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Mechanisms and Biological Consequences of Damage to Arterial Endothelial Cells by Peroxynitrous Acid

Priyashiel Elvina Parikh

A thesis submitted in fulfillment of the requirements for the award of the degree of Doctor of Philosophy

June 2015
Declaration

The work contained in this Thesis is original work conducted by the author at the Heart Research Institute, Sydney. It has not been submitted to any other institution for a higher degree and does not contain any materials previously published or written by another person except where due reference is made in the text.

Priyashiel E. Parikh BSc (Hons)
“The whole of science is nothing more than a refinement of everyday thinking...”

Albert Einstein
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Abstract

Cardiovascular diseases are the main source of morbidity and mortality in developed countries. Atherosclerosis is the main cause of cardiovascular diseases. In this disease, the blood vessels thicken due to build-up of cholesterol (such as from oxidised low-density lipoproteins (LDL)), which over time lead to plaque formation. In the early stages of atherosclerosis, native LDL can be oxidised by various oxidants, which may include peroxynitrous acid (ONOOGH). ONOOH is formed by the spontaneous reaction of nitric oxide and the superoxide anion. ONOOH is a potent oxidant capable of reacting with CO₂, low molecular mass antioxidants, DNA, proteins, lipids and carbohydrates. ONOOH reacts with CO₂ to form the nitrosoperoxycarbonate anion adduct (ONOOCO₂⁻). Due to their high reactivity and abundance, proteins are believed to be a major target of ONOOH.

The main hypothesis behind the studies reported in this Thesis is that ONOOH reacts with specific targets in endothelial cells with proteins a major target of damage, which causes cell damage and may propagate disease. ONOOH-mediated damage is expected to occur predominantly at sulphur-containing amino acids (both free and on proteins). The project aimed to investigate the extent and order of amino acid damage induced by ONOOH and ONOOCO₂⁻ on isolated peptides and protein. It also aimed to investigate the mechanisms and consequences of reaction of ONOOH and ONOOCO₂⁻ with endothelial cell proteins, both cytosolic and membrane-derived, using human coronary artery endothelial cells (HCAEC) as a model.

Previous studies have reported that ONOOH modifies multiple amino acids of bovine serum albumin (BSA), however the order and efficacy of this modification was unknown. The results outlined in Chapter 3 indicated that bolus ONOOH and ONOOCO₂⁻ (to a lesser extent) induced dose-dependent thiol oxidation on exposure to glutathione and BSA. ONOOH and ONOOCO₂⁻ also modified tyrosine, tryptophan, histidine and methionine residues in BSA. ONOOH also induced the formation of methionine sulphoxide and 3-nitrotyrosine (3NT) in a dose-dependent manner, and induced fragmentation of BSA. The results indicate that ONOOH modifies cysteine residues prior to methionine, tryptophan, tyrosine, histidine and phenylalanine on exposure to the model protein BSA.
There is a lack of knowledge relating to the pathways involved in cellular damage on short and prolonged exposure of HCAEC to ONOOH and ONOO\textsuperscript{2-}. The results reported in Chapter 4 indicate that 5 min incubation of HCAEC with high concentrations of bolus ONOOH and ONOO\textsuperscript{2-} induced significant loss of cell viability and thiol oxidation, with membrane thiol residues oxidised to a greater extent than the cytosolic thiol residues. 3NT formation was detected on selected HCAEC cytosolic and membrane proteins. As oxidant concentrations increased, the staining intensity of nitrated proteins on immunostaining with an anti-3NT antibody increased as well. Direct observations indicated that these oxidants did not induce other detectable protein modifications such as aggregation and/or fragmentation after 5 min incubation with the cells.

The results reported in Chapter 5 suggest that after prolonged exposure (4 h) of HCAEC to SIN-1 in the presence and absence of bicarbonate, which generated ONOOH and ONOO\textsuperscript{2-}, the latter species induced greater loss of cell viability than ONOOH. In this case, ONOOH and ONOO\textsuperscript{2-} seem to preferentially target cytosolic and membrane thiols respectively. ONOO\textsuperscript{2-} induced more dramatic thiol loss than ONOOH in the absence of an intact cell membrane. Both in the absence and presence of an intact cell membrane, membrane proteins were nitrated to a greater extent than cytosolic proteins. In the absence of an intact cell membrane, ONOO\textsuperscript{2-} appears to induce aggregation and/or fragmentation of proteins to a greater extent than ONOOH, as evidenced by a smearing of the protein bands following SDS-PAGE.

The mass spectroscopic data presented in Chapter 6 indicate that in HCAEC, ONOOH targets cytoskeletal proteins (e.g. actin alpha cardiac muscle 1, actin cytoplasmic 1 and 2 proteins, alpha actinin 4, β-actin, β-tubulin, myosin, nestin, talin-1 and vimentin), chaperone proteins (e.g. endoplasmic, heat shock cognate 71 kDa protein and translational endoplasmic reticulum ATPase), proteins that are involved in protein synthesis (e.g. elongation factor 1 – alpha 1, eukaryotic initiation factor 4A-1 and isoform 2 of clathrin heavy chain), mitochondrial proteins (e.g. mitochondrial ATP synthase beta subunit), membrane proteins (e.g. ABCG1) and signalling proteins (e.g. VEGF). It appears that ONOOH selectively modifies proteins, since certain proteins appear to be modified by ONOOH more than others.

The results outlined in Chapter 6 also indicate that due to HCAEC donor variations, some modifications may occur selectively in specific donors, which may be due to
genetic differences, age, racial background and/or health conditions. Overall these studies have provided novel data on the mechanisms and targets of ONOOH in endothelial cells and the susceptibility of cellular proteins to modification, which has important implications for the development of atherosclerosis, where oxidation and endothelial dysfunction play a key role in the disease pathology.

**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3NT</td>
<td>3-Nitrotyrosine</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ABCG1</td>
<td>ATP-binding cassette, sub-family G, member 1</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BS³⁻</td>
<td>Bis(sulfosuccinimidyl)suberate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CID</td>
<td>Collision induced dissociation</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CO₃⁻</td>
<td>Carbonate radicals</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>De</td>
<td>Decomposed</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5’-dithiobis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>ε</td>
<td>Extinction coefficient</td>
</tr>
<tr>
<td>ECL</td>
<td>Electrochemical luminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionisation</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidised glutathione</td>
</tr>
</tbody>
</table>
\( \text{H}_2\text{O}_2 \)  
Hydrogen peroxide

\text{HBSS}  
Hanks buffered salt solution

\text{HCAEC}  
Human coronary artery endothelial cells

\text{HCO}_3^-  
Bicarbonate anion

\text{HDL}  
High density lipoprotein

\text{HEPES}  
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

\text{NH}_4\text{HCO}_3  
Ammonium bicarbonate

\text{HO}^\cdot  
Hydroxyl radical

\text{HOCI}  
Hypochlorous acid

\text{HOSCN}  
Hypothiocyanous acid

\text{HRP}  
Horse radish peroxidase

\text{HSA}  
Human serum albumin

\text{iNOS}  
Inducible nitric oxide synthase

\text{k}  
Rate constant

\text{LC/MS/MS}  
Liquid chromatography tandem mass spectrometry

\text{LDL}  
Low density lipoprotein

\text{LDS}  
lithium dodecyl sulfate

\text{MALDI}  
Matrix-assisted laser desorption/ionisation

\text{MOPS}  
3-morpholinopropane-1-sulfonic acid

\text{MPTP}  
Mitochondrial permeability transition pore

\text{MS}  
Mass spectrometry

\text{MSA}  
Methanesulfonic acid

\text{mtNOS}  
Mitochondrial nitric oxide synthase

\text{MTT}  
3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

\text{NO}_2^-  
Nitro group

\text{NAD}^+  
Nicotinamide adenine dinucleotide phosphate

\text{NADPH}  
Reduced form of Nicotinamide adenine dinucleotide phosphate

\text{NaHCO}_3  
Sodium bicarbonate

\text{NaOH}  
Sodium hydroxide

\text{nNOS}  
Neuronal nitric oxide synthase

\text{NO}^\cdot  
Nitric oxide

\text{NO}_2^\cdot  
Nitrogen dioxide radical

\text{NOS}  
Nitric oxide synthase

\text{NOx}  
NADPH oxidase

\text{nH}_2\text{O}  
Nano pure water
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Conference Presentations arising from this Thesis

