2 monomer (Figure 3.12A). The data shown in Figure 3.12A represent lysate equivalent to a 5% cell suspension; however, the amount of oxidation of GSH (not shown) and Prx2 (Figure 3.12B) did not differ in lysate that was up to four-fold more dilute treated with the same amount of GlyCl per cell equivalent. Because the same amount of cell material was treated in both lysate and intact cell experiments, the effect of GlyCl on the two systems could be compared, based on complete reaction of GlyCl with the lysate compared with 20% consumption in an equivalent cellular system (Figure 3.5). Oxidation of GSH was much more efficient in the lysate (complete oxidation at 5 nmoles versus more than 100 nmoles). However, there was little difference for Prx2, which underwent 50% dimerisation with ~40 nmol GlyCl in both systems.
3.3.8 Testing for endogenous $\text{H}_2\text{O}_2$ production

A possible explanation for the oxidation of intracellular Prx2 by GlyCl is that the chloramine treatment caused the cells to generate increased amounts of endogenous $\text{H}_2\text{O}_2$. The hypothesis that $\text{H}_2\text{O}_2$ was responsible for the oxidation of Prx2 was explored; the amount of Prx2 oxidation was compared under conditions which either enhanced or inhibited any effect of endogenous $\text{H}_2\text{O}_2$. 

Figure 3.12 Prx and GSH oxidation in GlyCl-treated haemolysate.

A. Concentration-dependent loss of monomeric Prx2 and reduced GSH in 50 μl haemolysate (representing a 5% cell suspension) treated with GlyCl. Lysed cells were treated with GlyCl for 20 min; amounts of reduced Prx2 and GSH were determined by Western blotting and HPLC, respectively (n=2; values are means ± range). Similar results were obtained when the same GlyCl doses were applied to haemolysate which was up to 4 times more dilute. 

B. Western blot showing the dose-dependent increase in Prx2 oxidation by GlyCl. The same amount of haemolysate was treated with the molar amounts of GlyCl shown, in varying total lysate volume. Relative amount of monomeric Prx2 with each treatment was unaffected by the lysate dilution. Note that in control lysate, some Prx2 oxidation occurred during lysis, hence there is a higher starting level of dimer.
H$_2$O$_2$ generation. To inhibit H$_2$O$_2$ production by means of haemoglobin autoxidation, an erythrocyte suspension was pretreated with carbon monoxide to convert haemoglobin to carboxyhaemoglobin. To inhibit catalase and thereby enhance H$_2$O$_2$-induced oxidation of Prx, cells were pretreated with sodium azide. Neither treatment had any effect on GlyCl-induced oxidation of Prx2 (Figure 3.13), suggesting that endogenous H$_2$O$_2$ generation is not likely responsible for Prx2 oxidation in intact cells.

3.4 Discussion

Prx2 is a highly abundant protein in the human erythrocyte and is extremely sensitive to oxidation by hydrogen peroxide; Low et al [65] demonstrated that as little as 5 μM H$_2$O$_2$ caused complete oxidation of Prx2 within a minute in a dilute cell suspension. HOCl and chloramines are oxidising species that react rapidly with thiol compounds, and chloramines are especially reactive with low pK$_a$ thiols [10; 248; 249]. It was therefore hypothesised that erythrocyte Prx2 may be highly sensitive to HOCl and chloramines as well as H$_2$O$_2$, thereby acting as a sink for these oxidants and protecting the erythrocyte's vulnerable redox state.
Exposure of erythrocytes to HOCl and cell-permeable chloramines resulted in dose-dependent oxidation of Prx2 to its disulfide-bonded dimer. As observed with H$_2$O$_2$ treatment [65], there was no evidence of Prx2 hyperoxidation. The efficiency of Prx2 oxidation was compared to the oxidation of GSH. This was first studied in the absence of glucose, thus limiting glucose-dependent recycling of the antioxidants and allowing a direct comparison of the sensitivities. Prx2 was more sensitive than GSH when the cells were treated with HOCl or NH$_2$Cl, with complete dimerisation of Prx2 oxidation observed with $\sim$10 times less NH$_2$Cl than was required for all of the GSH to be oxidised. GlyCl treatment caused approximately proportionate oxidation of both Prx2 and GSH. An equivalent amount of GlyCl gave less oxidation than NH$_2$Cl or HOCl, even allowing for only 20% consumption over the treatment period. This could be related to the slow rate of entry of GlyCl and the reaction occurring throughout the incubation period, whereas the other oxidants were consumed quickly. TauCl had no effect on either antioxidant, which is consistent with this chloramine’s lack of cell permeability (observed here and by Grisham et al [45]). Even though Prx2 is an abundant protein in the erythrocyte ($\sim$5.6 mg/ml or 250 μM [250]), the GSH concentration is $\sim$10-fold higher [251]. On this basis, a decrease of similar proportions of reduced cellular GSH and Prx2 corresponds to $\sim$10 times more of the oxidant reacting with GSH. Even with the greater selectivity for Prx2 shown by NH$_2$Cl, the amounts of oxidant scavenged by Prx2 and GSH would be similar. Reactions of the oxidants with other thiol proteins are also likely to be competitive with GSH [37; 47]. The results do not, therefore, support the concept that Prx2 is a major sink for removing this class of oxidant.

Nevertheless, Prx2 was readily oxidised by HOCl or permeable chloramines, and this oxidation could be detrimental to erythrocytes, increasing their vulnerability to oxidative stress. The impact will depend on how efficiently oxidised Prx2 is recycled. This is brought about by the thioredoxin/thioredoxin reductase system, whereas GSSG is recycled by glutathione reductase. Both reducing systems use NADPH and their function in the erythrocyte depends on the pentose phosphate pathway and glucose availability. As expected, with glucose present there was substantially less oxidation of GSH due to recycling. It has been shown previously that in the absence of glucose, the majority of the GSH oxidised by HOCl is converted to GSSG [30] and the same would be expected for chloramines. There was some residual loss of reduced GSH in the presence of glucose. This was not due to impaired recycling as no GSSG accumulated and there was no recovery on extended incubation. Whereas glutathionylated protein accounts for some of the oxidised GSH, most of the GSH lost from treated cells could not be accounted for...
in the form of GSSG, glutathione sulfonamide, or mixed disulfides. Thus, it seems that the lack of regeneration of reduced GSH after chloramine treatment is due to oxidation to other products, including to a small extent of glutathionylation.

For each oxidant, the net oxidation of erythrocyte Prx2 was also less in the presence of glucose, indicating that the thioredoxin-dependent recycling mechanism was functional. With HOCl and NH₂Cl treatments, more Prx2 than GSH was oxidised in the metabolically active cells (Figure 3.8). However, as shown with the lower concentrations of NH₂Cl, reduced Prx2 was slowly recyclable and there was further recovery on prolonged incubation. This is consistent with the low level of thioredoxin reductase activity reported for erythrocytes [65]. At higher concentrations, at which all the Prx2 was oxidised, there was only partial recovery of reduced Prx2. A possible interpretation is that higher concentrations of NH₂Cl compromise the recycling mechanism, potentially by inhibiting thioredoxin reductase. It seems unlikely that the pentose phosphate pathway was inhibited as there was no GSSG accumulation in these cells.

Based on work [9] suggesting that the reaction between GlyCl and Prx2 is very slow, it was surprising that Prx2 was readily oxidised by GlyCl in the erythrocyte. The reaction of purified Prx2 with NH₂Cl is fast (it has been recorded as $1.5 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ [12]), but this is a thousand-fold less than with H₂O₂. Nevertheless, this value is in the range expected for a low pKₙ thiol [10; 48]. The difference in reactivity is likely to be due to the Prx structure facilitating the reaction with H₂O₂ and not NH₂Cl. These data show a similar trend to the findings with another thiol-reactive reagent, peroxynitrite, which reacts 10 times more slowly than H₂O₂ with Prx2 [5], and illustrate the high specificity of the Prxs for peroxides. The rate constants measured for GlyCl and TauCl with Prx2 are less than for GSH and a remarkable million fold lower than the value for H₂O₂. The reason for the low reactivity of these chloramines is not apparent. It is unlikely that higher reactivity simply requires the chloramines to be small and uncharged, as ethanolamine chloramine, which is only slightly larger than NH₂Cl and also neutral, was far slower to react with Prx2 [9].

The fact that erythrocyte Prx2 and GSH are oxidised to similar extents by HOCl is not surprising, as HOCl shows little discrimination between thiols [47]. NH₂Cl -induced oxidation in the intact cell correlates well with the rate constants. Conversely, the extent of Prx2 oxidation—relative to GSH—by GlyCl in the erythrocyte is greater than predicted from relative rate
constants, and other influences must be involved. The hypothesis that endogenous H$_2$O$_2$ could be responsible for GlyCl-induced oxidation of Prx2 in the cells was explored but it was demonstrated that this was not the case. However, it is possible that the inhibition of catalase was insufficient to observe an additional effect of endogenously-generated H$_2$O$_2$ on Prx2 oxidation; it may be necessary to monitor H$_2$O$_2$ production by more sensitive means to confirm or exclude this possibility more conclusively.

One other possible explanation for this apparent discrepancy in the relative oxidation of GSH and Prx2 could relate to recycling of the antioxidants. If, even in the absence of added glucose, sufficient glucose remains inside cells to allow rapid GSH recycling, GSH could appear less oxidised while Prx2 would be slowly recycled due to low TrxR activity in the erythrocyte [65]. The observation that GSH is much more readily oxidised in haemolysate is consistent with this hypothesis; however, further investigation is required to determine whether or not this is the case.

### 3.5 Summary

In conclusion, Prx2 is oxidised in erythrocytes treated with HOCl and chloramines, but the extent of oxidation is insufficient for it to provide major antioxidant protection against oxidative damage by these neutrophil-derived oxidants. The findings reported in this chapter support the notion that Prx2 has a highly specialised role in protecting the erythrocyte against H$_2$O$_2$-mediated oxidation. Oxidised Prx2 was recycled in the presence of glucose but depletion of Prx2 in addition to GSH in cells exposed to higher concentrations of HOCl and chloramines could be detrimental in enhancing their sensitivity to further oxidative stress.
Chapter 4. Effect of MPO-derived oxidants on Prx oxidation in endothelial cells

Results from this chapter have been included in:

Stacey, M.M., Vissers, M.C., Winterbourn, C.C. 2-Cys peroxiredoxins are oxidised by inflammatory oxidants in human endothelial cells (manuscript in preparation).

4.1 Introduction

The endothelium is arguably one of the most vulnerable tissues in terms of exposure to localised production and release of oxidants from activated neutrophils in the vasculature. Not only is the endothelium vulnerable to oxidative injury, but endothelial dysfunction is widely accepted to be involved in the initiation and progression of inflammatory diseases such as atherosclerosis [81; 82].

However, the principal protein targets and associated biochemical pathways impacted by neutrophil oxidants have not been identified. Revealing the major targets of these oxidants could lead to better understanding of the mechanisms of endothelial dysfunction and therefore better treatment of inflammation. This is especially relevant to diseases such as obesity, diabetes mellitus, cancer, atherosclerosis, hypertension and arthritis, in which chronic low-grade inflammation is a contributing – if not complicating – factor.

Chloramines are known to react preferentially with thiol residues, and in particular low-pKa thiols [47]. One family of enzymes with a conserved low-pKa thiol residue at the active site is the peroxiredoxin (Prx) family. Prx oxidation status has been proposed as a potential sensor or indicator of in vivo oxidative stress, and the sensitivity of Prxs to oxidation by H$_2$O$_2$ in a variety of cell types has been investigated [65; 148; 159-161]. As discussed in Chapter 3, Prx2 in the erythrocyte can be oxidised by exogenous addition of chloramines[12], but it is unknown whether 2-Cys Prxs are sensitive to chloramines in other cell types. This chapter aimed to
investigate the oxidation state and recycling of Prxs 1-3 in HUVECs treated exogenously with \( \text{H}_2\text{O}_2 \), HOCl and chloramines.

### 4.2 Experimental Approach

Chloramines were generated as described (Section 3.2). \( \text{H}_2\text{O}_2 \) and HOCl were diluted from stock solutions of known concentration.

To determine the concentration of oxidant consumed during cell treatment (through loss of oxidant in the supernatant) a spectrophotometric assay measuring the oxidation of 3,3',5,5' tetramethylbenzidine (TMB) was used [225]. This assay is described in Section 3.2.

Peroxiredoxin redox status was examined by immunoblotting (Section 2.13) [65], with antibodies against the specific Prx isoforms. This assay is described in Chapter 3 (Section 3.2). In addition, the amount of hyperoxidised peroxiredoxin (Prx-SO\(_{2/3}\)) was quantified using a method employed by Cox et al [161] (Section 2.14). This method exploits the fact that when cells are lysed in the absence of alkylating agents, any reduced, monomeric Prxs become oxidised by small amounts of peroxide in the extract buffers. Thus, any remaining monomeric Prxs consist only of the hyperoxidised form(s) (Figure 4.1).

![Figure 4.1 Method used to quantify the amount of hyperoxidised Prx in cells.](image)

Cells lysed in the absence of alkylating agents are exposed to sufficient \( \text{H}_2\text{O}_2 \) in buffers to dimerise any reduced Prxs. Thus, monomeric Prxs as detected by non-reducing SDS-PAGE and western blotting consist of the proportion of enzyme which was hyperoxidised and in the monomeric form prior to lysis.
Intracellular levels of reduced glutathione (GSH) were measured by derivatisation with MBB. Fluorescent adducts were separated by HPLC with fluorescence detection as described in Section 3.2).

Total protein thiols were assessed in cell lysates derivatised with MBB; proteins were precipitated to capture only the MBB-derivatised proteins, and any non-protein thiols (e.g. GSH) were removed in the supernatant. Proteins were resolubilised and the fluorescence measured by fluorescence spectroscopy; the amount of fluorescence in this assay is a measure of the MBB-derivatised proteins, and the oxidation of free thiols is detected as a loss of fluorescence compared to controls.

Thioredoxin reductase activity was assessed by spectrophotometric measurement of the NADPH-dependent conversion of DTNB to TNB (Figure 4.2). The change in absorbance at 412 nm was monitored for 5 min in 96-well plates and the average slope used to calculate

\[
\frac{\Delta c_{\text{TNB}}}{\Delta \text{min}} = \frac{\Delta \text{Abs}}{\Delta \text{min}} \times \frac{1}{\varepsilon \times l}
\]

\[
\frac{\Delta c_{\text{DTNB}}}{\Delta \text{min}} = \frac{\Delta c_{\text{TNB}}}{\Delta \text{min}} \times \frac{1}{2}
\]

\(c = \text{concentration (\(\mu\text{M} = \text{nmol/ml}\))}
\)

\(\text{Abs} = \text{absorbance (}\lambda = 412\text{nm})
\)

\(\varepsilon = 1.415 \times 10^{-3} \mu\text{M}^{-1}\text{s}^{-1}
\)

\(l = 0.75\text{ cm}
\)

**Figure 4.2** TrxR Activity was monitored spectrophotometrically. The NADPH-dependent conversion of DTNB to TNB is detected as an increase in absorbance at 412 nm.
approximate activity (using an approximate path length of 7.5 mm and equations from Figure 4.2). Baseline slope was calculated in the absence of added NADPH; final values are reported after subtraction of the baseline slope.

Cell viability was assessed by two different methods. Initially, the effect of oxidant treatment on cell viability was measured by exclusion of propidium iodide (PI), detected using flow cytometry (Section 2.17.1). This loss of membrane integrity was used as a general measure of viability in comparing the dose-dependent effects of each oxidant on HUVECs. To investigate the effect of specific treatments on cell viability and proliferation, reduction of MTT to formazan was measured (Section 2.17.2; Figure 4.3). This assay relied on the mitochondrial reductase activity in live cells, and required less cellular manipulation than the PI assay prior to analysis (i.e. no disruption of the monolayer prior to permabilisation, and fewer wash steps), allowing for more sensitive detection of treatment toxicity.

![Figure 4.3](image)

**Figure 4.3** In mitochondria of living cells, MTT is converted to a formazan product. The formazan crystals are detected spectrophotometrically and the absorbance compared between treated and untreated cells to determine the toxicity of the treatment.