Oral Presentations
P.E. Parikh, C.L. Hawkins and M.J. Davies
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20th Annual Meeting of the Society for Free Radical Research Australasia
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P.E. Parikh, C.L. Hawkins and M.J. Davies
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P.E. Parikh, C.L. Hawkins and M.J. Davies
Mechanisms and biological consequences of damage to arterial endothelial cells by peroxynitrous acid.
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San Antonio, Texas, USA, November 2013.

P.E. Parikh, C.L. Hawkins and M.J. Davies
Mechanisms and biological consequences of damage to arterial endothelial cells by peroxynitrous acid.
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Sydney, Australia, September 2013
Chapter 1: Introduction
1.1 Oxidative Stress

Under physiological conditions, cells generate oxidants within certain limits in order to maintain cellular homeostasis. Low levels of oxidants are involved in cell growth, cell stress adaptation, promotion of injury responses, modification of cellular phenotype and regulation of signal transduction (1, 2). Growth factors stimulate oxidant generation to modulate vital proliferative responses. Phagocytic cells generate oxidants as part of their defence mechanism to eradicate pathogens (3). Oxidant formation is balanced by the antioxidant defence systems. This balance is not always maintained in cells resulting in oxidant-mediated damage, which needs to be either repaired (e.g. DNA) or replaced (e.g. majority of oxidised proteins) (4).

Oxidants can be classified into two categories that include reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS are a family of molecules that consist of derivatives of molecular oxygen formed by partial reduction. ROS consist of two groups, which include free radicals and molecular oxidants. Free radicals include the superoxide radical anion (O$_2^{-}$), hydroxyl (HO$^•$), peroxyl (ROO$^•$), alkoxyl (RO$^•$) and hydroperoxyl (HO$_2$O$^•$) radicals. ROS molecules that are not free radicals include hydrogen peroxide (H$_2$O$_2$), ozone (O$_3$), hypochlorous acid (HOCI), hypothiocyanous acid (HOSCN) and singlet oxygen (1$O_2^•$). RNS are nitrogen-containing derivatives such as nitric oxide (NO$^•$), peroxynitrous acid (ONOOH), peroxynitrous anion (ONOO$^-$) and nitrogen dioxide radicals (NO$_2$$^•$) (5).

Under pathophysiological conditions, excessive or misplaced generation of ROS and RNS can occur, which induces oxidative and nitrosative stress respectively (2). These processes damage critical biological components such as DNA, proteins, carbohydrates and lipids. High levels of oxidants alter cell function, promote tissue injury, aging and can induce cell death (1, 4). Accumulating evidence suggests that excessive oxidative or nitrosative stress is associated with pathologies such as atherosclerosis, cancer, diabetes, hypercholesterolemia, hypertension, neurodegenerative disorders and congestive heart failure amongst others (4, 6, 7). Various sources and consequences of oxidant formation are shown in Figure 1.1.
1.2 Major Biological Oxidants

1.2.1 Nitric oxide

Nitric oxide (NO\textsuperscript{--}) is a free radical gas that is synthesised in biological systems by nitric oxide synthase (NOS) enzymes. NOS enzymes catalyse the reaction of oxygen and arginine to produce NO\textsuperscript{--} and citrulline. Since NO\textsuperscript{--} is hydrophobic, it can easily pass through cell membranes and can diffuse several cell diameters from its site of synthesis (8). NO\textsuperscript{--} is used as a cellular messenger and controls vascular tone and neuronal signalling (9). NO\textsuperscript{--} can also be involved in smooth muscle cell proliferation, inhibition of platelet adherence and aggregation (10) and inhibition of oxidation (11). In vivo, haemoglobin, myoglobin, myeloperoxidase and other peroxides catalyse the degradation of NO\textsuperscript{--} to nitrate (12, 13).

Under inflammatory conditions, the concentrations of NO\textsuperscript{--} can be increased and this can result in apoptosis and cell death. NO\textsuperscript{--} mediated apoptosis has been postulated to be...
involved in endothelial dysfunction and atherosclerosis (14). Elevated NO\(^\cdot\) generation within endothelial cells causes increased ROS generation, ONOOH formation, mitochondrial DNA and membrane damage and inhibition of mitochondrial protein synthesis. Cell death can occur via either mitochondrial damage or mitochondrial-independent mechanisms mediated by ONOOH (15).

1.2.2 Superoxide radical anion

Cellular metabolism generates superoxide (O\(_2\)^\(-\)) as a by-product (16). Intracellular O\(_2\)^\(-\) is generated from the mitochondrial, endoplasmic reticulum, and nuclear membrane electron transport chains and proteins such as haemoglobin, aldehyde oxidase and xanthine oxidase (17). Extracellular sources of O\(_2\)^\(-\) include phagocytic cells, endothelial cells and smooth muscle cells (18). Activated phagocytes such as leukocytes, neutrophils, eosinophils and macrophages generate O\(_2\)^\(-\) to kill invading viruses and bacteria (19). It is thought that lymphocytes and fibroblasts also generate O\(_2\)^\(-\), which participate in cellular signalling and growth regulation (20-22).

Intracellular O\(_2\)^\(-\) concentrations are kept low (10-100 nM) due to the action of superoxide dismutase (SOD), the major scavenger of O\(_2\)^\(-\). SOD catalyses the dismutation of O\(_2\)^\(-\) to H\(_2\)O\(_2\) (rate constant \(k_2 = 2 \times 10^9 \text{ M}^{-1}\text{S}^{-1}\)) (23). Therefore the high intracellular concentrations of SOD (5-10 \(\mu\)M) keep intracellular O\(_2\)^\(-\) concentrations low (24). O\(_2\)^\(-\) formation is increased by pathologies such as hyperoxia (25), xenobiotic metabolism (26), inflammation (27) and ischemia/reperfusion (28). When excessive O\(_2\)^\(-\) formation occurs, this oxidant, or species downstream of this, can induce cell injury, tissue damage and inflammation (29).