An enzyme-linked immunosorbent assay (ELISA) was performed using a BioCell® kit (Papatoetoe, New Zealand) to quantify the level of protein carbonyls in HUVECs (Figure 4.4). The protocol involved sample derivatisation with dinitrophenylhydrazine (DNP), followed by adsorption of the DNP-bound proteins to an ELISA plate. Immunodetection involved incubation with anti-DNP biotin-conjugated antibodies, followed by Streptavidin-linked horseradish peroxidase and finally chromatin reagent containing peroxide, which catalyses the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) to a blue product. The reaction was stopped by addition of sulfuric acid and colorimetric detection of the final yellow product at 450 nm was used to quantify the amount of DNP-bound protein from a standard curve.
Figure 4.4 Scheme for Protein Carbonyl ELISA.
Chapter 4: Effect of chlorinated oxidants on Prxs in HUVECs

4.3 Results

4.3.1 Prxs in HUVECs

HUVECs were found to express each of the three Prxs of interest (cytosolic Prxs 1 & 2, and mitochondrial Prx3). Using Western blot analysis to compare signal intensity of the Prxs against purified protein, Prx protein levels were determined to be in the range of several ng (~4-6) per µg total protein (Figure 4.5).

![Figure 4.5 Quantification of Prxs by Western blotting.](image)

Lysates containing known amounts of protein were separated by reducing SDS-PAGE alongside standardised amounts of pure Prx protein. Western blotting with antibodies against the specific isoforms of Prx was followed by chemiluminescence and densitometry to estimate Prx content in cells.

Untreated HUVECs were lysed in the presence of the alkylating agent, (NEM), and the proteins separated by non-reducing SDS-PAGE; under these conditions the Prxs migrated primarily at ~21-23 kDa, the MW of the reduced monomeric enzymes. Some dimeric (disulfide-linked, oxidised) Prx was detected in control (untreated) cells (Figure 4.6A, lane 1). Of the three Prxs investigated, Prx3 was most highly oxidised. Hyperoxidation of the Prxs was not observed in control cells [Figure 4.6A, (-NEM) lane 1].

4.3.2 Effect of H₂O₂ on Prx redox state

H₂O₂-treated HUVECs lysed in the presence of NEM showed some modest dimer accumulation, most evident with Prx1 (Figure 4.6A, left panel), but in cells treated with sublethal doses of hydrogen peroxide, Prxs accumulated primarily in the hyperoxidised form as opposed to the dimeric form. To isolate and quantify the effect of H₂O₂ on Prx hyperoxidation, cells were lysed in the absence of alkylating agents (Figure 4.6 A(right panel) & B). With increasing doses of
H$_2$O$_2$ accumulation of the hyperoxidised band was observed for all 3 Prxs, with maximal effect at 120 μM. The levels of hyperoxidation differed; Prx2 was most hyperoxidised, while Prx3 was less readily hyperoxidised at each H$_2$O$_2$ concentration.

**Figure 4.6 Prx redox state in H$_2$O$_2$-treated HUVECs.**

A Nonreducing western blots demonstrated the redox status of Prxs in HUVECs subjected to 10 minute treatments of H$_2$O$_2$ of varying doses. Cells treated in M199 were harvested in extract buffer with (left panel) or without (right panel) NEM present during lysis. In the left panel, monomer (M) is the sum of any reduced and/or hyperoxidised monomeric enzyme, and dimer is disulfide-linked oxidised Prx. In the right panel (-NEM), monomer (M) represents hyperoxidised species only, while dimer (D) in this assay represents all non-hyperoxidised species (sum of reduced and dimeric protein at time of lysis). B Prx hyperoxidation (% monomer) in the absence of NEM was quantified by non-reducing SDS-PAGE followed by Western blotting with antibodies against Prx1, Prx2 and Prx3 and the data (means ±SEM for at least 3 separate experiments) shown in B. Data were analysed by one-way ANOVA with Holm-Sidak multiple comparison (p ≤ 0.05). Significance is indicated where *all three* Prxs are significantly more hyperoxidised than untreated cells (*, above data points), and where there was a significant difference between two different Prxs (*, at right; significance for the latter comparison applies for all concentrations ≥ 120µM).
Analysis of the H$_2$O$_2$ remaining in the supernatant (Fox assay, Section 2.8) showed that for each concentration added to the cells, a maximum of 30% was consumed over the 10 min treatment (not shown). Thus, only a fraction of the H$_2$O$_2$ was responsible for mediating the Prx redox changes observed.

It has been reported that purified Prx1 does not hyperoxidise except in the presence of all catalytic components that enable turnover (Trx, TrxR, NADPH)[252]. Auranofin (AFN) is a gold-containing compound which at low concentrations inhibits TrxR [253], and thereby should inhibit the catalytic cycling of Prxs. In this study AFN induced a dose-dependent effect on HUVEC TrxR activity with close to 100% inhibition at 4 µM AFN applied for 20 min (Figure 4.7).
Concomitantly, there was a dose-dependent dimerisation of Prxs (shown as a loss of reduced monomer, Figure 4.7B), and this effect was significant for Prxs 1 & 2 at the same AFN dose which induced complete loss of TrxR activity. Western blotting of AFN-treated cells lysed in the absence of NEM confirmed that in the absence of TrxR activity, HUVECs were insensitive to $\text{H}_2\text{O}_2$-induced Prx hyperoxidation (Figure 4.7). Thus, it was possible to inhibit Prx hyperoxidation in HUVECs by AFN-induced interference of the Prx catalytic cycle.

To establish whether the Prx hyperoxidation induced by $\text{H}_2\text{O}_2$ in endothelial cells was reversible, cells were treated with 400 µM $\text{H}_2\text{O}_2$ in 6-well plates (a concentration that gave similar levels of oxidation to 120-200 µM in the smaller well format; Figure 4.6), then allowed to recover in fresh medium (Figure 4.8). After a 10 min treatment, all three Prxs were significantly hyperoxidised, and all showed regeneration of the reduced species within 6 h. After 2 and 4 h, Prxs 1 and 2 had undergone significant regeneration compared to the initial treatment time. Recycling of Prx3 was
more difficult to detect because it underwent less hyperoxidation. It appeared slower than for Prxs 1 and 2, but after 4 h the level of Prx3 dimer no longer differed significantly from control values.

4.3.3 Chloramine or HOCl-induced oxidation of Prxs

When HUVECs were treated with GlyCl, NH₂Cl and HOCl, there was a dose-dependent decrease of the Prx monomers (Figure 4.9), corresponding to dimer accumulation. Unlike treatments with H₂O₂, no hyperoxidised Prx species were formed (even in the absence of alkylating agents; not shown).

The three Prxs showed similar levels of oxidation for each oxidant examined. However, a major difference between the Prxs was the fact that Prx3 oxidation did not differ significantly from control levels with any of the concentrations of GlyCl employed, whereas Prx1 and 2 were oxidised when cells were treated with higher doses of GlyCl (Figure 4.9A). Prx2 was most sensitive to oxidation in cells treated with NH₂Cl; significant levels of oxidation were seen with as little as 10 μM (Figure 4.9B). HOCl-treated cells (20-100 μM) induced similar levels of oxidation in all three of the Prxs (Figure 4.9C). Prx2 (marginally the most highly oxidised of the three Prxs examined) was almost completely oxidised with: 500 μM GlyCl, 15 μM NH₂Cl, and 80 μM HOCl.

4.3.4 Chloramine consumption

Consumption of these oxidants upon treatment of cells differed considerably (Table 4.1). This was reflected in uptake over 30 min: loss of GlyCl from the treatment buffer was less than 10%, whereas ~70% of the NH₂Cl was consumed. If consumption is assumed to be proportional to chloramine concentration, this would imply that complete oxidation (in 10 min) of Prx2 required approximately 10 μM GlyCl and 4 μM NH₂Cl. By comparison, more than 50% of the HOCl was consumed within 10 min, with another ~25% consumed by 30 min (note that the apparent decreased rate of consumption between 10 and 30 min could be due to the production of other secondary oxidants which could not be distinguished in this system). The higher amount of HOCl required for Prx oxidation probably reflects its high reactivity with a range of substrates, and possible role in secondary chloramines or other relevant oxidation products.
Figure 4.9 Chloramine-induced oxidation of peroxiredoxins.

HUVEC cultured in 24-well plates were treated with the given concentrations of chloramine or HOCl in a 1-ml volume of HBSS for 10 min at 37°C. Cells were lysed in the presence of NEM to alkylate free thiols; the relative concentration of monomer was quantified by non-reducing SDS-PAGE followed by Western blotting with antibodies against A, Prx1; B, Prx2; and C, Prx3. Densitometry was used to determine the percentage of Prx present in the monomer position, indicating the reduced form of the enzyme. Data are means ± SEM for at least 3 separate experiments. Data which differed significantly from control (0 µM) are indicated; statistically significant differences were determined by one-way ANOVA with Holm-Sidak multiple comparison (*p<0.05; **p<0.005; ***p<0.001).
4.3.5 **Regeneration of reduced Prxs following dimerisation**

To determine whether chloramine-induced Prx oxidation was reversible, cells were treated with sufficient chloramine to dimerise most of the Prxs, then incubated in fresh media (Figure 4.10). This resulted in rapid reduction of all three Prxs within 10 min and complete recovery at 30 min. In comparison, cells treated with sufficient HOCl to induce high levels of Prx oxidation did not recover (not shown). Cell morphology appeared to deteriorate following HOCl treatment with loss of protein and no regeneration of Prx monomer.

Inhibition of TrxR activity can be sufficient to induce accumulation of Prx dimer (Figure 4.7B). Prx dimer accumulation in cells treated with chloramines or HOCl could result from an indirect effect of the inhibition of the reductase. This possibility was investigated by measuring TrxR activity in cells treated with each of the oxidants. TrxR appeared unaffected by either the chloramine or \( \text{H}_2\text{O}_2 \) treatments, but a dose-dependent decline in TrxR activity was observed with increasing concentration of HOCl (Figure 4.11). Therefore, loss of TrxR activity could contribute to HOCl-dependent (but not chloramine- or \( \text{H}_2\text{O}_2 \)-dependent) Prx oxidation.


Figure 4.10 Regeneration of reduced Prxs following chloramine-induced oxidation. HUVECs (grown in 6-well plates) were treated for 10 min with oxidant in HBSS; after treatment, cells were washed with PBS and either returned to M199 or lysed immediately for '0 min' time point) replaced. Following 10- or 30 minute recovery, cells were lysed in the presence of NEM. Prx oxidation was quantified by non-reducing SDS-PAGE followed by Western blotting with antibodies against Prx1, Prx2 and Prx3. Values are means ± SEM for 4-8 separate experiments. Statistically significant differences were determined by one-way ANOVA with Holm-Sidak multiple comparison; significant differences relative to untreated cells (*, p<0.005) and relative to treated cells at t=0 min(#, p<0.005) are shown. Prx1 was significantly regenerated after only 10 min recovery regardless of treatment (GlyCl or NH₂Cl). Following GlyCl treatment, Prx 2 was significantly regenerated after 10 min recovery. Prxs 2 and 3 underwent significant monomer regeneration by 30 min recovery when oxidation was induced by NH₂Cl.
4.3.6 Comparison of Prx oxidation with loss of reduced GSH

To assess whether the oxidation of Prxs in the presence of the oxidants was simply a reflection of broad-spectrum thiol oxidation in treated cells, loss of GSH was assessed (Figure 4.12). No significant decrease in GSH was observed following H$_2$O$_2$ treatments. Significant losses of GSH in chloramine-treated cells were observed, but only at concentrations of oxidant that gave almost complete Prx oxidation; thus, there was a greater loss of reduced Prx than GSH with these treatments. For instance, 25 μM NH$_2$Cl was the lowest concentration at which significant loss of GSH was observed, while Prxs 1-3 were fully oxidised. In contrast, HOCl treatments oxidised GSH over the same concentration range that caused Prx oxidation.

Figure 4.11 TrxR activity in treated HUVECs.

HUVECs grown in 24-well plates were treated with the given concentrations of oxidant for 10 min in HBSS, and then assayed for TrxR activity by NADPH-dependent reduction of DTNB. Statistically significant differences from untreated cells are shown (*, p<0.001 as determined by one-way repeated measures ANOVA with Holm-Sidak multiple comparison; n=3±SEM).
4.3.7 Oxidant-induced loss of viability

Uptake of PI was measured either immediately after treatment or 24 h after treatment, with cells maintained in fresh M199 during recovery (Figure 4.13). Whereas H$_2$O$_2$ caused no significant increase in fluorescence, cells treated with chloramines or HOCl did show a concentration-dependent increase in PI-positive cells, corresponding to a concentration-dependent loss of membrane integrity. However, only cells treated with NH$_2$Cl or the highest concentration of GlyCl were significantly less viable than control cells. No increase in PI was observed.
immediately after treatment except with HOCl treatments (which were not significantly different from controls), where results were similar to those seen at 24 h (Figure 4.13D) this suggests that while HOCl mediates a loss of cell membrane integrity immediately, NH$_2$Cl appears to cause a more delayed loss of viability in cells. It was also the most cytotoxic compound investigated, with 10 min treatments of 15-25 μM leading to cell death in up to 80% of cells.

**Figure 4.13 Dose-dependent loss of viability in HUVECs treated with different concentrations of oxidant.**

Cells were interrogated by flow cytometry for fluorescence from propidium iodide immediately or 24 h after treatment. Treatments were in HBSS; where 24 h samples were taken, cells were maintained in M199 following treatment. Data are from at least 3 separate experiments and represent means ±SEM; treatments which induced significantly higher cell death compared to untreated cells are indicated (*, p<0.05, by one-way ANOVA with Holm-Sidak multiple comparison).
4.3.1 Relationship between Prx oxidation and viability

Linear regression analysis revealed a strong relationship between Prx oxidation (dimer formation at 10 min) and cell viability (PI uptake 24 h after treatment) when all of the data for chloramines as well as HOCl were considered (Figure 4.14). Interestingly, there was no such consistent relationship between cell death and either GSH oxidation (not shown) or inactivation of GAPDH (from data extrapolated from [36]).

![Figure 4.14 Regression analysis of Prx oxidation and cell death measured by uptake of propidium iodide.](image)

The proportion of cells which were positive for PI at 24 hr post-treatment in HUVECs treated for 10 min with chloramines or HOCl correlated with the amount of Prx1, Prx2 or Prx3 oxidation in cells given the same treatment. Untreated control values were included in the analysis. Data are those illustrated in Figure 4.9 and Figure 4.13. Values are means from at least 3 experiments; R² values and significance were obtained by linear regression with analysis of variance.
Because of the strong correlation between cell death and Prx oxidation for all three Prxs, Prxs might be protecting cells against chloramine-induced toxicity. To investigate this hypothesis, endothelial cells were pre-treated with H$_2$O$_2$ prior to NH$_2$Cl treatment to hyperoxidise the Prxs and prevent their reactions with chloramines. Alternatively, pre-treatment with AFN inhibited TrxR, thereby preventing the Prx catalytic cycle (Figure 4.15).

**Figure 4.15 MTT measurement of HUVEC viability 24 hr after treatment.**
H$_2$O$_2$ (400 µM, 20 min), AFN (4 µM, 20 min) and NH$_2$Cl (15 µM, 10 min) treatments were applied either alone or in combination; where in combination, order of treatments is shown as a subscript in legend under graph (n=3; data are means ±SD). Following treatment(s) in HBSS, cells were returned to culture medium for 24 h, whereupon viability was measured using MTT assay as described under Methods. Significance was determined by one-way ANOVA with Holm-Sidak multiple comparison: #, data are significantly different from control, NH$_2$Cl alone, and H$_2$O$_2$ or AFN alone, p<.001; +, datum is significantly different from control and from H$_2$O$_2$, but not NH$_2$Cl alone, p<.005; *, paired treatments are significantly different from each other, p<.001.

Analysis of cell viability by the MTT assay demonstrated that cells treated with either NH$_2$Cl or H$_2$O$_2$ alone underwent no loss of viability. (Note that this assay was optimized in 6-well dishes, and that although larger treatment volumes were used to accommodate the increased cell number, greater concentrations of oxidant were tolerated by cells grown in these larger dishes; thus, a treatment of 15 µM NH$_2$Cl alone in this case induced no decrease in viability, unlike in the 24-well dishes used in other assays). However, HUVECs pretreated with H$_2$O$_2$ immediately before exposure to 15 µM NH$_2$Cl showed a dramatic decrease in cell viability. To determine
whether this phenomenon could be explained by a synergistic (and non-Prx-related) effect of the two treatments, the treatments were also reversed, with NH$_2$Cl treatment preceding H$_2$O$_2$. Whereas H$_2$O$_2$ followed by NH$_2$Cl treatments caused on average an 84% decrease in viability, only 35% loss of viability was observed when the order was reversed (Figure 4.15).

HUVECs pretreated with AFN and then NH$_2$Cl also had decreased viability (75% loss on average), the effect of which was similar to that seen with H$_2$O$_2$ and NH$_2$Cl. However, when the treatment order was reversed (NH$_2$Cl then AFN) viability remained low, with an approximate 65% decrease compared to control cells (Figure 4.15).

An alternative explanation for the increased cell death seen with the combinations of treatments could be that the cumulative oxidative damage induced by the treatments was responsible for inducing cell death. If this were the case, a decrease in total protein thiols (indicating selective oxidation of these residues) or an increase in protein carbonyls might be observed; this was investigated (Figure 4.16). However, the loss of total protein thiols did not relate to viability; on average each treatment alone or in combination induced no more than 25% loss of protein thiols compared to untreated cells (Figure 4.16A). Protein carbonyls did increase even with NH$_2$Cl treatment alone (~1.6-fold over control levels; Figure 4.16B), but none of the treatment combinations resulted in a further increase in protein carbonyls (only the NH$_2$Cl - H$_2$O$_2$ combination increased protein carbonyls to 170% of control levels, on average). Taken together, these results suggest that the cell death measured in HUVECs treated with AFN and NH$_2$Cl in combination, or H$_2$O$_2$ followed by NH$_2$Cl, could not be explained by cumulative oxidative damage to proteins in HUVECs.
Figure 4.16 Levels of protein thiols and protein carbonyls in HUVECs treated with exogenous oxidants.
Total protein thiols and protein carbonyls were measured by fluorometry and ELISA, respectively, in cells treated with NH$_2$Cl, H$_2$O$_2$ and AFN, alone or in combination. A Variations in total protein thiols, as measured by MBB with fluorescence detection are from 2 separate experiments. For untreated cells, mean fluorescence intensity was ~100 fluorescence units. Data are means ± range, and statistical analysis was by one-way ANOVA). B Most treatments caused an increase in the level of protein carbonyls compared to untreated cells. Untreated cells had ~0.2 nmol protein carbonyls per mg of total protein. Only the increased percentage of protein carbonyls induced by the combined treatment of NH$_2$Cl followed by H$_2$O$_2$ was statistically significant compared to the expected mean of 100% (one-sample t-test, p<0.05).
4.4 Discussion

This chapter demonstrates that in endothelial cells, 2-Cys Prxs are present and exist in a partially oxidised form. These enzymes appear to be in a dynamic state of redox cycling as dimerisation is enhanced when recycling by TrxR is inhibited. This implies that Prxs scavenge low levels of endogenously-generated H$_2$O$_2$. As shown in other cell types, this study of cultured HUVECs indicates that Prxs 1 and 2 are sensitive to oxidative inactivation by exogenous H$_2$O$_2$, while Prx3 is less so. In addition, for the first time it has been demonstrated that these 2-Cys Prxs are readily oxidised by chloramines and HOCl, with accumulation of Prxs in a different redox state to that seen with H$_2$O$_2$. Prxs are likely involved in scavenging chlorinated species in living cells, which is a novel role for this class of enzymes.

In most cells that have been investigated, with the exception of the erythrocyte, exposure to H$_2$O$_2$ leads to accumulation of the hyperoxidised form of the typical 2-Cys Prxs [160; 161; 252]. This is also the case in HUVECs, as demonstrated in this chapter. Prxs became hyperoxidised in a dose-dependent manner, reaching a maximum level of hyperoxidation with ~120 μM H$_2$O$_2$. The level of maximum hyperoxidation differed between the isoforms, with Prx3 showing resistance to hyperoxidation compared with Prxs 1 and 2. This difference has been observed in other cell lines as well as with purified Prx proteins [161], and a recent study has suggested a structural basis for this phenomenon [254].

In addition to a structural basis for the difference in sensitivity to hyperoxidation of Prx3 compared to the other 2-Cys Prxs, the mitochondrial localisation of this isoform renders it less accessible to exogenous doses of H$_2$O$_2$; this gives the enzyme an apparently lower sensitivity in a to H$_2$O$_2$ in a cell-based system. Nevertheless, it has been demonstrated that purified Prx3 also behaves in a manner consistent with cell-based studies [161], in that reaction with H$_2$O$_2$ is high, but hyperoxidation is not facilitated as seen with the cytosolic Prxs. It has been suggested that this could important for maintaining the antioxidant function (through catalytic cycling) of Prx3 in the mitochondria, where endogenous production of H$_2$O$_2$ is high, and reduction of the sulfenic acid is slow (reviewed in [255]).

The catalytically inactive hyperoxidised Prxs are reducible by sulfiredoxin (SrX), an enzyme with a slow catalytic rate constant ($k_{cat} = 0.18$/min [154]). In line with this (and also consistent with observations made in other cell types [137; 256], very slow regeneration (4-6 h) of the reduced
forms of the three Prx isoforms was observed. Although Srx is normally a cytosolic protein, Noh et al. [256] demonstrated that Srx translocates to the mitochondria under situations of oxidative stress, where it is able to reduce hyperoxidised Prx3. The slower rate of Prx3 reduction (relative to Prxs 1 and 2) shown here might be related to the primary subcellular localisations of the enzymes involved.

The possibility that our findings represent resynthesis of active Prx (combined with degradation of hyperoxidised protein) cannot be ruled out, but this seems unlikely. Although this could not be tested directly using cycloheximide because of toxicity issues, in other cell types Prx regeneration was due primarily to reduction by Srx rather than by de novo synthesis of the enzyme [137; 155]. Also, recovery experiments suggest that the total amount of Prx did not change significantly over the recovery period, since total band intensity was relatively constant.

Despite the propensity for hyperoxidation in the presence of peroxide, Prxs showed no hyperoxidation in the presence of the chlorinated oxidants investigated here. Instead, Prxs were oxidised to the dimeric forms following in vitro exposure to cell-permeable chloramines and HOCl. This is likely due to the slower rate of reaction between the enzyme and substrates; rapid reaction of oxidized Prx with a second molecule of \( \text{H}_2\text{O}_2 \) is required for the formation \( \text{Prx-SO}_2^- \) such that a disulphide is unable to first form between two adjacent Prx monomers. However, the reaction of Prx with chloramines molecules is so much slower that dimer formation likely occurs prior to the reaction between \( \text{Prx-SO}_2^- \) and a second chloramine (or HOCl).

Furthermore, Prx oxidation induced by either GlyCl or \( \text{NH}_2\text{Cl} \) was short-lived due to rapid recycling by the Trx-TrxR system. Thus, only the Prx-dependent portion of the catalytic cycle appeared affected by these chlorinated oxidants. Further evidence of this includes the lack of TrxR inhibition (Figure 4.11).

The oxidative effects of HOCl are broad-ranging [257], and less selective for thiol residues [48]. Compared with chloramines, HOCl did not inactivate Prxs as specifically. In addition to oxidising other thiols like GSH, inhibition of TrxR was observed with HOCl treatments. Prx recycling was not apparent, and in general cell recovery following HOCl treatment was not as thorough as with chloramines. Indeed, it has been suggested that the formation of chloramines
under physiological conditions might protect the body’s cells from the damaging effects of HOCl [71], and the oxidative effects investigated in this study appear to support this notion.

Of the oxidants examined, NH₂Cl was the most cytotoxic. NH₂Cl is roughly 10-fold more cell-permeable than GlyCl (Table 4.1) and the more rapid uptake of this oxidant might account for the difference in cytotoxicity. The toxic effects of HOCl on cells are immediate; cells which maintain membrane integrity throughout the 10 min treatment remain PI-impermeable at 24 h after the treatment.

There was a strong correlation between Prx oxidation and cell death when each of the chlorinated oxidants were considered. This could reflect protection or association of the Prxs with cell death pathways (either causal or otherwise), or that Prx oxidation causes cell death. Of note, the lack of GSH oxidation, overall loss of cellular thiols, or increase in protein carbonyls is good evidence that the effects observed were not a result of general thiol oxidation. The hypothesis that cell viability could be linked to Prx redox status was therefore investigated. Endothelial cells were pre-treated with H₂O₂ prior to NH₂Cl treatment to hyperoxidise the Prxs and prevent their reactions with chloramines. The combination of H₂O₂ and NH₂Cl (regardless of order) was more harmful than either treatment alone, but cells were far worse off when H₂O₂ preceded NH₂Cl treatment. This would suggest that if Prxs are hyperoxidised and therefore unavailable to be oxidised by chloramine treatment, cells are more vulnerable to cell death from other chloramine-mediated reactions in the cell. However, when cells were treated with a chloramine first, the regeneration of the reduced form of Prx was likely rapid enough that it became available to react with some of the H₂O₂; other antioxidant systems such as the GPxs and catalases would also likely compensate and help protect the cell from potentially damaging effects of H₂O₂.

Similarly, AFN treatment in combination with NH₂Cl caused a decrease in cell viability. However, the viability outcome did not depend on the order in which the treatments were applied. The increased cytotoxic effect of NH₂Cl when TrxR was inhibited could indicate a protective effect of NH₂Cl removal by cycling Prxs.
Prxs, along with GPxs and catalase, are considered to be highly important in protecting cells against $H_2O_2$, especially at low doses. It has been shown here that the Prx/Trx catalytic cycle is also effectively oxidised by chloramines in HUVECs. Prxs have been linked to the prevention of cell death pathways (reviewed in [258]), but their role is usually associated with the scavenging of reactive oxygen species, especially those generated endogenously [259]. The redox status of the 2-Cys Prxs is likely to have an impact on their biological function, and these functions could be very important in the context of inflammation. Specifically, Prxs could be playing a key role in preventing the activation of death pathways when cells are exposed to inflammatory oxidants. When Prxs were allowed to accumulate in the dimeric form (and reduction was prevented), cell death increased. Understanding the exact role of Prxs in cell death pathways will require further study involving manipulation of the expression levels of these enzymes.

Endothelial cells in the proximity of an inflammatory site in vivo would be exposed to inflammatory oxidants (including $H_2O_2$, HOCl and chloramines). It would be very interesting to confirm these findings in a physiologically relevant model by using activated neutrophils to generate the inflammatory oxidants for endothelial exposure. Preliminary attempts to investigate this very question were unsuccessful, since phorbol 12-myristate 13-acetate (PMA) -stimulated neutrophils were found to induce proteolysis in the endothelial cells (hence no valid Prx data was obtained). It was, however, highly intriguing that amines could protect against this proteolysis when added to the system. This suggested that formation of chloramines might have a protective role under these circumstances. It is possible that chloramines might either neutralise proteolytic enzymes, or activate a HUVEC response which is protective against the neutrophil proteolytic enzymes. Further investigation is necessary to elucidate which oxidants ($H_2O_2$, HOCl, or chloramines) are most relevant in a physiological model of inflammation in terms of both Prx oxidation and protection against proteolysis.

The sensitivity of Prxs, as well as their rapid oxidation/reduction facilitated by the Trx/TrxR system makes this machinery a very efficient and effective mechanism for specific removal of chloramines. Based on the susceptibility of Prxs to oxidative modification following short-term exposure of endothelial cells to these oxidants, these enzymes are of interest in relation to inflammation. In the context of inflammatory conditions, Prxs could be acting as important antioxidants and/or participating in the control of cell death pathways.
Chapter 5. Proteomic investigation of novel targets oxidised by chlorinated oxidants in endothelial cells

5.1 Introduction
It has been well established that chloramines and HOCl react preferentially with thiol residues. While HOCl is much more reactive than chloramines (e.g. HOCl reacts with GSH more than $10^4$ times faster than do chloramines), both favour reactions with cysteine and methionine residues [47]. Thus, HOCl and chloramines are suspected to affect thiol-dependent cellular events in cells or tissues exposed to these compounds.

The specific targets of chloramines and HOCl in intact cells are still unidentified, and this is further complicated by the fact these oxidants are not all equal in terms of their cell permeabilities, reactivities and therefore preferred targets [23; 43-45]. A variety of factors therefore influence how each of these compounds affects living cells: which pathways become activated or repressed, and which antioxidant molecules might act to protect the cells. This chapter explores the hypothesis that specific intracellular protein thiols are targets for HOCl and chloramine oxidation.

5.2 Experimental approach
The experiments described in this chapter employed IAF-labelling of oxidised protein thiols to visualise specific proteins which underwent reversible oxidation in treated cells. In HUVECs treated with chloramines, HOCl or $\text{H}_2\text{O}_2$, any reversibly oxidised thiol residues (i.e. those with disulfide bonds, with thiols in the sulfenic acid state or glutathionylated thiol residues) were reduced by incubation with DTT, and the resultant thiols labelled with 5-iodoacetamidofluorescein (IAF), as described under Methods (Section 2.23) and shown in Figure 5.1.

Prepared cell lysates were separated by one- or two-dimensional (2-D) SDS-PAGE and IAF-labelled protein spots were visualised by fluorescence imaging. In addition, positive protein identification in some instances was assessed by immunoblotting (Section 2.13).
Figure 5.1 Protocol for the specific labelling of disulfide-modified proteins in cell lysate.
First, cells were lysed in the presence of NEM to alkylate any reduced thiols present. Excess NEM was removed and samples further incubated with DTT to reduce any disulfides, then IAF was added to label those reduced thiols. Hence the fluorescent tag specifically labelled those proteins which had oxidised, disulfide (or glutathionylated) residues at the time of lysis. These proteins were subsequently separated electrophoretically and visualised using fluorescence detection.
Previously, $\text{H}_2\text{O}_2$-induced thiol oxidation has been investigated in Jurkat cells [260]. In this chapter, $\text{H}_2\text{O}_2$ was included as a relevant inflammatory oxidant (refer to Section 1.1.2.2), and also served to distinguish changes which were specific to chlorinated oxidants versus more general cellular responses to oxidative stress.

To identify proteins which underwent consistent changes in cells subjected to the various treatments, 3 replicate experiments were performed. Spots which underwent at least a 2-fold change in intensity were considered. For identification of these proteins, spots were excised from large 2D gels, and sent to the Centre for Protein Research, where they were analysed by LC-MS/MS (Methods are described in Section 2.20.2).

5.3 **Results**

To assess major oxidant-induced changes across the thiol proteome in HUVECs, cells were exposed to the oxidant (GlyCl, $\text{NH}_2\text{Cl}$, HOCl, $\text{H}_2\text{O}_2$, or HOSCN) in HBSS. Both dose-dependent and time-dependent oxidation of thiols was assessed.

5.3.1 **Time-dependent oxidation of HUVEC thiol proteins by chloramines and HOCl**

Each of the oxidants examined induced an increase in fluorescence intensity of a number of IAF bands in SDS-PAGE (Figure 5.2), indicating that these proteins contained oxidised thiols. Some of the bands appearing in GlyCl- or $\text{NH}_2\text{Cl}$-treated samples increased marginally in intensity with increasing treatment time. Rather than protein bands appearing to undergo oxidative modification later in the time course, susceptible proteins seemed instead to be oxidised at early time points (before 30 min), with some intensification of the signal over time (consistent with the oxidants slowly permeating the cells). Many changes were obvious after the earliest (10 min) time point. It is noteworthy that occasionally band(s) appeared which were inconsistent between experiments, and confined to only one gel lane of a single experimental replicate; these bands were considered artefacts of the experimental technique, but occasionally do appear in the figures shown in this chapter. Additionally, some lanes occasionally appeared overall more intense than others. Because total protein was normalised prior to the addition of DTT, the differences seen were either due to unequal loss of protein in subsequent spin column steps (removal of excess DTT, IAF), or inconsistent labelling efficiency across each of the samples. Coomassie staining of the gels suggested that most often the latter, rather than the former, was responsible for this observation.
Compared to chloramine treatment, HOCl-induced protein oxidation was apparent at earlier time points and increased treatment had virtually no detectable effects on protein intensity; changes in band intensity were obvious from the earliest time point (10 min). This is consistent with HOCl being rapidly consumed and quickly reacting in the cell, inducing broad oxidative changes quickly.

Two bands changed markedly compared to control in all of the treatments (GlyCl, NH₂Cl, and HOCl). Immunoblot analyses confirmed that these bands co-localised with GAPDH and the Prxs, which appeared at ~37 and ~22 kDa, respectively. It was not possible to determine which Prx band was labelled most strongly in this IAF assay because the enzymes did not separate sufficiently, but as described in Chapter 4, Prxs 1, 2 and 3 are readily oxidised to disulfides by exposure to these chlorinated compounds.
5.3.2 Disulfide modifications induced by hypothiocyanous acid

Hypothiocyanous acid (HOSCN) is a known thiol oxidant which like chloramines is likely to be generated during inflammation as a result of the neutrophil oxidative burst [261; 262]. HOSCN very slowly penetrates HUVECs compared with the other oxidants examined ([223] and Section 4.3.4), and long treatments (3 h) with high doses (300 µM) have been shown to induce significant loss of total reduced thiols in endothelial cells [223].