1.2.3 Hydroxyl radical

Hydroxyl radicals (HO\(^\cdot\)) are highly reactive oxygen-centred radicals. Due to its high reactivity, HO\(^\cdot\) are considered cytotoxic to plants, animals and microorganisms living in the presence of oxygen (30). Ionising radiation dissociates the O-H bonds of water molecules to form HO\(^\cdot\) and hydrogen atoms H\(^\cdot\) (31). HO\(^\cdot\) can also be generated via the Fenton reaction where H\(_2\)O\(_2\) reacts with iron (II) (Fe\(^{2+}\)) to form hydroxide (OH\(^-\)) and HO\(^\cdot\) (32). O\(_2\)^\(-\) can act as a reducing agent for the resulting iron (III) (Fe\(^{3+}\)) producing a catalytic process (33). HO\(^\cdot\) reacts unspecifically with almost every biomolecule including
proteins, carbohydrates, DNA and lipids at diffusion-controlled rates. When HO’ are generated, these radicals therefore react with any molecules that are located less than a few nanometers from its site of production due to this high reactivity, and they cannot migrate significant distances (31, 34). Excessive HO’ are believed to be generated under numerous stress conditions and are implicated in various cellular disorders including inflammation (35), embryo teratogenesis (36), herbicide effects (37), cell death (38) and eradication of microorganisms (39).

1.2.4 Hydrogen peroxide

Hydrogen peroxide (H₂O₂) can easily diffuse both intra- and intercellularly. The majority of O₂− generated in vivo undergoes either spontaneous or catalysed dismutation by SOD to form H₂O₂. Apart from these processes, H₂O₂ is also generated in vivo by the action of numerous oxidase enzymes such as amino acid oxidases and xanthine oxidase (16, 40). The thyroid glands produce tyrosine-based hormones such as triiodothyronine and its prohormone thyroxine that are involved in the regulation of metabolism. Iodine is crucial for the generation of these hormones. Under physiological conditions, H₂O₂ is generated in the thyroid gland so that it can used by peroxidase enzymes to iodinate (a process whereby iodine is added) the thyroid hormones (41). H₂O₂ is also an important substrate for other peroxidase enzymes, including myeloperoxidase (42).

1.2.5 Hypochlorous acid

Hypochlorous acid (HOCl) is a highly reactive species that can react with many molecules such as DNA, proteins and lipids (43). HOCl is involved in both oxidation and chlorination reactions. Stimulated neutrophils produce O₂•− and H₂O₂ and release myeloperoxidase (a haem enzyme) from intracellular granules (44). Monocytes and some macrophages also express myeloperoxidase (45). Myeloperoxidase catalyses the generation of HOCl from H₂O₂ and chloride ions. This enzyme also oxidises peroxidase substrates to radical intermediates (46). Under most conditions, HOCl is the major oxidant produced by neutrophils to kill a wide range of invading pathogens (42). HOCl formation has also been detected in numerous pathologies such as kidney disease (47) and human atherosclerosis (48).
1.2.6 Hypothiocyanous acid

Human peroxidases including myeloperoxidase (49), lactoperoxidase (50), eosinophil peroxidase (51) and thyroid peroxidase (52) can catalyse the reaction of thiocyanate (SCN⁻) with H₂O₂ to generate hypothiocyanous acid (HOSCN). In human plasma, SCN⁻ is normally present at low micromolar levels (10-50 µM) (53) while in heavy smokers significantly elevated levels (up to 250 µM) of SCN⁻ have been detected (54). HOSCN has potent antibacterial properties, which enables it to play a crucial role in the human immune system (55, 56). HOSCN mainly reacts with low molecular mass and protein thiol residues (57). Under inflammatory conditions, the immune cells generate a substantial amount of HOSCN (53). SCN⁻ may act as a protective agent in cystic fibrosis and cardiovascular diseases by diverting myeloperoxidase from HOCl to HOSCN formation (53).

1.2.7 Peroxynitrous acid

O₂⁻ and NO’ react spontaneously with each other at near the diffusion-controlled limit (ca. 10⁻¹⁰ M⁻¹ s⁻¹) (58, 59) to form the peroxynitrous anion (ONOO⁻) (Scheme 1.1) (60). ONOO⁻ is present in equilibrium with peroxynitrous acid (ONOOH) (pKₐ = 6.8 at 37 °C) (61, 62). ONOOH is a potent oxidant capable of oxidising various biological targets (63, 64). The term peroxynitrite is often used to refer to the sum of ONOO⁻ and ONOOH.

\[ \cdot \text{NO} + \text{O}_2\cdot^- \rightarrow \text{ONOO}^- \]

Scheme 1.1 Reaction of nitric oxide with superoxide radicals to form peroxynitrous anion.

ONOO⁻ is a relatively stable species at high pH. At physiological pH, ONOO⁻ has a very short half-life (about 10 ms) as it undergoes protonation to generate ONOOH, which decomposes rapidly. ONOOH is not a free radical, as it has no unpaired electrons but is far more reactive than its precursors. ONOOH also undergoes homolysis and rearrangement reaction to form NO₂⁺, HO’ and nitrate anion (Scheme 1.2) (62). These radicals are also powerful oxidants capable of inducing molecular and tissue damage and enhancing oxidative stress (65).
Kinetic studies have suggested that ONOOH reacts with target molecules via two possible routes (63). First, ONOO' or ONOOH react directly with their targets via an overall second order process. An example of this process includes thiol oxidation (63). In the alternative route, ONOOH first decomposes to form NO_2' and HO', which then react with their targets (63). In the latter process, the generation of radicals is rate limiting. Consequently, the latter process is first order in ONOOH and zero order in target. For example, this is the case of tyrosine nitration and lipid peroxidation (64, 66, 67).

NO' is a neutral and hydrophobic species that can pass through cell membranes whereas O_2'^- is in an anionic form at neutral pH. Consequently ONOOH is mostly generated at the sites of O_2'^- formation. ONOOH can traverse cell membranes by passive diffusion via anion channels (Figure 1.2) and react with many crucial biomolecules (68). Local pH and the microenvironment affect the reactions of ONOOH. Hydrophobic membrane compartments appear to favour ONOOH-mediated nitration while aqueous environments favour ONOOH-mediated oxidation (69).

![Figure 1.2 Cellular diffusion of superoxide, nitric oxide and ONOOH in a model blood vessel system](image)

**Figure 1.2** Cellular diffusion of superoxide, nitric oxide and ONOOH in a model blood vessel system [taken from (2)].
1.3 Peroxynitrous acid formation in vivo

ONOOh formation depletes NO\(^{-}\), a potentially cardioprotective molecule. ONOOH has been implicated as a toxic chemical that causes tissue injury. However it has also been proposed to act as a protective agent that improves cellular and organ function (70). Therefore the balance of these contrasting reactions can be shifted towards either injury or protection depending on the availability of substrates (71).

Under physiological conditions, low levels of ONOOH are generated and may have a protective effect. Phagocytes such as macrophages, monocytes and neutrophils form ONOOH as a bactericidal agent (72). In human coronary artery endothelial cells, sublethal concentrations of ONOOH are reported to maintain redox homeostasis by activating the nuclear factor erythroid 2-related factor 2 (NRF2). NRF2 is a transcription factor and an important regulator of cell homeostasis, which upregulates the expression of enzymes involved in ROS detoxification. Exposure to ONOOH results in the overexpression of NRF2-regulated genes and induces autophagy, a lysosomal degeneration pathway important for cell survival, differentiation, development and homeostasis (73). In addition, low concentrations of ONOOH stimulate extracellular signal-regulated kinase phosphorylation and protein kinase c. These proteins are key mediators of cell proliferation (74).

During pathologies such as inflammation, excessive NO\(^{-}\) and O\(_2\)\(^{-}\) production occurs resulting in excessive ONOOH formation. High concentrations of ONOOH are cytotoxic as a result of damage to DNA, proteins and lipids via multiple reaction pathways (69). There is accumulating evidence to suggest that ONOOH formation is involved in numerous pathologies including atherosclerosis, myocardial infarction, chronic heart failure, diabetes, cancer and neurodegenerative disorders such as Alzheimer’s disease and Parkinson’s disease (2). Therefore investigation into the biological effects of ONOOH is an important area of research with potentially wide-ranging clinical implications.
1.4 Biological sources of peroxynitrous acid

Within biological systems ONOOH is generated via multiple pathways. Two important sources include endothelial cells and phagocytes. The following subsections discuss how these cell types generate ONOOH.