Treatment with 300 µM HOSCN for up to 1 h caused a strong intensification of the IAF signal from many protein bands, the band intensities increased in a time-dependent manner (Figure 5.3). Thus, compared to the thiol-targeting agent HOSCN, sublethal exogenous doses of chloramine or HOCl appeared to induce oxidation in a more select group of thiol proteins.

![Image of thiol oxidation in HOSCN-treated HUVECs](image)

**Figure 5.3 Thiol oxidation in HOSCN-treated HUVECs.**
Lysates from HUVECs treated with HOSCN (300 µM) were prepared after 10-60 min treatment and treated as in Figure 5.2. Control lane was treated with HBSS for 60 min. Gel is representative of 2 separate experiments.
Chlorinated oxidants appeared more likely to induce specific—rather than broad-ranging—oxidative modifications to thiol proteins. The effect of HOSCN was not investigated further in this thesis.

5.3.3 Effect of prolonged incubation in HBSS alone

In order to determine the specific effects of chlorinated oxidants, treatments were given in HBSS, and it was necessary to determine the oxidative implications of prolonged treatment in HBSS alone (i.e. in the absence of added oxidant). Indeed, a band which appeared in the region of the Prxs was one of the few that visibly intensified over time (Figure 5.4). Overall, however, the thiol proteome appeared to undergo few redox changes as a result of incubating HUVECs in HBSS for up to 1 h.

Figure 5.4 Effect of incubation in HBSS on HUVEC thiol oxidation.
HUVECs were incubated in HBSS alone for up to 1 h. A band which migrated at the same position as Prx is indicated. These results are representative of 2 separate experiments.
5.3.4 Effect of cell-impermeable taurine-chloramine on disulfide formation in HUVECs

A recent study by Klamt et al identified Cys-oxidation of cofilin as a major oxidative event in taurine-chloramine (TauCl) treated lymphoma cells [263]. However, previous studies in our lab have shown that TauCl – despite its capability to activate phosphorylation cascades via the EGF receptor – has low cell permeability and induces very little cytotoxicity [51]. The finding by Klamt and colleagues was therefore intriguing, but no changes in 1D SDS-PAGE after IAF labelling were seen inTauCl-treated HUVECs (Figure 5.5). The dose-dependent effect of 10 min treatments of TauCl (100-500 µM) induced no major changes in IAF labelling, and likewise treatment with 500 µM TauCl for up to 1 h did not appear to induce any changes above and beyond those seen with HBSS alone (Figure 5.4). Only treatment with TauCl in M199 (which would allow transchlorination to generate other cell-permeable oxidants [50]) induced disulfide modifications (not shown). This, in combination with differences in TauCl cell permeability with the different cell types, might explain the findings of Klamt et al. The specific effects of TauCl on HUVEC disulfide modification was not investigated further in this thesis.

5.3.5 Dose-dependent oxidation of HUVEC thiol proteins by chloramines, HOCl and H₂O₂

IAF labelling was performed immediately following 10 min treatments with GlyCl (up to 500 µM), NH₂Cl (up to 25 µM), and HOCl (up to 50 µM) in HBSS, or H₂O₂ (up to 200 µM) diluted in M199 (Figure 5.6). Wherever possible, treatments were administered in complete medium; however, chloramines and HOCl (but not H₂O₂) react with components in the media, and furthermore can result in the production of secondary amines. Therefore, these oxidant treatments were applied in the absence of culture medium (HBSS was used instead) Similar to the time course experiments, two major bands which appeared more strongly with the oxidant treatments ran in the expected positions corresponding to GAPDH (~37kDa) and Prx (~22 kDa). In addition, several bands appeared strongly between 10 and 40 kDa which clearly underwent a dose-dependent oxidation with several of the treatments. Over the concentration ranges examined, GlyCl appeared to induce the greatest number of disulfides, despite its slow uptake by cells (refer to Section 4.3.4) compared to NH₂Cl and HOCl (Figure 5.6). HOCl appeared to induce more broad-ranging thiol oxidation, and strong induction of thiol oxidation was seen with GlyCl, consistent with the time course studies.
It was more difficult to discern changes in the upper MW region of the gel, where bands were less well resolved. With the various oxidants employed, there was a tendency for overall intensification of the IAF signal in the upper MW region of the gels, but individual bands underwent changes less distinctly than those in the lower MW region of the gels. Several bands appeared to intensify in the 75-100 kDa region of the gel. A more intense signal in the upper MW region of the H$_2$O$_2$-treated samples was suspected to be due to a small amount of contamination from serum proteins (not visible by protein stain but highlighted and intensified by IAF binding to these proteins), as these samples were extracted from cells treated in complete medium.

![Image](image.png)

**Figure 5.5** HUVECs treated with TauCl were monitored for disulfide-modified proteins. Dose- and time-dependent effect of TauCl treatment on HUVECs was monitored by IAF labelling of oxidised thiols. Lanes shown are from a single gel from one experiment; gel has been cropped for visualisation. Lanes 6 & 7 are the same lane (10 min with 500 μM TauCl), represented in both the dose curve and time courses. Results are representative of 2 separate experiments.
Figure 5.6 Thiol proteins in HUVECs which underwent disulfide modification during 10 min exogenous oxidant treatment. HUVECs grown in 6-well plates were treated with the given concentrations of oxidant (μM concentrations are shown) and the oxidised thiols specifically labelled with IAF (as shown in Figure 5.1). Cells were harvested after 10 min treatment in HBSS (or M199 where H₂O₂ treatment was used). Several bands appear randomly in some lanes (e.g. lanes 3 and 8 from left-hand gel); these are suspected to be due to small amounts of contamination from oxidised serum proteins, and these appeared more regularly in H₂O₂-treated samples (for which cells were treated in full M199 medium). Results are representative of 2-3 separate experiments.
5.3.6 Visualisation of oxidised proteins by two-dimensional IAF labelling

Although many bands were visualised by 1D SDS-PAGE, these were often not distinct, and were furthermore localised to regions of the gel where many proteins migrate; this would make these bands near-impossible to excise for positive identification. Therefore, to further investigate the specific changes occurring in HUVECs treated with chlorinated oxidants or H$_2$O$_2$, cells grown in 10-cm dishes were prepared for the 2-D IAF method (Methods Section 2.23).

Oxidant concentrations were selected based on results reported in Chapter 4 and earlier in this chapter. In order to identify physiologically-relevant redox changes (i.e. proteins which are involved in a functional cell response to the oxidants), concentrations of oxidant were selected based on the following criteria (for chloramine and HOCl treatments):

1) No significant loss of reduced GSH;
2) At least 25% decrease in reduced Prxs 1 and 2 (relative to untreated cells);
3) At least 2-fold increase in cell death at 24 h (as measured by PI in Chapter 4);
4) No severe morphological change (or endothelial disruption) after 10 min treatment; and,
5) Moderate increase in IAF labelling after 10 min treatment by 1-D SDS-PAGE method.

For H$_2$O$_2$-induced redox changes, a concentration of 200 µM H$_2$O$_2$ was selected for ease of comparison with results reported by Baty et al [260].

Because the most interesting protein oxidation changes appeared within 10 min, all oxidant treatments were removed after 10 min. By restricting the treatment time, fewer changes occurred due to the incubation in HBSS, and these experiments virtually eliminated the possibility that changes in spot intensity could be due to variations in protein expression levels. In addition, changes occurring after short treatment times would more likely lead to the identification of proteins which could be involved in cell signalling and/or rapid antioxidant protection (by rapid reaction with the oxidant) in HUVECs, rather than being involved in more downstream effects or cell death pathways.
Three replicate experiments were performed in which cells were treated with the doses shown in Figure 5.7; cell lysates were labelled and prepared as described (Figure 5.1). 2D gels were imaged and one replicate experiment was transferred to PVDF for immunoblotting (blots were allowed to air-dry and were stored for later use). Images representative of the three separate experiments are shown in Figure 5.7.

Visual comparison of the IAF gel images showed that the proteins migrated with a high degree of fidelity. Analysis of the gels showed many differences between treatments. There was intensification of spots and the appearance of novel spots in the treatment versus control gels, and several treatments induced changes in the same regions of the gels, especially when comparing the chloramine and HOCl treatments. GlyCl appeared to induce the most pronounced differences compared to control gels. Overall, H₂O₂ induced the fewest changes, particularly upon inspection of the lower MW region of the gel.
Chapter 5: Novel thiol targets of chlorinated oxidants

A  Control (HBSS)

B  250 µM GlyCl

C  15 µM NH$_2$Cl

D  60 µM HOCl
E 200 µM H₂O₂

Figure 5.7 Two-dimensional electrophoresis with IAF labelling of oxidised thiols.
HUVECs grown in 10-cm plates were treated with the given concentration of oxidant; cells were harvested after a 10 min treatment in HBSS, and the oxidised thiols specifically labelled with IAF (as shown in Figure 5.1). Proteins were focused to IPG strips (pH 5 – 8) and subsequently separated in the second dimension on Criterion gels. IAF-labelled proteins were visualised by fluorescence detection. Gels are representative of three separate experiments.
5.3.7 Identification of proteins undergoing disulfide modification

Replicate gels were aligned and spot matching performed using PDQuest™ analysis software with visual confirmation and manual spot matching where necessary. For each treatment, 400-500 spots were identified as distinct protein spots. The software was used to identify changes in spot intensity which were significantly different from the control gels, either increasing or decreasing in intensity by at least 2-fold. The protein changes numbered up to 50 for each treatment. They were visually validated for consistency of spot appearance and change between replicates, and many were omitted from further analysis based on one or more of following exclusion criteria:

1) Spot was very faint;
2) Spot was part of a large IAF signal which appeared to be a cluster of spots;
3) Spot was in a particularly protein-dense region of the gel;
4) Spot was inconsistent between gels; or,
5) Spot was in an area of the gel which had high levels of streaking.

Of the spots which changed significantly with one or more of the treatments, a selection of 18 spots was compiled for further analysis (Appendix A). To maximise protein loading for spot excision and subsequent protein identification, equal amounts of protein from all three replicate experiments with control and GlyCl samples were separated by 2-dimensional electrophoresis using 17cm IPG strips and large gels (Section 2.24).

Gels were imaged and visual mapping used to pinpoint the 18 spots of interest (Figure 5.9) on the large gels; although the proteins separated slightly differently on the large-scale gels compared to the smaller Criterion gels, the spot pattern was consistent and the locations of the spots of interest were confidently established; these were excised and sent to the Centre for Protein Research for analysis and identification (Section 2.25).
Figure 5.8 Spots excised from large 2-dimensional SDS-PAGE gels.
Proteins labelled with IAF were visualised by fluorescence as shown. Spots were excised from GlyCl-
treated sample (A) and Control (HBSS) sample (B) and correct excision verified by fluorescence.
Spot numbers shown correspond to spots and proteins from Appendix A & Table 5.1.
Mascot search results were reviewed; all spots had some keratin contamination and keratin matches were not considered positive hits. The protein pI's were expected to differ somewhat due to oxidation, phosphorylation, or other protein modifications which are known to modulate the pI of a given protein. On the other hand, proteins with vastly different MWs than estimated from the gel spot were generally dismissed. The remaining protein matches are listed in Table 5.1. Where more than 2 protein matches were returned, those with scores <30 were excluded from the summary table.

The three faintest spots (spots 12, 15 and 16) were the only ones which had no positive protein matches. All other 15 spots yielded at least one positive protein ID. However, the protein ID from spot 13 was protein S100-A6, the amino acid sequence of which contains no cysteine residues. Thus, although Protein S100-A6 may co-localise with spot 13, the IAF signal undoubtedly arose from labelling of an unidentified protein, and protein S100-A6 is not considered to have undergone disulfide modification as a result of treatment.

5.3.7.1 GAPDH

As shown in Figure 5.9, GAPDH appears as a string of spots which intensified with each of the treatments. This protein also appeared as a major band by the 1D method (Figure 5.2), and confirmed by Western blotting of 1D IAF blots. In addition, an early experiment in which spots from this region (a string at ~ 37 kDa) were excised and identified confirmed that GAPDH was the major protein identified (data not shown). It has been previously demonstrated that GAPDH is susceptible to oxidative modification by H₂O₂ treatment of Jurkat cells [260], and that GAPDH is very sensitive to inactivation by chloramines and HOCl [37; 50]. Therefore, the observation that GAPDH was sensitive to thiol oxidation in this chapter was consistent with previous reports.

5.3.7.2 Peroxiredoxins (spots 3, 4, 5 and 17)

Several spots did contain peptide sequences which matched those of Prxs. Spot #3 was identified as Prx2, and immunoblotting of a GlyCl-treated sample confirmed that one of the Prx2 spots did co-localise with the region from which Spot #3 was obtained (Figure 5.10). In agreement with this, spot intensity analysis showed that IAF labelling increased in this region with chloramine or HOCl treatment (consistent with the formation of a disulfide upon oxidation) but decreased with H₂O₂ treatment (consistent with hyperoxidation).
Spots 4 and 17 were selected from the GlyCl-treated and Control gels, respectively, and an attempt was made to map these spots, obtaining the ‘same’ spot from each gel. Indeed, identification yielded several strong protein matches, of which Prx3 and GPx1 were the hits with the 2nd and 3rd highest rankings in both analyses. Immunoblotting of membranes confirmed that Prx3 protein was localised to this region of the gel (Figure 5.10). Although these findings give confidence in the analysis, it does not necessarily hold true that all of the proteins identified were oxidised and IAF-labelled. Only low fidelity matches were obtained from spot 5, with 2 peptides identified from Prefoldin subunit 3 and one from Prx1. Nevertheless, Western blotting again confirmed that Prx1 does migrate to that region of the gel, so despite the weakness of the match Prx1 could indeed be the primary protein of high IAF intensity at this spot (Figure 5.10). It is worth noting that most of the Prx1 protein does not focus on these strips (pI > 8.0), so the spots represented in the western blots shown in Figure 5.10 represent a small subset of the total Prx1, likely modified (e.g. by phosphorylation) resulting in a lower pI.
### Table 5.1 Proteins identified from Mascot search of amino acid sequences detected by MS/MS.

Spot numbers correspond to those excised from gels as shown in Figure 5.7 and Table 5.1.

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<tr>
<th>Spot</th>
<th>Trend, relative to Control</th>
<th>Protein Match</th>
<th>Strength of Match</th>
<th>M&lt;sub&gt;i&lt;/sub&gt; (kDa)</th>
<th>pI</th>
<th># Cys</th>
<th>Thiol modifications</th>
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<td>Annexin A1</td>
<td>1322</td>
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<td>5.67</td>
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<td>( pI )</td>
<td># Cys</td>
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<td>Protein Match</td>
<td>Strength of Match</td>
<td>M, (kDa)</td>
<td>pI</td>
<td># Cys</td>
<td>Thiol modifications</td>
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<td>2</td>
<td>18.81</td>
<td>10.6</td>
<td>4.94</td>
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* Observed from 2D gel; calculated from standards  
# Basal isoelectric point (www.phosphosite.org) for protein identified  
SP Other authors’ experimental results, as reported in SWISS-2DPAGE database (http://expasy.org/ch2d/)  
queries matched = number of tryptic peptides identified which may be obtained from the identified protein  
CAM = carbamidomethylation of Cys residue detected  
Matches in **bold** are further discussed in the text (5.3.7 & 5.4)
5.3.7.3 Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A; spots 7, 8, 9, 10 & 11)

One of the more remarkable changes observed by the 2-dimensional analysis was a major series of spots at ~20 kDa which consistently increased with all treatments (though particularly in GlyCl-treated cells, and less obviously in gels from H₂O₂-treated cells). The increase in these spots ranged from an average of up to 10-fold increase for GlyCl and NH₄Cl-treated cells, up to 6-fold in HOCl samples and 3-fold in H₂O₂-treated samples. Several spots from this ‘string’ were selected for identification, including spots 7 through 11 (Figure 5.11). All of these spots were identified as consisting predominantly of peptidyl-prolyl cis-trans isomerase A, also known as cyclophilin A (CypA). This identification was confirmed by immunoblotting the 2-D membranes with antibodies against CypA (Figure 5.12A & B).

![Figure 5.11 Spots excised from GlyCl-treated gel and identified as CypA by LC-MS/MS.](image)

Western blotting of 1-dimensional IAF gels confirmed that CypA co-localised with a major IAF-labelled band appearing at ~18 kDa, and a band at this in position increased in fluorescence intensity (Figure 5.12C) with all of the treatments investigated, including GlyCl, NH₄Cl, HOCl, H₂O₂, and HOSCN. The protein expression did not appear to change significantly, as the signal detected by Western blotting didn’t vary, but IAF intensity at that location increased, consistent with an increase in thiol oxidation of CypA. The IAF band did not appear in cells incubated over time in HBSS alone.
CypA is a highly abundant protein with known activities as both a cis-trans isomerase and chaperone, with involvement in protein folding (reviewed in [264]). In addition, CypA has been implicated in stress tolerance [265] and can undergo S-glutathionylation of cysteines 52 and 62 [266], which could be the modification(s) responsible for the changes observed herein.

5.4 Discussion

Obvious changes in protein thiol oxidation were apparent in SDS-PAGE with all treatments, and these were further characterised by the 2-D proteomic approach. The methodologies used in this chapter were sensitive enough to identify a number of redox changes to thiol proteins from cells treated with chloramines, HOCl or H₂O₂. While many changes were detected, major changes were detectable primarily in the region of the gel containing proteins of MW below 50 kDa. There were many similar changes between GlyCl, NH₂Cl, and HOCl treatments in terms of the general trend (increase or decrease in intensity, and appearance of novel IAF-labelled spots.

Figure 5.12 Immunoblots for CypA with 2-dimensional and 1-dimensional IAF.
Immunoblotting for CypA confirmed co-localisation with spots identified as CypA by LC-MS/MS (A & B; box surrounds the same region of blot and gel). C, In addition, a major IAF-labelled protein band which increased with treatment was found to co-localise with CypA in 1-dimensional SDS-PAGE analysis. Shown here are an IAF gel (GlyCl and NH₂Cl dose curves from Figure 5.6), the CypA immunoblot from the same gel and an overlay of these two.
compared to control); differences may relate to the variable permeabilities and treatment doses of these oxidants. HOCl and chloramines appeared to induce more extensive thiol oxidation than H₂O₂ though undoubtedly more substantial changes would likely be observed with higher H₂O₂ doses (as illustrated by the 1-D dose-response gels Figure 5.2).

A number of the protein spots which were identified as undergoing significant thiol oxidation following treatment with exogenous inflammatory oxidants are interesting in the context of oxidative stress and cell function. The various proteins and their known and potential oxidative modifications are discussed below.

5.4.1 Peroxiredoxins spots (3, 4, 5 and 17)

The results presented in this chapter affirm the earlier findings of Prx susceptibility to chlorinated oxidants and point to the Prxs as being among the major thiol targets of these oxidants as well as H₂O₂. The identification of Prxs by mass spectrometry was bolstered by Western blotting data showing that Prxs do indeed co-localise to the areas of the gels from which the spots were excised.

When exposed to H₂O₂, Prxs can become hyperoxidised, and consistent with this (and with observations by Baty et al [260] in Jurkat cells), spot #3 decreased in intensity following treatment with 200 µM H₂O₂. However, this does not occur with the chlorinated oxidants, which cause dimerisation of Prxs via disulfide formation, as described in Chapter 4, and hence the IAF signal intensified following treatment with these oxidants.

In Chapter 4, it was demonstrated that Prx3 is less sensitive than Prxs 1 or 2 to hyperoxidation in HUVECs, and spots 4 & 17 (from the same region of control and GlyCl-treated gels) here were both shown to include Prx3 protein. The increase in spot intensity with H₂O₂ treatment was consistent with the results in Chapter 4, in which the amount of dimeric Prx3 with 200 µM H₂O₂ was substantial with little accumulation of hyperoxidised protein.

Prx1 has a pI of approximately 8.3 and would not be expected to focus on the IPG strip of pH 5-8. Nevertheless, the pI may vary as a result of post-translational modifications such as phosphorylation. Two spots (#4 & 5) were identified to contain Prx1 peptides, and spot 5 did
appear to colocalise with a minor spot which was also recognised by the Prx1 antibody. This could be due to post-translational modification, and one possibility is phosphorylation, as it has been demonstrated that the 2-Cys Prxs are regulated by tyrosine and threonine phosphorylation [267; 268]. Further investigation would be required to determine whether this was indeed the case.

5.4.2 Cyclophilin A

The increases in spot intensity for spots #7 through 11 (corresponding to cyclophilin A) were among the most remarkable changes observed by the IAF method. The high abundance of this protein was likely partly responsible for the intensity at this location on the gels, but this protein nevertheless appeared to become oxidised with all of the oxidants investigated.

CypA has been implicated in a number of cellular processes: cell division, transcriptional regulation, protein trafficking, signalling, ROS scavenging, Na\(^+\)-Ca\(^{2+}\) exchange, pre-mRNA splicing, molecular chaperone functions and stress tolerance (reviewed in [269]). It has even been suggested to reduce 2-Cys Prxs in plants [270], by a similar mechanism to that used by the thioredoxin system. Other authors have also suggested a link between CypA and various Prxs [271; 272].

Further study is required to determine how CypA is oxidatively modified by inflammatory oxidants (e.g. via glutathionylation as has previously been suggested [266]). It would also be of interest to determine whether or not there is any interaction between CypA and the Prxs, since these were among the most notable proteins which underwent changes in this chapter.

5.4.3 Nucleoside diphosphate kinase B (spots 9, 11)

Nucleoside diphosphate kinase B (also known as C-myc purine-binding transcription factor PUF) was a second protein identified in both spots 9 and 11. Nucleoside diphosphate kinases are responsible for maintaining cellular levels of nucleoside triphosphates (reviewed in [273]). These enzymes also have roles in apoptosis, signal transduction and transcription, with strong links to metastasis in cancer [274; 275]. In spot 11, the peptide containing Cys109 was found to be bound to NEM, suggesting that this residue was at least partially in the reduced state following treatment with GlyCl. The same six peptides matched both isoforms 1 and 3 of this transcription
factor, but the MW of isoform 1 more closely matches that estimated from the gel. This isoform has 2 Cys residues, but only the peptide containing Cys109 was detected.

Studies have suggested the function of this enzyme is under redox control. Song et al [276] demonstrated that intermolecular disulfide formation as a result of $\text{H}_2\text{O}_2$-induced oxidation caused inactivation of the enzyme’s function. Lee et al [277] confirmed that Cys109 could be oxidised in various ways in response to oxidative stress induced by peroxide: inter- and intra-molecular disulfides, glutathionylation and sulfonic acid formation on this residue were detected by Mass Spectrometry. Interestingly, the authors also found that this enzyme was able to bind Trx1, an important protein in the Prx catalytic cycle. These findings suggest that this protein’s redox status under conditions of oxidative stress should be investigated further.

5.4.4 Cofilin 1
Cofilin-1 was the major protein identified in spot 6, but was also identified in spots 7 and 8 alongside CypA. Although the expected pI for this protein was higher than observed, this protein has previously been found to migrate at a pI similar to what was observed here [278]. Cofilin-1 was one of very few from which a peptide was identified which was bound to IAF, and the Cys modified was Cys147.

Cofilin-1 translocates to the mitochondria, where it has been shown to mediate apoptotic events [279; 280]. Interestingly, cofilin-1 was a major target of oxidation in taurine-chloramine (TauCl)-treated cells [41]; the authors demonstrated that oxidation of all 4 cysteine residues was required for cofilin mitochondrial translocation and subsequent induction of apoptosis.

5.4.5 Parkinson disease protein 7 (Spot 4)
Parkinson disease protein 7 (also known as oncogene DJ-1) was the major protein identified in spot 4. Although several proteins were identified from this spot including Prx3 and GPx1, DJ-1 should not be ignored. DJ-1 has been shown to respond to oxidative stress, and to be oxidised under pathophysiological conditions including Parkinson disease [281] and Alzheimer disease [282]. In the present study, Cys46 was found to be NEM-alkylated in the peptide containing that residue. The other two cysteine residues (Cys53 & Cys106) are accessible for oxidation and are important for the protein’s functions; Cys53 oxidation to sulfonic acid inhibits DJ-1 chaperone
activity [283], while Cys106 can become oxidised to a sulfenic acid residue which appears important for translocation of this protein into the mitochondria [284-286]. The oxidation of Cys106 has been observed as an acidic shift by 2-dimensional electrophoresis, into the pI range observed from which spot 4 was excised [285].

It is worth cautioning that this particular spot was obtained from a protein-rich region of the gel, and the fact that several strong matches were obtained from spot 17 (the same region on the Control gel) suggest that this is the case. A previous study by Kinumi et al [284] showed co-localisation of DJ-1 and Prx3 [284], which does corroborate the findings reported here. They reported that this co-localisation was particularly pronounced when both proteins had been oxidised prior to reduction and separation by 2-D electrophoresis. A similar phenomenon could be responsible for the co-localisation of these two proteins in the spot from the GlyCl gel but not the control gel.

5.4.6 Glutathione-S-Transferase P (Spot 17)
Glutathione-S-Transferase P (πGST) was the strongest match for spot 17. Like DJ-1, πGST has been identified from a seemingly protein-rich region of the gel, as spot 17 returned several proteins. Several of these (including Prx1, Prx4 and GPx1) have oxidisable Cys residues, so any one or combination of these proteins could potentially be contributing to the IAF signal at this location. Nevertheless, πGST is of interest.

As a member of the GST superfamily, πGST catalyses the attack of GSH thiol on electrophiles, thus having a role in scavenging cellular products of oxidative stress as well as detoxifying against xenobiotics [287; 288]. Recently, however, πGST has been explored by the lab of Fisher and colleagues [289-291] in its capacity to reduce 1-Cys Prx (Prx6 in humans); these studies have suggested that πGST participates directly in the recycling of Prx6, a cytosolic antioxidant enzyme which is suspected to play an important role in endothelial wound healing [292]. This antioxidant system (Prx6-πGST-GSH) could therefore be relevant in the context of endothelial cells exposed to inflammatory oxidants.
5.4.7 Glutathione peroxidase 1 (spots 4 & 17)
Like Prxs, the glutathione peroxidases (GPxs) are considered major antioxidant enzymes in cells, competing with Prxs as well as catalases to catalyse the reduction of hydroperoxides, in a glutathione-dependent manner as shown below:

\[
ROOH + 2 \text{GSH} \rightarrow \text{GSSG} + \text{ROH} + 2 \text{H}_2\text{O}
\]

GPx1 is a seleno enzyme which also contains 5 Cys residues. Its catalytic mechanism, however, involves selective oxidation of the active site selenol with subsequent reduction by GSH [293; 294]. However, the high reaction rate of this enzyme with GSH [295], (as well as the bioavailability of cellular GSH under conditions used in these treatments) mean that this enzyme would be unlikely to accumulate in a state where the active site is glutathionylated, (reviewed in [295]), despite the fact that this intermediate has been detected by Mass Spectrometry [296]. On the other hand, it is possible that one of the other 4 cysteines could be oxidised.

Nevertheless, GPxs and Prxs are both peroxidase enzymes that react with \(H_2O_2\) with similar rate constants [9]. It would therefore be interesting to investigate the possibility that chloramines, which appear capable of oxidising the active-site cysteine of Prxs, might also preferentially react with the selenol of GPx1.

5.4.8 Annexin A1/A2 (Spots 1 & 2)
Annexin A1 was the protein identified from both spots 1 and 2, which decreased significantly with all treatments applied. Annexin A2 was also identified in spot 1. Annexin A1 interacts with membranes and phospholipids in a calcium-dependent manner. Annexin A1, along with other members of the Annexin family, have been extensively investigated and implicated in diseases ranging from cancer to atherosclerosis, and in cell processes from cell growth and differentiation to apoptosis; studies investigating the role of Annexins have been extensively reviewed [297; 298].

Interestingly, Wong et al [299] observed that 10 min treatments with Endothelin-1 induced loss of Annexin A1 protein in pulmonary artery smooth muscle cells, as identified by 2D-PAGE and mass spectrometry; this observation is similar to what is reported here for chloramine/HOCl/
H$_2$O$_2$ treatments. Wong and colleagues determined that the decrease in Annexin A1 was due to carbonylation and subsequent degradation of Annexin A1 in cells treated with Endothelin-1. In Chapter 4, NH$_4$Cl was shown to increase protein carbonyl levels in HUVECs, and it would be interesting to investigate whether Annexin A1 was a target of carbonylation.

Various studies have also investigated the relevance of the various Annexin cysteine residues, with some indication that these may be important in activity and/or regulation of the Annexins. Specifically, Cys8 of Annexin A2 has been shown to undergo S-glutathionylation in TNF-α-stimulated HeLa cells [300], possibly via H$_2$O$_2$ generated. This cysteine residue has been shown to be important in the binding of Annexin A2 to tissue plasminogen activator (tPa)[301]; additionally, derivatisation or alkylation of the same residue appears to inhibit the activity of the Annexin A2-S100A10 complex [302].

The identification of Annexin A1 and/or A2, as well as these proteins’ susceptibility to be modified by cysteine oxidation and/or carbonylation, make this protein a likely target for modification by chlorinated oxidants. It has been suggested that Annexins may be key players in a number of cell signalling pathways and cell responses to stimuli [299], which could be very interesting in the context of inflammation.

### 5.4.9 Small ubiquitin-related modifier 3 (or 4) (SUMO3/SUMO4) (Spot 18)

Spot 14 was a faint spot that nevertheless was found to decrease significantly in intensity following GlyCl or H$_2$O$_2$ treatment. Although little is known about cysteine modifications to the identified protein, small ubiquitin-related modifier 3 (SUMO3), its function makes it an interesting candidate in terms of protecting cells under conditions of oxidative stress.

Like ubiquitination, sumoylation (binding of SUMO to target proteins), is a process which has been shown to regulate various cell processes, including signal transduction pathways [303]. Loss of SUMO3 could correspond to increased sumoylation of proteins, as previously observed in cells exposed to oxidative, electrophilic, heat or metabolic stresses [304-306].
5.4.10 Beta-2-microglobulin precursor (Spot 14)

Beta-2-microglobulin was the strongest match for spot 4, but with only 3 peptides identified the sequence coverage of this protein amounted to only ~17%. Nevertheless, this component of the class 1 major histocompatibility complex (MHC) is known to undergo disulfide formation as a post-translational modification. Most studies, however, have focused on this protein's role in immune response (reviewed in [307]) and as a marker of acute kidney injury (reviewed in [308]), with a number of studies also implicating beta-2-microglobulin in various cancers [309-312].

If beta-2-microglobulin is indeed modified under conditions where cells are exposed to inflammatory oxidants, this might be a novel role for this immune-linked protein.

5.4.11 Interpretation of these results

5.4.11.1 Limitations

Identification of a spot with increased IAF intensity may indicate a protein which has undergone disulfide modification and/or glutathionylation, while likewise a decrease can indicate the loss of such a modification, for example through further oxidation (to a sulfinic or sulfonic acid residue). Alternatively – though not particularly relevant in this chapter – a change in intensity could indicate a change in protein abundance. While the methods used herein minimised any effect of protein expression by limiting most treatment times to 10 min, there remain instances where an increase in intensity could be due to protein disassociation, for instance.

There remains the confounding factor of shifts in pI or MW. Some proteins may, whether or not in conjunction with changes in disulfide modification, undergo changes in pI, for example through phosphorylation or dephosphorylation. This shift in pI, causing the protein to migrate to a pH on the IPG strip during focussing, can easily be sufficient for the IAF spots to no longer ‘match up’ between treatment gels. Covalent bonding – or alternatively, the disruption of such bonds – could likewise cause shifts in MW between samples. In such an instance, spots could appear to change in intensity (or appear/disappear altogether) without any involvement of thiol modification.

Overlapping spots, or proteins that migrate to the same region on the gel present another challenge, which can lead to false identifications of proteins which actually undergo changes. In
the data presented in this chapter, this phenomenon likely occurred with some of the spots which were identified as proteins containing no Cys residues.

Thus, the relevance of proteins identified by the spot excision method does need to be interpreted with caution. Regardless of the reason for the change, this method does provide a means by which to identify proteins which may undergo a change in response to treatment.

Confirmation of the proposed redox modifications reported here could include employing an alternative approach to specifically look for Cys modifications on the proteins of interest. For example, proteins could be labelled with isotope-coded affinity tags (ICAT) followed by mass spectrometry [313] to investigate different redox modifications.