1.4.1 Endothelial cells

The healthy endothelium has anticoagulant, antiplatelet and fibrinolytic characteristics and modulates vascular tone by the release of either dilator or constrictor molecules (75). The endothelium also regulates vascular homeostasis, inhibits and activates proliferation and migration of smooth muscle cells and controls thrombogenesis and fibrinolysis. The endothelial cells preserve the balance between vasodilation and vasoconstriction. When this balance is disturbed, endothelial dysfunction occurs leading to damage to the arterial wall (75). In healthy coronary arteries endothelium dependent vasodilation is a critical regulator of blood pressure, however during endothelial dysfunction, vasodilation decreases and vasoconstriction increases. NO’ is a critical molecule that mediates vasoprotective effects such as vasodilation and inhibition of smooth muscle cell growth (76).

NOS enzymes catalyse the oxidation of L-arginine to L-citrulline forming NO’ and citrulline. In humans, NOS enzymes exist in different isoforms including constitutive [e.g. endothelial (eNOS) (77), neuronal (nNOS) (78, 79), mitochondrial (mtNOS) (80)] and inducible (iNOS) (81). All of these isoforms generate NO’. The constitutive forms of NOS are believed to produce pico- to nano-molar levels of NO’, while the inducible form of NOS produces micromolar concentrations (10). Low levels of NO’ production by the constitutive isoforms of NOS are considered to have beneficial effects since NO’ is a major regulator of vascular biology (10).

In normal blood vessels eNOS is constantly expressed whereas in atherosclerotic lesions eNOS expression is downregulated while iNOS expression is upregulated. Overexpression of eNOS has been shown to accelerate lesion formation (82). Extensive production of NO’ by iNOS has been detected in early and advanced lesions (83). Inflammatory cells such as macrophages and T lymphocytes in lesions have been shown
to produce NO’ through iNOS. NO’ produced by iNOS has been associated with cellular damage, inflammation and apoptosis (83).

NOS are catalytically active only in their dimeric form. A zinc-thiolate centre is present at the dimer interface to stabilise this enzyme and is crucial for this enzymes activity. ONOOH oxidises the zinc-thiolate centre and releases zinc (84). As a result, the eNOS dimer is disrupted and the enzyme becomes uncoupled. This results in the production of O$_2^{-}$ rather than NO’ by eNOS. This latter event contributes to oxidative stress in endothelial cells and has been associated with cellular damage, inflammation and apoptosis (85).

Vascular endothelial cells also express various isoforms of NADPH oxidase (NOx), a membrane bound enzyme complex. NOx transfers electrons from NADPH to molecular oxygen and during this process O$_2^{-}$ is generated. Depending on the subcellular location of NOx, O$_2^{-}$ is generated either inside organelles or extracellularly (86). Apart from NOx, the mitochondrial respiratory chain of endothelial cells also generates O$_2^{-}$ via partial reduction of oxygen inside the mitochondrial matrix due to leakage of electrons from the respiratory chains (87). NO’ and O$_2^{-}$ production within endothelial cells causes intracellular ONOOH formation.

1.4.2 Phagocytes

Activated macrophages eradicate microorganisms and remove tumour cells and damaged tissue at sites of inflammation. Macrophages express NOS and NOx enzymes, which generate NO’ and O$_2^{-}$ respectively and the resulting ONOOH is used to kill invading pathogens (72). Ischiropoulos and colleagues discovered that freshly isolated rat alveolar macrophages simultaneously generate both NO’ and O$_2^{-}$ radicals (88). The rate of reaction between NO’ and O$_2^{-}$ is more than 3.5 times faster than that between O$_2^{-}$ and SOD facilitating the production of ONOOH (89). Thus although SOD is present within macrophages at high concentrations, this enzyme fails to effectively scavenge all of the O$_2^{-}$ formed (2).

Monocytes are a type of white blood cell that is involved in the innate immune system. A key role of monocytes is replenishing macrophages and dendritic cells under physiological conditions (90). In the presence of inflammatory signals, monocytes
diffuse rapidly to sites of infection, where they differentiate into macrophages and dendritic cells and induce an immune response. Numerous cytokines and vasoactive hormones activate monocytes to generate NO$^\cdot$ by increasing the expression of iNOS (91). Upon stimulation, monocytes also undergo a respiratory burst and release O$_2^{\cdot-}$ and H$_2$O$_2$ (92). It has been shown that activated monocytes can generate RNS via reaction of O$_2^{\cdot-}$ and NO$^\cdot$, which nitrate the tyrosine residues of apoliprotein B-100 and trigger lipid peroxidation in LDL particles (93).

Neutrophils (polymorphonuclear leukocytes) are the most abundant white blood cells in mammals. They play an important role in the innate immune responses and are known to adhere to vessel walls, undergo emigration and chemotaxis at infection foci and ingest and digest microbes (94). It has been reported that neutrophils generate ONOOH (95). Under inflammatory conditions, neutrophils are activated by inflammatory markers such as interleukin 1, tumor necrosis factor alpha and interferon gamma, which increase generation of NO$^\cdot$ and O$_2^{\cdot-}$, the precursors of ONOOH (96, 97). Low concentrations of ONOOH cause increased neutrophil adhesion to targets, increased cell spreading on its substrate and enhancement of their capability to eradicate microbes (98). The next Section discusses the cellular reactivity of ONOOH.

1.5 Cellular Reactivity of ONOOH

Within biological systems ONOOH reacts with carbon dioxide (CO$_2$), low molecular mass antioxidants, nucleic acids, proteins, carbohydrates and lipids. Based on kinetic rate constants and the cellular concentration of targets, the proposed fate of ONOOH in the cytosol of cells is shown in Figure 1.3. The assumed concentrations and rate constants are carbon dioxide 1.5 mM, $k = 4.6 \times 10^4$ M$^{-1}$s$^{-1}$; ascorbate 0.5 mM, $k = 1 \times 10^2$ M$^{-1}$s$^{-1}$; glutathione 10 mM, $k = 1.35 \times 10^3$ M$^{-1}$s$^{-1}$; uric acid 0.1 mM, $k = 4.7 \times 10^2$ M$^{-1}$s$^{-1}$; proteins 15 mM, $k = 5 \times 10^3$ M$^{-1}$s$^{-1}$; metal- and selenium containing proteins 0.5 mM, $k = 1 \times 10^5$ M$^{-1}$s$^{-1}$ (63). It is clear from these calculations that proteins are a major target of ONOOH in the cell cytosol followed by CO$_2$ and low molecular mass antioxidants. The following subsections discuss how ONOOH is believed to react with these biological components.
1.5.1 Carbon dioxide

The bicarbonate anion (HCO$_3^-$) is a highly abundant component of the extracellular fluid. HCO$_3^-$ is present at ca. 25 mM in human blood plasma and 12 mM in cytosolic fluids (72) and is present in equilibrium with CO$_2$. The normal plasma concentration of CO$_2$ is therefore about 1.3 mM. In blood the HCO$_3^-$/CO$_2$ system is one of the key pH buffering systems (99).

An important reaction in biological systems involves ONOO$^-$ reacting rapidly with CO$_2$ to form nitrosoperoxycarbonate anion adduct (ONOOCO$_2^-$) (Scheme 1.2), which has an estimated lifetime of less than 1 µs. The second order rate constant for the formation of this species is $4.6 \times 10^4$ M$^{-1}$s$^{-1}$ at pH 7.4 and 37 °C. This is one of the fastest known and most biologically relevant reaction for ONOOH (100). ONOOCO$_2^-$ decomposes rapidly into nitrogen dioxide radicals (NO$_2^-$), carbonate radicals (CO$_3$^-) and other reactive derivatives. NO$_2^-$ and CO$_3$^- are strong one-electron oxidants (63, 101). The HCO$_3^-$ present in plasma increases the one-electron oxidation of cysteine, tryptophan and tyrosine residues (102-105) and activates the nitration of tyrosine and tryptophan (66, 106).

Figure 1.3 Biological targets of ONOOH in cytosol based on kinetic rate constants and concentration factors [taken from (63)].
Apart from ONOOH and ONOOCO$_2^-$, the radicals generated directly via homolysis of these species are oxidising and nitrating agents. Despite over two decades of research, it is still unclear as to whether ONOOH or ONOOCO$_2^-$ is the most damaging species in vivo. As a result, it is crucial to investigate the involvement of HCO$_3^-$/CO$_2$ in ONOOH-mediated reactions. It has been postulated that radicals generated directly via ONOOH homolysis are thiol oxidising agents while the radicals generated via ONOOCO$_2^-$ are nitrating agents (107).

Scheme 1.2 Multiple reaction pathways of ONOOH.

1.5.2 Low molecular mass antioxidants

Low molecular mass antioxidants participate in the defence mechanisms of cells and organisms (108). ONOOH reacts with low molecular mass antioxidants such as glutathione (GSH), which is a ubiquitous thiol-containing tripeptide (glutamate-cysteine-glycine) found in every cell type and is present in the cytosol, nucleus, endoplasmic reticulum and mitochondria (109). GSH is a major contributor to cellular redox systems (109). ONOOH oxidises the thiol group of GSH via an oxygen consumption pathway with a rate constant, $k$, of 1350 M$^{-1}$s$^{-1}$ at pH 7.4 and 37 °C. Although the rate constant for the reaction between GSH and ONOOH is not especially high (62), the high intracellular concentration of GSH makes this an efficient scavenger of ONOOH (110). Therefore GSH is believed to play a pivotal role in cellular defence against this potent oxidant (111).
The reaction of ONOO\(^{-}\) with protonated thiol residues is postulated to be a second-order process, which occurs via two-electron oxidation and forms an unstable sulphenic acid (RSOH) (Scheme 1.3a) (103). Subsequently ROSH reacts with another thiol residue to form a disulphide (RSSR). Radicals from ONOOH such as HO\(^{•}\) and NO\(_2^{•}\) react with thiols to form RS\(^{•}\) (Scheme 1.3b) (103). RS\(^{•}\) can dimerise to generate RSSR or can react with oxygen to form a peroxyl radical. In addition, RS\(^{•}\) can also react with a thiolate (RS\(^{-}\)) to form disulphide radical anion (RSSR\(^{•−}\)) which in turn can react with oxygen to form RSSR and O\(_2^{•−}\) (103).

Intracellular GSH levels can determine how susceptible cells are to ONOOH-mediated damage. It has been suggested that in endothelial cells, macrophages and SMC, oxidative stress occurs due to depletion of intracellular reduced GSH (112), with depletion of GSH increasing ONOOH-mediated tissue injury and toxicity.

### Scheme 1.3 ONOOH-mediated thiol oxidation pathways [taken from (103)].

#### 1.5.3 Nucleic acids

ONOOH can induce multiple DNA base modifications such as nitration, hydroxylation and deamination, and also induce DNA strand breakage (113). These modifications can culminate in cell death by either apoptosis or necrosis. As ONOOH is intrinsically more reactive than NO\(^{•}\), ONOOH is at least 100 times more potent compared to NO\(^{•}\) in inducing DNA strand breakage (113). ONOOH or radicals generated from it, can modify
the sugar phosphate backbone of DNA by removing a hydrogen atom from the deoxyribose moiety causing the opening of sugar rings and formation of DNA strand breaks (114, 115).

ONO OH can diffuse across cells, reach the nucleus and oxidatively modify the nucleobases and sugar phosphate backbone of DNA (114, 115). Out of the four nucleobases, guanine has the lowest oxidation potential, hence is the most reactive with ONOOH (116). Oxidation of guanine produces 8-oxoguanine (115) and causes guanine fragmentation. 8-oxoguanine is a stable product, which is used as a biomarker for detecting DNA damage and oxidative stress. Apart from ONOOH, other oxidants can also form 8-oxoguanine (114, 115). ONOOH can also nitrate guanine to form 8-nitroguanine, which induces the generation of abasic sites that can be cleaved by endonucleases to produce DNA single strand breaks (114-116). Both 8-oxoguanine and 8-nitroguanine can also react further with ONOOH, though this is unlikely to be a major reaction in vivo.

1.5.4 Proteins

ONO OH reacts with proteins via three different pathways (63). First ONOOH reacts rapidly with sulphur-containing amino acids such as cysteine and methionine. Secondly, ONOOH reacts rapidly with prosthetic groups, in particular transition metal centres (63). Thirdly, radicals derived from ONOOH such as HO’, CO3⁻ and NO2⁻ can react with amino acids, with the most susceptible to oxidation being those that contain sulphur (e.g. cysteine and methionine) and aromatic rings (e.g. tyrosine, tryptophan, histidine and phenylalanine) (63). Because these amino acids have the lowest oxidation potentials, they tend to react with the higher rate constants. Other amino acids and peptide bonds can also be a target for these radicals, but these reactions are less rapid (63).

Literature data indicates that the major modifications induced by ONOOH to proteins include thiol oxidation and tyrosine nitration (117). Other protein modifications induced by ONOOH include tryptophan oxidation and nitration, methionine oxidation and formation of dityrosine and carbonyl groups. High concentrations of ONOOH are also known to cause protein aggregation, fragmentation and crosslinking (118, 119). The following subsections discuss how ONOOH modifies various amino acids present in proteins.
1.5.4.1 Cysteine

Cysteine is the most easily oxidised amino acid and reacts the fastest with ONOOH. As a result, cysteine residues are one of the major targets of ONOOH in proteins. ONOOH oxidises cysteine residues to cystine by targeting the thiol groups (RSH) with this causing the formation of covalent crosslinks between peptide chains (120). Thiols are present at the active site of various enzymes and the oxidised form of cysteine (cystine) helps maintain the native confirmation of many proteins (61).

Modification of reactive cysteine residues can change protein structure and function as well as enzymatic activity (121). The pKₐ of the majority of thiols present in proteins is about 8.5, as a result they tend to be less reactive at intracellular pH. Due to their local environment, some thiols have lower pKₐ and are present as thiolate anions (RS⁻) at physiological pH, which are more reactive than the neutral form. Consequently RS⁻ are more easily oxidised than neutral thiols and react with ROS/RNS to form sulphoxidation products such as sulphenic, sulphinic and sulphonic acids, inter- and intraprotein disulphides or nitrosothiols (122).