Some of the limitations of this method include difficulty in detecting changes in crowded regions of the gel, in addition to favoured detection of high copy-number proteins. Improvements to the method used in this chapter would include efforts to obtain better resolution of particular areas (MWs & pIs) of interest by selecting IPG strips and gel gradients that maximise the resolution of a smaller subset of the proteome. This could act to better focus on major areas of signal change and/or separate out areas of high protein abundance.

5.4.11.2 Advantages

The sensitivity of this method is bolstered by the fact that very small amounts of protein can be easily detected by fluorescence. While this is not always sufficient for positive protein identification, subtle changes can be detected in not only the most highly abundant proteins [314]. In fact, far too little protein is used in these assays to detect the protein spots by gel staining (Coomassie or silver stains) apart from only the most abundant proteins (e.g. actin and GAPDH).

The methodologies used in this Chapter were optimised for detection of proteins below ~50 kDa; band and spot resolution from 1D and 2D experiments were good below this portion of the gel, allowing the detection of changes in IAF intensity that were both dose-and time-dependent (by 1D), and treatment-dependent (by 2D).
5.4.12 Chlorinated oxidants as thiol-targeting agents

When proteins were separated by 1D SDS-PAGE, IAF labelling of oxidised thiols demonstrated both a time- and dose-dependent increase in labelling of specific thiol proteins. From these experiments, it appeared that thiol oxidation was much more specific than the broad-spectrum oxidation seen with HOSCN (Figure 5.3). Indeed, it has been reported that HOSCN causes a significant loss of reduced thiols as determined by MBB derivatisation [223], and these results are consistent with the rate constants for the reactions of thiol proteins with HOSCN, as determined by Skaff et al [262] and Nagy et al [315]. By comparison, NH$_2$Cl does not induce overall thiol oxidation to the same extent (Section 4.3.1).

Most of the spots which were selected for analysis were spots which underwent changes with each of the oxidants (chloramines, HOCl and H$_2$O$_2$). There were a few exceptions to this: spot 3 (discussed in 5.4.1), 12 and 18, in particular. Unfortunately spot 12 yielded no likely protein identifications, but the protein at spot 18 was identified as SUMO3, and its decrease in intensity was detected only with GlyCl and H$_2$O$_2$ treatments.

5.4.13 Summary and future directions

A number of proteins identified by LC-MS/MS in this study have previously been shown to have involvement in a variety of cellular processes which could be relevant in conditions where cells are exposed to inflammatory oxidants. Specifically, several proteins are known to be involved in regulation of apoptosis (Cofilin-1, Annexin A1, πGST, GPx1, SUMO) and/or signal transduction pathways (Cofilin-1, DJ-1, SUMO3). In addition, the Prxs and GPx1 are known peroxidases that have important antioxidant roles in cells.

Of the proteins identified, some are especially interesting and worthy of further investigation. DJ-1 and Cofilin-1 have a high likelihood of being involved in cell responses to inflammatory oxidants; both have previously been shown to undergo cysteine modification, and both appear to undergo mitochondrial translocation following oxidation. In addition changes in cofilin-1 activity have been demonstrated in TauCl-treated Burkitt’s lymphoma cells [263] (though this is unlikely in HUVECs treated with TauCl, since few changes were evident in Figure 5.5).
For similar reasons, Annexins A1 and A2 should be further investigated, especially in the context of GlyCl and H$_2$O$_2$, the two treatments which induced a reduction in IAF signal in the region where this protein was identified. As discussed in Section 5.4.8, a number of modifications of these proteins have been reported and these proteins could have interesting roles in cell signalling pathways.

Sumoylation of proteins is not a modification that was originally considered in the context of the inflammatory oxidants investigated in this chapter. However, the results suggest that the SUMO3 protein itself may undergo redox changes, and it would therefore be interesting to investigate the possibility of altered protein sumoylation under these conditions.

CypA was arguably the most prominent change observed in these experiments, and it is likely that this protein may undergo glutathionylation of one or more cysteine residue(s), a redox modification of this protein which has previously been shown [266]. Of note, a number of studies in various species have suggested that CypA may interact with – and even reduce – Prxs (or their analogs) [270-272].

Prxs were in fact one of the other most notable changes; a band corresponding to the MW of Prxs was a prominent change even in 1-dimensional analysis. The activity, redox status and reversibility of Prxs in HUVECs treated with inflammatory oxidants are explored in Chapter 4. Other antioxidant proteins worthy of further analysis in the context of inflammatory oxidants include GPx1 and πGST, which contain oxidisable residues (selenocysteine and cysteine, respectively) in their active sites.

Interestingly, a number of the spots identified in this study were also identified by Klamt et al, in a similar study which investigated oxidative changes in response to TauCl treatment in lymphoma cells [263]. The proteins which were found to undergo oxidative changes in both studies included Cofilin-1, DJ-1, GST, Prxs 1 and 2, GAPDH. It therefore appears that a number of major protein changes are commonly initiated by treatment with these chlorinated compounds suggests that similar pathways are likely initiated by several different chlorinated inflammatory oxidants, and that these could be key in mediating cellular responses to inflammation.
Chapter 6. Induction of HO-1 expression by monochloramine

6.1 Introduction

Haem oxygenase 1 (HO-1) is responsible for catalysing the degradation of free haem, generating carbon monoxide, biliverdin and free iron (equation 1, [316]). The effective removal of the pro-oxidant haem from cells is an important antioxidant activity of HO-1 and, in addition, its products biliverdin/bilirubin and CO also have antioxidant, anti-inflammatory and anti-apoptotic activity [62; 185; 317; 318]. Thus, increased expression of the HO-1 enzyme in cells appears to have important cytoprotective and anti-apoptotic effects.

\[
\text{haem} + \text{NADPH} + \text{H}^+ + 3\text{O}_2 \rightarrow \text{biliverdin} + \text{Fe}^{2+} + \text{CO} + \text{NADP}^+ + \text{H}_2\text{O} \quad (1)
\]

HO-1 expression appears to be regulated in a variety of ways; its expression may be activated by several redox-sensitive transcription factors (Nrf2; activator protein 1, AP-1; cAMP response element binding protein, CREB; and NFκB) [194; 319]. Of these transcription factors, Nrf2 is arguably the most well-studied in terms of its induction of HO-1 expression. Normally bound to its repressor Keap1 in the cytosol, Nrf2 is targeted for ubiquitination and degradation under normal physiological conditions. Upon stimulation of cells with a variety of oxidative or electrophilic agents, Nrf2 is released, and subsequently undergoes nuclear translocation. Activated Nrf2 interacts with antioxidant response elements (ARE), cis-acting elements in the promoter regions of various antioxidant genes, thereby inducing their expression [320]. HO-1 is among these genes, as are thioredoxin 1, peroxiredoxin 1, NAD(P)H:Quinone oxidoreductase, GSH synthesis enzymes, and others.

Activation of Nrf2 and increased HO-1 expression have emerged as events which are of great interest in the context of inflammatory disease, and appear to confer protection against tissue injury during inflammation [212; 321; 322]. Atherosclerotic lesions have been found to have high levels of HO-1 expression [323], and Nrf2-dependent induction of HO-1 expression has been demonstrated in cells exposed to inflammatory oxidants including HOCl and taurine chloramine.
monochloramine \((\text{TauCl})\) [55; 72; 220; 324]. A growing body of evidence suggests that HO-1 has an important role in protecting against atherosclerosis [204-206; 323; 325].

This chapter aimed to investigate the direct effect of monochloramine \((\text{NH}_2\text{Cl})\) on HO-1 expression, and to explore the hypothesis that Nrf2 translocation and HO-1 expression could be induced in cultured cells treated exogenously with this inflammatory oxidant.

### 6.2 Experimental Approach

In the work described in this chapter, the effect of \(\text{NH}_2\text{Cl}\) on Nrf2 translocation and HO-1 expression was investigated in both human umbilical vein endothelial cells (HUVECs) and Hepa 1c1c7 cells (a murine hepatocyte cell line). The techniques employed included immunofluorescence and immunoblotting assays.

Nrf2 localisation in cells was investigated by Western blotting and immunofluorescence using antibodies against Nrf2. Hemin and/or sulforaphane were used as positive controls, and Hoechst counterstaining was used to visualise nuclei.

Because both HUVECs and Hepa 1c1c7 cells are adherent cells, an in-plate nuclear extraction procedure was possible (Figure 6.1); this procedure was used to enrich the nuclear fraction for enhanced detection of Nrf2 and HO-1, and to thereby verify the intracellular localisation of these proteins.

Hepa 1c1c7 cells were less sensitive to the treatments employed than HUVECs, and this was probably related to the fact that confluent dishes of Hepa cells contained 2-3 times as many cells (and a greater amount of protein) as an equivalent dish with HUVECs (refer to Table 2.3). Therefore, higher concentrations of oxidant were employed when treating Hepa 1c1c7 cells compared to HUVECs.
Chapter 6: Monochloramine-induced HO-1 expression

Figure 6.1 In-plate nuclear extraction procedure employed for HUVEC and Hepa 1c1c7 cytosolic and nuclear isolation.

1. Cell treatment is removed; cytosolic extract buffer (low salt, 0.5% NP-40) is added; cells swell and lyse, releasing cytosolic proteins.

2. Cytosolic extract is collected, leaving nuclei which remain adhered to the culture dish.

3. High-salt nuclear extraction buffer is added; nuclei are scraped using a rubber policeman; high-speed centrifugation is used to pellet insoluble material.
Chapter 6: Monochloramine-induced HO-1 expression

6.3 Results

6.3.1 Nrf2 & HO-1 in HUVECs

6.3.1.1 HO-1 expression and nuclear levels of Nrf2 in HUVECs grown in 12% FBS

In untreated cells grown under normal culture conditions (with 12% FBS), western blotting of nuclear and cytosolic fractions showed that Nrf2 protein was detectable only in the nucleus, and HO-1 only in the cytosolic fraction (Figure 6.2A & B). Despite its predicted mass of 66 kDa, Nrf2 appeared as a band running at ~130 kDa, which is consistent with observations from other studies [220; 236; 326; 327], and the band corresponding to HO-1 migrated as expected at ~32 kDa.

Haemin and sulforaphane (SFN) are two compounds that have been identified as initiators of HO-1 via Nrf2 activation in other cell types [328; 329]. Therefore, this study examined the ability of haemin and SFN to induce Nrf2 localisation and HO-1 expression in HUVECs. Western blotting analysis indicated that Haemin induced no apparent increase in nuclear levels of Nrf2 in HUVECs, and neither haemin nor SFN induced an increase in HO-1 signal (Figure 6.2C). It was noted that basal HO-1 expression appeared high. Both Haemin and SFN are known to induce HO-1 expression via Nrf2 nuclear translocation [328; 329], yet neither effect was seen in HUVECs in normal culture conditions.

6.3.1.2 HO-1 expression and Nrf2 localisation in serum-deprived HUVECs

In an attempt to reduce the basal levels of HO-1 expression and Nrf2 nuclear localisation, HUVECs were serum-deprived 24 h before treatment in M199 containing 2% FBS (M199 2% FBS), with no obvious detriment to cell morphology or proliferation. Morphologically, cells appeared normal regardless of FBS concentration used, but Western blotting of whole cell lysate confirmed that after 24 h of serum deprivation the level of HO-1 expression in HUVECs was lower (by approximately ½) than that of cells grown in the presence of 12% FBS (Figure 6.3).
Figure 6.2 Nrf2 localisation and HO-1 expression in HUVECs.
Proteins from HUVECs – untreated or incubated with 50 µM haemin – were separated by reducing SDS-PAGE following nuclear and cytosolic fractionation and Western blotted with antibodies against A, Nrf2, or B, HO-1. C HUVECs were untreated, or incubated 12 h with either 50 µM haemin or 20 µM SFN, then whole cell lysate was prepared and separated (20 µg per lane) by reducing SDS-PAGE followed by Western blotting with antibodies against HO-1. Dashed line separates bands from different regions of the same blot.
6.3.1.3 SFN-induced nuclear accumulation of Nrf2 and expression of HO-1 in serum-deprived HUVECs

To determine whether the known effects of SFN could be induced in HUVECs, serum-deprived cells were treated with 20 μM SFN for 12 h in M199 containing 2% FBS. Western blotting confirmed nuclear accumulation of Nrf2 as well as increased expression of HO-1 (Figure 6.4).

Nevertheless, accumulation of Nrf2 could not be detected by immunofluorescence (Figure 6.5). Untreated HUVECs, or HUVECs treated with SFN were analysed by immunofluorescence as described (refer to Chapter 2, Section 2.2.2). Cells were counterstained with Hoechst (left panel) and Nrf2 visualised using secondary antibodies conjugated to Alexa-488 (middle panel). Right panel with overlay of the two fluorescence channels indicates that Nrf2 appeared highly nuclear even in control cells, and no difference in fluorescence intensity could be detected when comparing control versus SFN-treated cells.
Figure 6.4 Nuclear accumulation of Nrf2 and cytosolic HO-1 protein levels in serum-deprived HUVECs treated with sulforaphane.

After treatment, HUVECs grown in 2% FBS underwent nuclear and cytosolic isolation; fractionated lysates (30 µg protein per lane) were separated by reducing SDS-PAGE and Western blotting was performed using antibodies against Nrf2, Parp1 and HO-1. Sulforaphane (SFN) treatment was 20 µM, applied for 24 h in M199 containing 2% FBS, while control wells were untreated. Duplicate lanes represent separate treatments of different wells. Nuclear fractions were separated on two separate gels and probed for either Nrf2 or Parp1. In addition to Parp1 signal, Coomassie R250 staining of the PVDF was used to confirm equal protein in each sample.

Figure 6.5 Immunofluorescence of HUVECs was investigated using antibodies against Nrf2 with Hoechst counterstaining.

Cells were grown on glass coverslips and serum-deprived (M199 containing 2% FBS) from 24 h before treatment. SFN treatment was 20 µM, for 12 h while control cells were maintained in reduced-serum medium. Cells were fixed after treatment, then coverslips with adhered cells were incubated with antibodies against Nrf2; secondary antibodies were conjugated to Alexa488. Coverslips were mounted onto glass slides for visualisation by fluorescence microscopy. Hoechst staining was used to visualise nuclei.
Monochloramine-induced HO-1 expression

6.3.1.4 Monochloramine-induced HO-1 expression in HUVECs

To determine whether HO-1 expression could be induced by treatment with chloramines, HUVECs were treated with NH\textsubscript{2}Cl or GlyCl for 10 min in HBSS, then returned to full M199\textsubscript{2%} FBS for 24 h. NH\textsubscript{2}Cl, but not GlyCl, induced increased HO-1 expression at 24 h post-treatment (Figure 6.6). Therefore, the remaining experimental investigations in this chapter focused on the effects of the chloramines on Nrf2 translocation and HO-1 expression.

6.3.2 Nrf2 localisation in Hepa 1c1c7 cells

Due to high basal levels of nuclear Nrf2 in HUVECs (along with a lack of detectable increase after treatment), nuclear localisation of Nrf2 was investigated in Hepa 1c1c7 cells, a cell line which has historically been selected for its highly inducible phase 2 response enzymes by various stresses [330-333]. In Hepa 1c1c7s – unlike in HUVECs – there was a clear observable difference between Nrf2 immunofluorescence in control cells versus those treated with SFN. In untreated cells, immunofluorescence corresponding to Nrf2 was low, and primarily localised to a small region adjacent to the nucleus, with very low fluorescence in the nuclear region. Conversely, cells treated with SFN showed a high degree of Nrf2 fluorescence throughout the cell, including in the nuclear region (Figure 6.7). Because of the strong induction of Nrf2 translocation with SFN, Hepa 1c1c7 cells were used for most of the experiments described in this chapter.
6.3.3 Effect of monochloramine on Nrf2 and HO-1 in Hepa 1c1c7 cells

Hepa 1c1c7 cells treated with NH₂Cl exhibited no nuclear translocation of Nrf2 when investigated by immunofluorescence; the pattern of fluorescence in these cells was indistinguishable from control cells (Figure 6.7, bottom row). This result was verified using the Nrf2 Western blot assay. Indeed, no accumulation of Nrf2 in whole cell lysate was observed when cells were treated with NH₂Cl (Figure 6.8). SFN, however, did induce time-dependent nuclear accumulation of Nrf2, consistent with release of Nrf2 from Keap1; maximum accumulation was observed at 3-6 h treatment with SFN.
Interestingly, HO-1 expression did increase, as with the SFN positive control. Maximum expression of HO-1 in cells treated with either compound was seen at 12-24 h post-treatment (Figure 6.8). A mere 10 min treatment with 50 μM NH₂Cl induced an approximate 9-fold increase in HO-1 expression 12 h after treatment compared to untreated cells. Thus, while the SFN positive control induced both Nrf2 translocation and HO-1 expression as expected, by the same detection methods NH₂Cl induced a high level of HO-1 expression in the absence of Nrf2 translocation.