Oxidation of thiol residues by ONOOH is rapid and typically occurs a thousand times faster (in terms of rate constant) than with H₂O₂ (103). Thus the second order rate constant for the reaction of ONOOH with cysteine, glutathione and the single thiol of albumin are 4500 (61), 1350 (62) and 2700 (61) M⁻¹s⁻¹ at pH 7.4 and 37 °C respectively. Rate constants of up to 10⁷ M⁻¹s⁻¹ have been reported for the reaction of ONOOH with the highly reactive RS⁻ of peroxiredoxins, glyceraldehyde 3-phosphate dehydrogenase, creatine kinase and tyrosine phosphatase (63).

ONOOH mediated oxidation of thiols is believed to have cytotoxic effects. Firstly, ONOOH mediates oxidation of low molecular mass thiols such as cysteine and GSH. This process depletes crucial intra- and extracellular scavenging mechanisms that act as a defence against oxidative damage. As a result, important macromolecules such as DNA, enzymes, structural proteins, polysaccharides and membrane phospholipids are more susceptible to modification and destruction. Secondly, ONOOH oxidises protein thiols and sulphur containing cofactors such as coenzyme A, lipoic acid, thioredoxin and phosphopantetheine. This latter process can disturb metabolic pathways and membrane
associated functions, which play a crucial role in important metabolic and biosynthetic pathways (61).

Oxidation of cysteine residues by ONOOH inactivates numerous enzymes which have been implicated in cellular energetic processes such as glyceraldehyde-3-phosphate dehydrogenase (123), creatine kinase (124), NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochrome c reductase (complex III) and ATP synthase (complex V) of the mitochondrial respiratory chains (125, 126). In contrast, in some cases, oxidation of cysteine residues of an enzyme can induce enzyme activation, such as the activation of matrix metalloproteinases as part of ONOOH-mediated toxicity in heart disease (127).

At physiological pH, ONOO\(^-\) reacts with thiol residues to form small amounts of S-nitrosothiols (128). It has also been reported that ONOO\(^-\) reacts with thiols at acidic pH to form the corresponding S-nitrosothiols. The yield of S-nitrosothiols was strongly pH-dependent (129). S-nitrosothiols are suggested to be intermediates in signal transduction and a slow carrier of NO\(^-\) (130). S-nitrosothiols can release NO\(^-\) via site-specific regulatory mechanisms, which can occur through reaction with thioredoxin and O\(_2\)\(^-\) (131, 132). S-nitrosothiols generation may provide a buffering system, which can modulate the bioavailability of NO\(^-\) (133). S-nitrosothiols may modulate the activity of various enzymes and has been implicated in redox signalling (134-136).

1.5.4.2 Tyrosine

Tyrosine residues comprise 3-4 mol % of the total amino acids in proteins. The amphipathic phenol side chains can interact with water, undergo hydrogen bond formation and participate in cation-π and nonpolar interactions (137). Due to the phenolic functionality, tyrosine residues can accept phosphate groups as a result of the action of protein tyrosine kinases, and be involved in electron transfer processes via tyrosyl radical formation \((E^{\prime \prime} = +0.94 \, V)\) (138). Within a protein, both solvent exposed (5 %) and buried (95 %) tyrosine residues coexist (139).

There is no direct reaction of tyrosine with ONOOH. However ONOOH-derived secondary radicals such as HO\(^-\), CO\(_3\)\(^-\), oxo-metal complexes, lipid peroxyl radicals and NO\(_2\)\(^-\) can react with tyrosine to form 3-nitrotyrosine (3NT) (107, 140, 141). 3NT
formation occurs via a two-step radical process whereby a one-electron oxidant such as CO$_3^{3-}$ abstracts a hydrogen atom from tyrosine residues to generate tyrosyl radicals (Tyr$^*$) which then react with NO$_2^*$ at a near diffusion controlled rate to form 3NT (Scheme 1.4). In addition to ONOOH, myeloperoxidase can also generate 3NT (142), therefore 3NT formation is not a specific biomarker for ONOOH formation in vivo.

\[
\text{TyrH} + \text{CO}_3^{3-} \rightarrow \text{Tyr}^* + \text{HCO}_3^{-}
\]

\[
\text{Tyr}^* + \text{NO}_2^* \rightarrow \text{Tyr-NO}_2
\]

(3-nitrotyrosine)

Scheme 1.4 3NT formation via ONOOH derived radicals.

The biological half-life of NO$_2^*$ has been estimated to be greater than 100 ms favouring its combination reaction with Tyr$^*$ (143). CO$_2$ has been reported to act as a catalyst in ONOOH-mediated protein tyrosine nitration, and to enhance nitration yields (106). These reactions are also catalysed by low molecular mass metal ion catalysts e.g. Fe$^{3+}$-EDTA and metal containing enzymes e.g. Cu, Zn SOD (140).

In cells under basal physiological conditions, protein tyrosine nitration appears to occur. Immunochemical and proteomic-based studies suggest that tyrosine nitration is a selective process in vitro and in vivo, with not all tyrosine residues in a protein nitrated, and not all proteins nitrated (143). Protein tyrosine nitration appears to occur preferentially on a subset of proteins and within those proteins, generally one or two tyrosine residues are modified. This selectivity depends on the nature and site(s) of oxidising or nitrating species generation, the physiochemical properties of the microenvironment and the structural features of the protein (143). In addition nitration appears to depend on the exposure of the aromatic ring to the surface of the protein, the position of the tyrosine residue in a loop structure and possible interactions with nearby negative charges (144).

Addition of a nitro group (-NO$_2$) on carbon 3 of tyrosine residues causes significant structural and functional changes, which can alter protein function as well as cell and tissue homeostasis. The nitration of tyrosine residues can disrupt NO$^*$ signalling.
pathways and change important properties of the amino acid such as phenol group $pK_a$, redox potential, hydrophobicity and volume (143). A significant reduction in the $pK_a$ of the $-\text{OH}$ group occurs (from 10.0-10.3 to 7.2-7.5 for free tyrosine and 3NT in water respectively) (145). The nitro group is a bulky and hydrophobic substituent that might form local steric hindrances, activate conformational changes and disrupt tyrosine phosphorylation. The nitrotyrosine/nitrotyrosyl radical couple have $E'_o = 200 – 300 \text{ mV}$, which is more positive than that of tyrosine/tyrosyl radical. Consequently tyrosine nitration may change tyrosine-dependent intramolecular electron transfer processes in proteins (146).

Possible biochemical outcomes of tyrosine nitration include changes in protein activity (either loss- or gain-of-function), facilitation of immunogenic responses, hindrance of tyrosine kinase dependent pathways, changes to protein assembly and polymerisation, activation of protein degradation and involvement in the generation of proteasome-resistant protein aggregates (145). Tyrosine phosphorylation is a crucial event because activation of tyrosine kinases occurs at the beginning of many cell-signalling pathways, and is also critical in the control of cell cycles. Nitration of important tyrosine residues by ONOOH can inhibit phosphorylation of proteins involved in cell signalling pathways (147). A fundamental question has been how changes to biological properties of nitrated proteins are associated with pathophysiological conditions (143).

In conditions that significantly increase oxidant and NO$^+$ formation such as inflammation, protein 3NT formation can be enhanced several fold (143). For over two decades, 3NT has been used as an established biomarker of ONOOH-mediated cell, tissue and systemic “nitrooxidative stress”. Nitrated proteins have been detected in at least 50 human and 80 animal diseases (2). Thus 3NT formation has been detected in human coronary artery atherosclerotic lesions (148), neonatal lung injury (149), multiple sclerosis (150), endotoxemia, ischemic lung injury and in inflammatory cell associated diseases (2). Free plasma 3NT has also been used as a biomarker of oxidative stress and inflammation. In normal healthy human plasma free 3NT could not be detected, however high 3NT levels has been detected in patients during renal failure, chronic smoking, sepsis and in atherosclerotic plaques (151-153). These pathologies are also linked with significant endothelial dysfunction.
Furthermore, incubation of rat thoracic aorta segments with high concentrations of free 3NT (100 and 250 µM) that are believed to be clinically relevant has been shown to cause three- to four-fold increases in TUNEL-positive endothelial nuclei compared to respective controls (154). This study clearly indicates that free 3NT can induce selective endothelial dysfunction via DNA damage and/or apoptosis.

1.5.4.3 Tryptophan

Nitration of tryptophan residues by ONOOH occurs via tryptophanyl radical formation (105) and the products generated include 1-, 2-, 4-, 5-, 6- and 7- nitrotryptophan (155-158) (Scheme 1.5). NO$^\cdot$ can add onto the 1-nitrogen site to form 1-nitrosotryptophan (156) via a process known as nitrosation (157). Apart from nitroso and nitration products, multiple oxidised species are also generated when ONOOH reacts with tryptophan residues, including kynurenine (159), N-formylkynurenine (66), hydropyrroloindole, oxindole-3-alanine (160), hydroxytryptophan and dihydroxytryptophan (66) (Scheme 1.5).

Reaction of human copper-zinc SOD (CuZnSOD) with ONOOH in the presence of sodium bicarbonate caused loss of a single tryptophan residue (Trp32) and a significant loss (30%) of enzymatic activity. 6-nitrotryptophan was the major product formed (161). 6-nitrotryptophan has also been detected in PC12 cells treated with ONOOH by using an anti-6-nitrotryptophan antibody with many peptides containing 6-nitrotryptophan originating from multiple proteins detected (162). This was the first study which identified multiple nitrated tryptophans present on proteins in PC12 cells exposed to ONOOH and compared the susceptibility of nitration between tyrosine and tryptophan residues.
1.5.4.4 Histidine

Histidine residues are modified by ONOOH via a radical mechanism to form histidinyl radicals. This mechanism is believed to be at least partially responsible for the inactivation of CuZnSOD by ONOOH. A study by Alvarez and coworkers showed that the reaction of ONOOH with human recombinant CuZnSOD caused the formation of a protein radical adduct detectable by electron paramagnetic resonance spin trapping experiment with a second order rate constant of $(9.4 \pm 1.0) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ at 37 °C. The electron paramagnetic resonance signal of the protein radical adduct was consistent with the generation of histidinyl radical. The authors of this study therefore proposed that ONOOH reacts with CuZnSOD to form NO$_2^-$ and copper-bound hydroxyl radical species. The latter species further reacts with histidine residues to generate histidinyl radicals (Scheme 1.6) (164).
1.5.4.5 Methionine

Methionine residues can participate in antioxidant defence mechanisms (165) and modulate cellular functions via reversible oxidation and reduction (166). In methionine the nucleophilic sulphur atom is prone to oxidation by ONOOH. ONOOH reacts with methionine with a second order rate constant of $\sim 10^2 \text{M}^{-1}\text{s}^{-1}$. The products formed from oxidation by ONOOH include methionine sulphoxide and nitrite with lower amounts of ethylene and dimethyl disulphide (167). Methionine sulphoxide formation is not however specific to ONOOH, and other oxidants such as $\text{H}_2\text{O}_2$, $\text{HOCl}$, $\text{HO}^-$, ozone, chloramines and $\text{O}_2^{-}\text{.}$ can also induce methionine oxidation via either direct- or indirect pathways (168).

If methionine oxidation is not repaired by the enzymatic activity of methionine sulphoxide reductases (169, 170), the hydrophobicity and conformation of a protein can be altered. The biological activity of the oxidised proteins can also be lost. ONOOH-mediated oxidation of methionine has been reported in $\alpha$1-antitrypsin inhibitor and glutamine synthetase, resulting in the inactivation of these enzymes in vitro (166, 171).

1.5.4.6 Transition metal ions

The reactions of ONOOH with transition metal centres are some of the fastest known reactions of ONOOH (63). In particular haem and non-haem iron species, copper and manganese ions are known to react with ONOOH, resulting in the formation of a secondary oxidising species at the metal ion centre and NO$_2^-$. If the oxidising species is
formed at a metal ion active site in an enzyme, it may react with neighbouring amino acids and result in a loss of function (63). This is the case for manganese SOD and prostacyclin synthase (172) where reaction of ONOOH with the metal ion centre causes modifications of nearby tyrosine residues.

Hemoglobin and myoglobin are abundant haem proteins in red blood cells and muscles respectively (173). It has been proposed that these proteins serve as a sink for ONOOH. ONOOH oxidises oxyhemoglobin and oxymyoglobin to form methemoglobin and metmyoglobin respectively (174). This process occurs via an intermediate ferryl complex formation (175, 176). It has been proposed that in myoglobin, the haem center protects the globin from being nitrated (177). Due to high abundance of hemoglobin and myoglobin in the blood vessels and muscles (cardiac and skeletal), these proteins could be an important target of ONOOH (177).

1.5.5 Carbohydrates

Glycosaminoglycans (GAG) are linear or branched heteropolysaccharides comprised of repeating disaccharide units. Common GAGs include chondroitin sulphate, dermatan sulphate, heparan sulphate, keratan sulphate, hyaluronic acid and heparin. GAG chains (except heparin and hyaluronic acid) can have their reducing ends linked to the oxygen atom of a serine residue or the nitrogen atom of an asparagine residue of proteins to form proteoglycans (178). These proteoglycans are major components of the extracellular matrix and are involved in cell adhesion and migration and dynamic processes that participate in interactions between the GAG chains and other extracellular matrix components (e.g. laminin, collagen and fibronectin) (178).

ONOOH reacts with carbohydrates such as GAG through a mechanism that is consistent with the formation of HO’ (179, 180). Extensive polymer fragmentation was detected when GAGs were exposed to ONOOH (180). Bolus ONOOH modified hyaluronan, heparin, chondroitin, dermatan and heparan sulphates in a concentration-dependent manner. ONOOH induced cleavage at disaccharide intervals that led to formation of specific polymer fragments. It has been reported that the reaction of ONOOH with ECM (derived from vascular smooth muscle cell line and porcine thoracic aorta) induced the release of ECM GAGs and proteins in a concentration-dependent manner (181). ONOOH-mediated fragmentation was detected when hyaluronan, heparin and
chondroitin, dermanatan and heparan sulphates are exposed to ONOOH derived secondary radicals such as $\text{HO}^\cdot$ and $\text{CO}_3^\cdot$ (182).

1.5.6 Lipids

Lipids are structural components of cell membranes and are pivotal signalling molecules. An important feature of ONOOH-mediated cytotoxicity is its capability to induce lipid peroxidation in membranes (183), liposomes and lipoproteins by removing a hydrogen atom from polyunsaturated fatty acids (PUFA) thereby generating lipid carbon and hydroperoxyl radicals, conjugated dienes and aldehydes in the presence of CO$_2$ (184). The lipid radicals can in turn attack surrounding PUFAs, thereby forming more radicals, which promote radical chain reactions and membrane lipid degeneration. These changes induce membrane permeability and fluidity changes, which can have major biological consequences including cytotoxicity to cells (185).