Figure 6.8 Nrf2 accumulation and Haem oxygenase-1 expression in Hepa 1c1c7 cells. Cells were grown until subconfluent in 6-well plates; treatment with 50 μM NH₂Cl was applied in HBSS for 10 min, after which cells were washed and either immediately lysed (0 h) or returned to MEM for the indicated times (shown in hours). SFN treatment was in MEM; lysis after 10 min (0 h) or up to 24 h is indicated (SFN treatment was removed only immediately prior to lysis). Whole cell lysates (25 μg total protein per well) were separated by reducing SDS-PAGE and western blotted with the indicated antibodies. A Western blots are representative of 2 separate experiments; B data are means ±range (n=2); values which were significantly different from control are shown (*, p<0.05, as determined by one-way ANOVA with Holm-Sidak multiple comparison).
To determine whether the results seen with Hepa 1c1c7 could be replicated in HUVECs, the experiments were repeated using the endothelial cells. Treatment with the SFN positive control induced an effect similar to that seen in Hepa 1c1c7 cells; Nrf2 expression was induced after as little as 10 min following addition of SFN (0 h time point; Figure 6.9, right panel), and HO-1 expression increased with maximal levels of expression at the latest time points (12-24 h). Nrf2 detected by Western blotting increased up to 6-fold in SFN-treated HUVECs, with up to 6-fold increased HO-1 expression (Figure 6.9).

Importantly, NH$_2$Cl treatment induced HO-1 expression in HUVECs; as seen with Hepa 1c1c7 cells, this occurred in the absence of any detectable Nrf2 activation. HO-1 expression was highest at 24 h (expression was at least 2.5 and up to 7 times higher than the level in untreated cells) following treatment with NH$_2$Cl. Nrf2 levels remained low at all time points, with very low
signal by Western blotting. There was some variability in signal between lanes (which could relate
to slight differences in loaded protein), but this was neither consistent (between the experimental
replicates) nor significant, with no more than 75% variability in the signal compared to the
control lanes (0 & 24 h). Thus, the level of HO-1 expression induced by exogenous treatment of
HUVECs with NH₂Cl was at a level similar to (or even in excess of) that induced by SFN. The
fact that this HO-1 expression occurred in the absence of detectable Nrf2 activation was
remarkable.

6.3.4 Nuclear accumulation of Nrf2 in NH₂Cl-treated cells

To more closely monitor whether or not Nrf2 underwent any nuclear accumulation with NH₂Cl
treatment, cells were fractionated to enrich the nuclear fraction, thereby optimising the detection
of Nrf2. Two time points (3 and 6 h post-treatment) were selected for these analyses, because
SFN treatment had induced the highest accumulation of Nrf2 protein in Hepa 1c1c7 cells at
these time points (refer to Figure 6.10).

In nuclear fractions of Hepa 1c1c7, the Nrf2 signal intensity increased only with SFN treatment,
and was evident at both the 3 h and 6 h time points (Figure 6.10). HO-1 expression in cytosolic
fractions increased noticeably (though not significantly) 6 h post-treatment, consistent with the
time course experiment (Figure 6.8).
Chapter 6: Monochloramine-induced HO-1 expression

It was again verified that these effects could be observed in HUVECs. The increases in Nrf2 and HO-1 levels were consistent in the two cell types, but were more pronounced in the Hepa 1c1c7 cells compared to the HUVECs (particularly at the earlier time point), even with the SFN positive control. Nevertheless, increase in HO-1 expression was consistent in the two cell types. Results indicated that there was no significant nuclear accumulation of Nrf2 in either cell type treated with NH₂Cl, despite pronounced increases in HO-1 expression.

Figure 6.10 Western blotting of nuclear and cytosolic fractions to investigate intracellular localisation of Nrf2 in Hepa 1c1c7 cells and HUVECs.

Cells were cultured on 100-mm dish until confluence; treatment with 50 or 15 µM NH₂Cl (Hepa 1c1c7s or HUVECs, respectively) was applied in HBSS for 10 min, after which cells were washed and returned to media larger for the indicated times. HUVECs were pre-incubated in M1992%FBS 24 h before treatment, and returned to the reduced-serum medium during recovery (or remained in this medium for SFN treatment). Hepa 1c1c7 cells were maintained in MEMα. Cytosolic and nuclear fractions were isolated and 15 µg protein separated by reducing SDS-PAGE. A Western blotting was performed with antibodies against Nrf2 & HO-1. B Densitometric analysis of 1c1c7 cells (grey bars) represents means ± range for 2 separate experiments, while HUVEC data (white bars) are from a single experiment. Significant differences were assessed by one-sample t-test (expected mean = 1.0; *, p<0.05).
Chapter 6: Monochloramine-induced HO-1 expression

HO-1 expression increased with NH₂Cl treatment, and was particularly evident at the 6 h time point in both the primary HUVECs and Hepa 1c1c7 cell line; the increase in HO-1 at 6 h was more pronounced when the cytosolic fraction (Figure 6.10) was analysed compared to the whole cell lysate (Figure 6.8 & Figure 6.9); this was likely due to either improved detection from the fractionated sample, or to slight differences in cell response associated with the different plate format used in the two analyses (or a combination of these two factors).

6.4 Discussion

The results shown in this chapter demonstrate, in two distinct adherent cells types (a mouse hepatocyte cell line and a primary human endothelial cell), that NH₂Cl induces a pronounced increase in HO-1 expression independent of Nrf2 activation/translocation. It is particularly noteworthy that as little as 15 µM NH₂Cl applied for 10 min were required to induce a roughly 4-fold increase in expression of HO-1 6 h after treatment.

The induction of HO-1 by NH₂Cl is similar to observations by others showing induction by inflammatory oxidants including TauCl and HOCl [55; 72; 220; 324]. In those studies, maximal HO-1 expression was observed between 4-12 h, depending on treatment and cell type; where mRNA levels were measured by RT-PCR, peak transcript levels were observed after 6-8 h treatment. Thus, the time course for HO-1 induction observed in this study is consistent with previous reports which employed other inflammatory oxidants.

Each of the above-mentioned studies of the effect of inflammatory oxidants concluded that HO-1 expression was induced by Nrf2 activation and binding to the ARE of the HO-1 promoter gene. Maximal levels of Nrf2 protein levels, and/or nuclear localisation were generally observed between 6-12 hrs, with increases seen as early as 30 min in some instances [72]. Reported results varied greatly, however, in terms of the time scale investigated; Sun Jang et al [55] reported Nrf2 nuclear localisation and ARE-binding at only the 12 h time point (it is unclear whether they investigated earlier time points), and Kim et al [72] reported ARE binding and nuclear Nrf2 protein levels only up to 60 min. Indirect evidence provided by these authors did suggest that Nrf2 activation was required for HO-1 expression induced by HOCl and TauCl; oxidant-induced HO-1 expression was inhibited in cells either expressing a dominant-negative form of Nrf2 [219] or transfected with Nrf2 siRNA [72].
One important difference between the experiments outlined in this chapter versus other studies discussed herein is the fact that all previous studies investigated the effect of inflammatory oxidants in cells maintained in medium. This means that trans-chlorination was possible in their systems prior to uptake by the cells, and that the actual oxidant responsible for the observed effects is unknown, and may or may not be relevant under physiological conditions. Relatively high doses of oxidant (~500-700 μM TauCl or up to 300 μM HOCl) were required to see the effects reported. By contrast, in this study, cells were treated in HBSS to pinpoint the effect of monochloramine alone on endothelial cells and low doses (15-50 μM, depending on cell type) were employed for only 10 min, after which the oxidant was removed and cells were returned to full medium. Therefore, this chapter represents a novel study whereby the specific downstream effect on HO-1 expression of an inflammatory oxidant was investigated. Certainly, this study explored more specifically the effects of one particular oxidant, and highlighted the sensitivity of the cellular HO-1 response to an exogenous stimulus.

Contrary to the studies mentioned above, the HUVECs and Hepa 1c1c7 cells investigated in this chapter showed no evidence of Nrf2 activation by NH₂Cl, with no evidence of nuclear accumulation or increased protein levels of Nrf2 within 24 h of treatment. Further experimentation would be required to rule out the involvement of Nrf2 entirely, and/or to investigate other potential pathways involved in mediating the increase in HO-1 expression. Nevertheless, studies exploring HO-1 induction in a variety of cell types, and by a variety of stimuli, have suggested a number of different pathways by which HO-1 expression appears to be regulated. Although the contribution of other transcriptional controls to the increase of HO-1 was not assessed, a number of possibilities do exist.

Recently, binding of the Bach1 transcriptional repressor to enhancer regions of the HO-1 gene has been shown to inhibit transcription of the gene [334-337]. It has also been suggested that Nrf2 and Bach1 compete for binding to the enhancer regions which control HO-1 expression, thereby controlling the level of gene expression [338] (reviewed in [3]). Interestingly, it appears that in the absence of Nrf2 induction, inhibition of Bach1 might be sufficient for HO-1 induction by low basal levels of nuclear-localised Nrf2 [338].

An interesting and compelling example of HO-1 induction in the absence of any Nrf2 activation has emerged from studies investigating the effects of cigarette smoke and heavy metals. The zinc
finger proteins Egr-1 and SP-1 have both been shown to bind to an enhancer region controlling HO-1 transcriptional activation when cells are exposed to cigarette smoke or heavy metals, as has AP-1 [188; 339; 340]. Convincingly, Baglole et al [198] showed that human lung fibroblasts exposed to cigarette smoke exhibited increased HO-1 expression mediated by NF-κB and AP-1 and this was shown to be independent of Nrf2 activation.

Other studies, too, have highlighted the implication of these and other transcription factors in HO-1 expression, though not always with the exclusion of Nrf2. Lipopolysaccharide-induced HO-1 expression has been attributed to AP-1 [341] as well as NFκB [187]; the latter transcription factor has also been linked to HO-1 expression in aging livers [342]. The STAT1/STAT3 heterodimer has also shown some promise as an inducer of HO-1 expression in response to both vascular endothelial growth factor (VEGF) signalling and hyperoxia [221; 343], and numerous studies have pointed to a role for Heat Shock Factors (HSF) in HO-1 expression (though not in response to hyperthermia in humans or mice; reviewed in [8]). A summary of the most well-studied transcription factors involved in positively regulating HO-1 expression is illustrated in Figure 6.11.

In addition to its antioxidant activities in endothelial cells (reviewed in [197]), HO-1 expression has been associated with protection of the endothelium against tissue injury during inflammation (reviewed in [3]); inhibition of apoptosis [321], as well as decreased expression of TNF-α-induced adhesion molecules [344; 345] support this. Production of CO by HO-1 also inhibits NADPH oxidase activity in activated neutrophils and macrophages, thus potentially decreasing superoxide production and potentially limiting the damaging effects of the oxidative burst to the endothelium [55; 346-348]. Further evidence of this was demonstrated in mice overexpressing HO-1 in cardiac tissue, which had reduced inflammatory cell infiltration and reduced oxidative damage in response to ischemia/reperfusion injury [349]. Decreased availability of free haem as a result of HO-1 activity prevents the assembly of the functional NADPH oxidase enzyme [350], and bilirubin is apparently capable of interfering with the enzyme assembly and activation as well [351]; these effects are reviewed in [111].

A number of associations have been made between HO-1 expression and protection against atherosclerosis, a disease with a strong inflammatory component. Studies have shown enhanced expression in foam cells from atherosclerotic plaques [323], and the protective effects of HO-1
Chapter 6: Monochloramine-induced HO-1 expression

142

have been well documented through studies involving either modulation of HO-1 levels by various methods [207-211; 352; 353]. Indeed, several studies have shown HO-1 expression to be negatively correlated with development of experimental atherosclerosis [205; 206; 325].

Additionally, the overproduction of CO by increased HO-1-expression and activity has been suggested to have an important anti-inflammatory function in endothelial cells. Inhibition of NADPH oxidase activity in PMA- or lipopolysaccharide (LPS)-stimulated neutrophils and

Figure 6.11 Simplified schematic of the transcriptional regulation of HO-1 expression.
AP1, Nrf2, Hsf1 and NFκB transcription factors have been shown to control transcriptional levels of HO-1 by binding to various sites in the upstream promoter region. (NFκB may act indirectly through unidentified DNA-binding partners). MAPK signalling has been widely implicated in the nuclear translocation of these transcription factors, acting either directly (i.e. via direct phosphorylation of the TF; arrow with solid line) or indirectly (arrow with dotted line). These signalling cascades are activated by various biological stresses and stimuli (middle ring), apparently induced by physiological conditions (outer ring). Adapted from [8]
macrophages (respectively) has been demonstrated by HO-1-mediated CO production [55; 346-348]. This results in decreased production of superoxide (and therefore other reactive downstream oxidants), and has been proposed as a feedback mechanism to reduce the harmful effects of inflammatory oxidants. Taken together, these studies provide overwhelming evidence for a protective, anti-inflammatory role for HO-1 in endothelial cells, at both the molecular level and in the prevention of pathological inflammatory disease. The results presented in this chapter illustrating the effect of NH$_2$Cl on HO-1 expression are striking, and may represent an important way by which cells respond to and limit the potentially damaging effects of the neutrophil oxidative burst.
Chapter 7. General Discussion

7.1 Summary
Chlorinated oxidants have been implicated in various cell processes, from the activation of signalling pathways (e.g. MAPK signalling and NFκB transcription factor activation) in endothelial cells, to the expression of inflammatory cytokines in various cell types, and they are known thiol-targeting agents. Most studies to date which have investigated the effects of chloramines and HOCl in cells have treated cultured cells with the oxidants in culture medium (though there are some exceptions to this [36; 51]). Because transchlorination reactions and methionine scavenging can occur in full medium, the interpretation of those chloramine treatments performed in medium is limited. To better understand the specific cellular reaction targets at a site of inflammation by the various oxidants produced, this thesis has used an endothelial cell model to explore both specifically and broadly the potential oxidation targets of HOCl and two cell permeable chloramines, GlyCl and NH₂Cl, and to compare these to the effects of H₂O₂.

In this study the effects of GlyCl, NH₂Cl and HOCl on Prx redox state, TrxR activity, and intracellular GSH concentrations were investigated, and the effects were compared to those of H₂O₂. Startling differences were observed between the effects of H₂O₂ and the chlorinated oxidants; while Prx becomes readily hyperoxidised by H₂O₂, the chlorinated oxidants induced Prx oxidation to the dimer form, and this was a major event in both erythrocytes and endothelial cells. Loss of GSH was a comparatively minor effect in HUVECs (but not erythrocytes), while TrxR activity was inhibited only with HOCl. This study also identified a potential link between Prx dimer accumulation and cell death. In addition, an investigation of sensitive protein thiols in cells treated with the various oxidants highlighted that a number of changes occur with any of the inflammatory oxidants used, though there were some notable differences in the effects of the various oxidants. A number of these changes were selected for identification, yielding a list of interesting proteins which can be investigated for their roles in signalling and antioxidant protections in the context of inflammation. Finally, HO-1 expression was shown to be strongly and sensitively induced by low doses of NH₂Cl in the absence of obvious Nrf2 accumulation or activation.
7.2 Inflammatory oxidants and Prxs

7.2.1 Conformational changes in Prxs differ depending on oxidant treatment

The 2-Cys Prxs investigated in this study form disulfide-linked homodimers when oxidised, and are reduced via the thioredoxin system. In the presence of high amounts of \( \text{H}_2\text{O}_2 \), Prxs are known to undergo hyperoxidation to the sulfenic or sulfonic acid; the slow-acting enzyme sulfiredoxin reduces the sulfenic form of the enzyme. Prxs have been well studied in terms of \( \text{H}_2\text{O}_2 \)-induced oxidative stress (reviewed in [354; 355]). In addition, the roles of Prxs in disease have been well characterised through studies involving knockout mouse models, and increased expression and/or hyperoxidation in various inflammatory diseases ([174; 356-359]; reviewed in [355]). These findings, in addition to a number of Prx protein interaction partners which have been identified to date (reviewed in [355]) have highlighted the complex roles of these enzymes in vivo. It has therefore become clear that Prxs are important antioxidant enzymes likely involved in a number of signalling events, and yet few studies have investigated other potential substrates of these enzymes, or considered other oxidants which could be mediating the Prx redox state and expression changes.

This study was the first to investigate ability of chlorinated oxidants to induce redox changes in Prxs in an intact cell system. The findings in this investigation have uncovered a remarkable difference between the oxidation of Prxs by chlorinated oxidants compared to peroxide-induced oxidation. Namely, while exogenous \( \text{H}_2\text{O}_2 \) induces inactivation of Prxs in cells via hyperoxidation of the peroxidatic cysteine, chlorinated oxidants do not. Instead, Prxs accumulate in the readily-reduced dimeric form, and therefore remain catalytically active and capable of continuous scavenging of oxidants. The reaction of Prxs with chlorinated oxidants is much slower than the reaction with peroxides [12], and this probably relates to the different outcome between oxidation by the two classes of oxidant (since hyperoxidation is known to occur only when all components of the catalytic cycle are available [252][32]). The reaction of the thiolate residue with \( \text{H}_2\text{O}_2 \) is rapid and so is the reaction with the sulfenic acid; this is suspected to be more rapid than the conformational change required for dimerisation in situations where local \( \text{H}_2\text{O}_2 \) concentrations are sufficiently high, thereby inducing the hyperoxidised enzyme form (reviewed in [149; 158; 268; 354; 360]). When Prxs react with chlorinated oxidants, the sulfenyl chloride is likely the initial reaction product (as seen with other thiols [47; 248]). Subsequently, the reaction between the sulfenyl chloride (or the sulfenic acid formed by hydrolysis) and further
oxidant may not be favoured over resolution by disulfide formation, and hence the observation of accumulated dimer.

Despite the fact that reduction of the disulfide-linked Prxs was very fast in chloramine-treated endothelial cells, short-term accumulation of the dimer was nevertheless observed. This means that chloramines react sufficiently fast in cells for accumulation of dimer before reduction by the unaffected Trx-TrxR system. Furthermore, Prxs 1-3 were identified from spots excised from 2D gels which underwent a significant change in intensity in HUVECs treated with chloramines and HOCl (Chapter 5). This suggests that the oxidation of Prxs, though admittedly slow, results in dimer accumulation and appears to be a significant oxidative event in cells treated with these compounds.

A major consequence of these findings is therefore that, to date, chlorinated oxidants are the only treatments in which accumulation of Prx dimers has been reported in cultured cells. This is with the exception of Prx dimer accumulation seen in the erythrocyte, where Prx scavenges H$_2$O$_2$ non-catalytically due to slow TrxR activity [65].

Because Prxs do exist in various oligomeric states, these enzymes have been suspected to act as redox switches; the inter-conversion of the typical 2-Cys Prxs between monomeric, dimeric and higher-order oligomeric structures has been hypothesised to regulate functional changes in the enzymes, possibly participating in the regulation of cell signalling pathways [361; 362] (reviewed in [4]; Figure 7.1). The results presented here provide a mechanism by which Prxs might undergo a functional switch in cells presented with different oxidants; accumulation of the dimer (or loss of the reduced monomer) might confer an adaptive response to inflammatory conditions by participating in different signalling cascades. This switch is probably more relevant to chloramine than HOCl treatments. Chloramines are less reactive and more discriminatory than HOCl [10; 46; 47]. HOCl reacts with a greater range of cellular targets (refer to Section 5.3.5). In this study, it inhibited TrxR activity and inducing greater loss of GSH relative to Prx oxidation. It is therefore probable that the mechanisms leading to cell death differ between treatments with chloramines versus HOCl.
If the results of this study are indicative of the *in vivo* situation during inflammatory conditions, then chronic elevation of Prx dimer would be expected at inflammatory sites. The elevated expression of Prxs as reported in a variety of pathological disease (discussed above) might be an adaptive response to bolster the antioxidant protection afforded by these enzymes under situations of heightened oxidative stress.

### 7.2.2 Potential role of Prx in apoptosis

Prxs could potentially participate in apoptotic signalling cascades. The strong correlation between Prx dimer accumulation and increased cell death reported here support this possibility, and the lack of such a correlation between cell death and other oxidation targets (including GSH, GAPDH, total thiols and protein carbonyls) supports the notion that this is a noteworthy relationship.
The oxidants examined in this study are cytotoxic and HOCl and NH₂Cl in particular are known to induce apoptosis [34; 45]. The role of Prxs in apoptosis has been suggested by other authors; in particular, the protective ability of Prx3 and Prx5 (an atypical 2-Cys Prx) to scavenge mitochondrial H₂O₂ has been suggested to indirectly modulate apoptosis [363-365]. Generally, the role of Prxs in inhibition of apoptosis is attributed to their ability to scavenge H₂O₂ (and/or ONOO⁻), thereby preventing the induction of cell death pathways by these oxidants [366]. This could also be the case in chloramine-treated cells, whereby doses in excess of those easily scavenged by antioxidant systems including the Prxs react with other targets which results in initiation of apoptotic pathways. Alternatively, Prxs themselves could be involved directly in the regulation of apoptotic pathways; inhibition of pro-apoptotic pathways by monomeric Prx and/or enhancement of such pathways by the dimeric enzyme would have the observations reported herein.

Evidence toward this latter possibility exists in the observation that active cycling of the Prxs appeared important for protection of HUVECs against oxidant-induced loss of viability. In this study, modulating the oxidation state of Prxs prior to treatment with chloramines caused a substantial increase in cell death. The relationship between cell death pathways and Prxs needs to be further explored to determine how the oxidation and/or oligomeric state of the various Prxs might be regulating these pathways.

7.3 Novel thiol targets of inflammatory oxidants

Short-term treatment of HUVECs with chloramines induced no detectable loss of total protein thiols (Chapter 4), but analysis by one-dimensional (1-D) and 2-D SDS-PAGE with IAF labelling (Chapter 5) highlighted specific thiol protein oxidation following treatment with H₂O₂, HOCl or chloramines. This demonstrates that targets of these oxidants are highly specific. Other studies have investigated the effects of inflammatory oxidants on the thiol proteome using similar methodologies in Jurkat cells treated with H₂O₂ [260] and in lymphoma cells treated with TauCl [41].

Interestingly, a number of the proteins identified in this study as undergoing oxidative modification have also been identified in the studies mentioned above. GAPDH, GST and the Prxs (Prxs 1 & 2 in both [41] and [260] and Prxs 1-3 in the present study) have consistently appeared to undergo oxidative changes in all of the studies. The reappearance of these proteins
in this work is consistent with high sensitivity to oxidative stress and with them having a major role in cellular antioxidant protection. Admittedly, these proteins are all present in relatively high abundance, which would facilitate a positive identification, but the recurrence of these proteins in all three studies suggests a common role in cell responses to oxidative stress induced by inflammatory oxidants. As discussed above, the Prxs specifically were investigated in this thesis and are likely to play a major role in cellular responses and antioxidant defence during inflammation.

DJ-1 and coflin-1 were also identified in both this study and in the study which investigated TauCl-induced thiol changes [41]. (It is worth mentioning that in the current study TauCl was found to be cell-impermeable and did not induce thiol oxidation in HUVECs, but others have reported TauCl permeability in other cell types [54; 367; 368]; alternatively, these observations can be explained by transchlorination due to inclusion of other amines in the treatment system). While both DJ-1 and coflin-1 are known to be involved in promoting apoptosis following Cys oxidation and mitochondrial translocation [279; 280; 284-286], the study by Klamt et al [41] focused on coflin-1. They confirmed that this protein has a major role in mediating apoptosis in their lymphoma cell model, and have shown that this activity is dependent on the oxidation of its four cysteine residues. This oxidation is likely reversible and would presumably be transient in cells until the oxidant stress has been removed; this needs to be verified and could provide further information on this protein’s functional role as a redox switch in vivo.

A number of other proteins with known involvement in apoptotic pathways were also identified in this study (namely, Annexin A1, πGST, GPx1 and SUMO). In light of the fact that apoptotic pathways are of interest in cells treated with chloramines in terms of possible links to the peroxiredoxins (as discussed in Section 7.2.2, above), these proteins provide a basis for these investigations. Both the role of each protein in chloramine-induced apoptosis, in addition to potential Prx binding could easily be explored.

GSH-related systems also appear important in inflammation, likely in the context of cellular antioxidant protection. GSH was identified as undergoing dose-dependent oxidation in erythrocytes and HUVECs exposed to chlorinated oxidants (Chapter 3 & Chapter 4). In screening for oxidative changes to thiol proteins several related proteins were identified as undergoing significant thiol oxidation in cells treated with inflammatory oxidants: πGST and
GPx1. Further investigation of these enzymes might uncover whether these enzymes might be contributing to or protecting against oxidative damage during inflammation.

One of the most impressive – and novel – targets of thiol oxidation observed in this study involved CypA. The host lab has now identified this protein as undergoing major changes in a number of contexts and cell types, and is currently exploring how it is oxidatively modified in order to better understand its role in the context of inflammatory oxidants. It is also being investigated for any interactions with Prxs, since this enzyme has been shown to reduce Prxs in plants [270].

### 7.4 Chloramine-induced HO-1 Expression

Haem oxygenase 1 is another major antioxidant in cells, which has strong links to inflammatory disease and the protection against oxidative stress, as discussed in Chapter 6. The current study found that HO-1 expression was significantly induced in Hepa-1c1c7 cells and in HUVECs with maximal protein levels observed between 12 and 24 hr following a 10 min treatment with NH₂Cl. In corroboration with recent studies suggesting that TauCl and HOCl are also capable of activating HO-1 [55; 72; 220; 324], these data suggest that chlorinated oxidants may be responsible for inducing HO-1 expression at inflammatory sites. Indeed, HO-1 expression has been observed in atherosclerotic lesions [323], and mounting evidence suggests that the enzyme has anti-inflammatory effects (reviewed in [3]). There is therefore potential for HO-1 expression to be induced as a protective response to pro-inflammatory stimuli including chloramines.

There was no evidence of Nrf2 accumulation or translocation in mouse hepatocyte or human endothelial cells in which NH₂Cl induced an increase in HO-1 expression. This is in contrast with reports that Nrf2 activation is the primary mediator of HO-1 expression, though it has been suggested that repression of the transcription repressor Bach1 might be sufficient for basal nuclear Nrf2 binding to the ARE, inducing HO-1 expression (reviewed in [3]). Further investigation is required to definitively ascertain whether or not Nrf2 is involved in mediating NH₂Cl-induced activation of HO-1. Inhibition of Nrf2 by siRNA in treated cells should allow the determination of the degree to which HO-1 expression depends upon Nrf2 activation in these cells. Use of an Nrf2⁻/⁻ cell line would also help to address this question. While there was no sign of increased Prx1 expression (Prx1 is another gene induced by HO-1), other proteins which can be transcriptionally induced by Nrf2 could be assessed.
Nevertheless, a clear induction of Nrf2 translocation and accumulation was observed in cells treated with the isothiocyanate sulforaphane, a well-characterised inducer of the phase II response which is known to induce HO-1 transcription in an Nrf2-dependent manner (reviewed in [369]). The ability to detect Nrf2 activation following SFN treatment suggests that the methods used were sensitive enough to detect this, but it cannot be ruled out that any Nrf2 activation was below a threshold of detection by these methods. Little-to-no Nrf2 activation in response to NH$_2$Cl treatment might suggest that other transcription factors are involved, and these could be identified by process of elimination using known inhibitors, siRNA or mutant cell lines.

It is interesting that both Prx1 and HO-1 have been identified in this study as key antioxidant proteins which are sensitive to chloramines and HOCl. Aside from the fact that both proteins undergo inducible expression via Nrf2 activation, both HO-1 and Prx1 are haem-related enzymes; while the former uses haem as a substrate, the latter is inactivated by haem [370; 371]. Co-localisation and co-induction of HO-1 and Prx1 in rat hemorrhagic brain have consequently been proposed to constitute a cellular response whereby Prx1 function (as an antioxidant and in promoting cell proliferation pathways) could be maintained [372]. The authors suggested that the two enzymes might interact in the context of intracerebral haemorrhage, and it would be interesting to investigate this possible interaction in the context of inflammation.

The sensitivity of response in cells treated with NH$_2$Cl was particularly surprising given the low dose used, and the short time course over which the cells were exposed. Although NH$_2$Cl was the only oxidant explored in this study in terms of HO-1 expression, it would be interesting to assess other chlorinated oxidants under the same conditions, since previous studies have utilised high concentrations and much longer treatment periods [55; 72; 219; 220]. The sensitivity of this response investigated in the current study, in addition to the known involvement of HO-1 in anti-inflammatory activity, make this enzyme a very interested subject for future research into chloramine- and HOCl-induced endothelial responses.

### 7.5 General Conclusions

In summary, this study has shown that Prxs and HO-1 are important antioxidant systems in cells exposed to inflammatory oxidants, and has uncovered thiol modifications which could be important in conditions of inflammation. It has provided the impetus for future investigation
and characterisation of various thiol redox systems – and potential interactions between them – in the context of inflammation.

This study has provided novel insight into the mechanisms of chlorinated oxidant-induced damage to the endothelium. Characterisation of Prx oxidation in endothelial cells and erythrocytes under various oxidant stresses, identification of thiol targets of exogenous treatments with chlorinated oxidants, and exploration of HO-1 induction have not only revealed valuable information about the targets of chloramine-induced oxidative stress, but has also provided new insight into the workings of these various antioxidant systems and signalling pathways. The investigation of thiol targets of chlorinated oxidants has provided a means by which new associations, pathways and mechanisms of inflammation-induced damage to the endothelium might be uncovered.

Future directions to elaborate on the findings reported here should include the design of a more physiologically relevant system. For instance, the use of an activated neutrophil system as a source of oxidants could be used to validate both the relative importance of the various inflammatory oxidants in, and the effects on major thiol targets discussed here.

Importantly, this study has employed physiologically relevant doses of chlorinated oxidants and has demonstrated that even with short time courses, a number of thiol-based changes occur in an exposed endothelium. Further understanding of these modifications will hopefully lead to the development of therapeutics to interfere with or augment appropriate cellular pathways to diminish endothelial dysfunction and damage during inflammatory disease.
References


References


References


References


Spots identified as undergoing significant change with one or more treatments, relative to Control.
One gel image for each treatment is shown, representative of three separate experiments. Spot numbers are arbitrary and correspond to those used in protein identification (Figure 5.9 and Table 5.1).

<table>
<thead>
<tr>
<th>Spot</th>
<th>Control</th>
<th>GlyCl</th>
<th>NH$_2$Cl</th>
<th>HOCl</th>
<th>H$_2$O$_2$</th>
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<tr>
<td></td>
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</tr>
<tr>
<td>Spot</td>
<td>Control</td>
<td>GlyCl</td>
<td>NH₂Cl</td>
<td>HOCl</td>
<td>H₂O₂</td>
</tr>
<tr>
<td>------</td>
<td>---------</td>
<td>-------</td>
<td>-------</td>
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<td>------</td>
</tr>
</tbody>
</table>
| 3    | pH = 5.0
25.8 kDa |       |       |      |      |
| 4    | pH = 5.53
26.1 kDa |       |       |      |      |
| 5    | pH = 6.06
24.7 kDa |       |       |      |      |
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<tr>
<th>Spot</th>
<th>Control</th>
<th>GlyCl</th>
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<th>HOCl</th>
<th>H$_2$O$_2$</th>
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</table>
| 6    | pH = 6.13  
22.4 kDa | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) |
| 7    | pH = 5.71  
20.8 kDa | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) |
| 8    | pH = 5.97  
21.5 kDa | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) |
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<th>Spot</th>
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<th>HOCl</th>
<th>H$_2$O$_2$</th>
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</table>
| 9    | pH = 6.06  
20.9 kDa | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) |
| 10   | pH = 6.28  
20.8 kDa | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) |
| 11   | pH = 6.36  
20.7 kDa | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) |
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<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
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<td><img src="image6.png" alt="Image" /></td>
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<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
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<td>Spot</td>
<td>Control</td>
<td>GlyCl</td>
<td>NH₂Cl</td>
<td>HOCl</td>
<td>H₂O₂</td>
</tr>
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</tbody>
</table>
| 15   | pH = 5.66  
15.5 kDa |
| 16   | pH = 5.75  
15.2 kDa |
| 17 (same as 4) | pH = 5.53  
26.1 kDa |
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<th>Control</th>
<th>GlyCl</th>
<th>NH$_2$Cl</th>
<th>HOCl</th>
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<td>![Image]</td>
<td>![Image]</td>
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</tbody>
</table>

- pH = 4.94
- 18.8 kDa