1.6 Consequences of peroxynitrous acid formation

1.6.1 DNA damage

3-morpholinosydnonimine-$N$-ethylcarbamide (SIN-1) has been widely used to generate ONOOH slowly over an extended period of time (186). Exposure of J774 macrophages and rat aortic smooth muscle cells to either authentic ONOOH or SIN-1 results in DNA strand breakage (113). Treatment of calf thymus DNA and human skin epidermal keratinocytes DNA with ONOOH or SIN-1 showed similar results (187). ONOOH has also been shown to cause DNA fragmentation and apoptosis in rat thymocytes indicating that ONOOH can nick DNA and trigger apoptotic process, which leads to genomic degradation (188). Endogenous components such as GSH and metallothionein (an endogenous protein thiol compound that binds metal ions) may regulate the reactivity of ONOOH and its reactions with DNA. In endothelial cells, GSH decreased ONOOH-mediated DNA strand breakage (189). Metallothionein appears to protect against DNA strand breakage by preferential reactivity with ONOOH (190).

The generation of DNA strand breaks is an important feature of ONOOH-mediated cytotoxicity as it triggers the activation of poly(ADP-ribose) polymerase (PARP) (113, 189). PARP is an abundant nuclear protein that acts as a DNA nick sensor, nucleotide
polymerising and protein-modifying enzyme. Activated PARP binds to DNA breaks and cleaves NAD\(^+\) into ADP-ribose and nicotinamide. ONOOH can overactivate PARP causing rapid depletion of intracellular NAD\(^+\) levels, a slower rate of glycolysis, decreased electron transport chain activity and thereby reduced ATP generation. These events lead to cell dysfunction and cell death via either apoptosis or necrosis (191-194).

Exposure of human endothelial cells to ONOOH dose-dependently suppressed mitochondrial respiration, caused DNA strand breakage and PARP activation. It has also been suggested that PARP activation by ONOOH in these cells is associated with the progression of endothelial dysfunction (195). Therefore ONOOH-mediated PARP activation has been associated with cell injury and cell death in pathologies such as inflammation, shock, pancreatic islet cell destruction, diabetes and neurodegenerative disorders (189).

1.6.2 Mitochondrial damage

Mitochondria are involved in cellular signalling, cellular differentiation, necrosis and apoptosis, calcium homeostasis, and the control of many biosynthetic pathways and modulation of the cell cycle and cell growth. Necrosis and apoptosis, which are two forms of cell death, are closely associated with mitochondrial dysfunction (196). Mitochondrial malfunction has been implicated in many diseases including diabetes, atherosclerosis, ischemic heart diseases, stroke, aging and neurodegenerative diseases (2).

Mitochondria can generate NO\(^\cdot\) through the action of a calcium-sensitive mitochondrial NOS (197, 198). NO\(^\cdot\) can also be produced from the electron transport chain dependent reduction of nitrite (125). O\(_2\)\(^{\cdot-}\) can be generated from the partial reduction of oxygen inside the mitochondrial matrix due to leakage of electrons from the respiratory chains (199). Intramitochondrially generated NO\(^\cdot\) and O\(_2\)\(^{\cdot-}\) can react spontaneously to form ONOOH and it has been proposed that most of intramitochondrially generated ONOOH reacts with targets in close proximity to its site of production (125).

ONOOH generated either in extramitochondrial compartments or within these organelles, can induce oxidative damage to the mitochondria via various mechanisms (2). ONOOH and ONOO\(^-\) generated outside of mitochondria can also diffuse into the mitochondria (68) and scavenging of ONOOH by cytosolic thiols, carbohydrates and metabolic
intermediates appears to protect the mitochondrial respiratory chain from ONOOH-mediated damage (200).

Low concentrations of mitochondrial ONOOH have been postulated to be beneficial and may regulate mitochondrial physiology. For instance, low concentrations of intramitochondrially generated ONOOH may control calcium homeostasis by stimulating a specific calcium release pathway. Oxidation of key thiols by ONOOH appears to activate pyridine nucleotide-mediated calcium release from intact mitochondria with this preventing an overload of mitochondrial calcium levels (201, 202).

ONOOh induced mitochondrial toxicity can arise both from direct oxidative reactions and radical mediated damage (where ONOOH reacts with CO$_2$ to form CO$_3^{2-}$ and NO$_2^-$). The latter pathway appears to be favoured as mitochondria are the main organelles in cells where decarboxylation reactions form CO$_2$(125, 203).

High ONOOH concentrations can induce significant oxidative damage and disrupt mitochondrial function. Exposure of mitochondria to ONOOH leads to extensive protein modification, cross linking and lipid peroxidation (204) and inactivation of enzymes of the electron transport chain such as complex I (NADH dehydrogenase) (205), complex II (succinate dehydrogenase) (206), complex III (cytochrome c reductase) (207) and complex V (ATP synthetase) (Figure 1.4) via thiol oxidation, tyrosine nitration and oxidation of iron sulphur centers (203).

In the inner membranes of mitochondria, oxidative stress, calcium overload, increased phosphate concentrations and depletion of adenine nucleotide can result in opening of the mitochondrial permeability transition pore (MPTP). MPTP opening has two major consequences; firstly the intramitochondrial membrane no longer preserves the barrier to protons, causing dissipation of the proton motive force, uncoupling of oxidative phosphorylation and disruption of mitochondrial ATP formation. Secondly, opening of the MPTP allows the free passage of protons, cofactors and ions into and out of the mitochondria. This leads to disturbances in metabolic gradients between mitochondria and the cytosol, release of cytochrome c and calcium-dependent apoptotic proteins and mitochondrial swelling, eventually resulting in mitochondria rupture, caspase activation and cell death via either apoptosis or necrosis (208-210). ONOOH can promote MPTP opening and apoptosis (211).
ONOOH also disrupts or blocks the activity of aconitase, creatine kinase, mitochondrial DNA, mitochondrial membranes, Mn-SOD and increases proton leak (208). The Krebs cycle is a series of chemical reactions that generate energy in cells. Aconitase, which is situated in mitochondrial matrix, is an important component of the Krebs cycle. ONOOH oxidatively damages the iron-sulphur center situated in the active site of this enzyme thereby inhibiting its activity (212) and the iron released from aconitase may augment mitochondrial oxidative stress (213).

Creatine kinase maintains the cellular levels of ATP. This enzyme is readily and rapidly inactivated by ONOOH via oxidation of critical thiols of this enzyme (124). Inhibition of creatine kinase may affect mitochondrial energy metabolism and may indirectly enhance calcium accumulation in the cytosol due to changes in ATP-dependent calcium transport into mitochondria. It has been suggested that mitochondrial creatine kinase could be one of main targets of ONOOH oxidation in intact mitochondria (214).

Mn-SOD is an important antioxidant enzyme that detoxifies $O_2^{-}$ in the mitochondria. ONOOH inactivates this enzyme by modifying a critical tyrosine residue (Tyr 34) (215). In particular, the manganese center of Mn-SOD reacts rapidly with ONOOH causing the formation of nitrating species, which nitrate the critical tyrosine. $CO_3^{2-}$ and $NO_2^{-}$ can also
nitrate and disrupt the activity of Mn-SOD (216), resulting in inhibition of detoxification of locally produced $O_2^{-}$ (217). This process may further augment ONOOH generation.

Nicotinamide nucleotide transhydrogenase is a vital mitochondrial protein that forms NADPH from NADH and NADP$^+$. ONOOH has been shown to oxidise, nitrate and inactivate this protein disrupting NADPH formation (218). NADPH depletion results in a decreased mitochondrial capability to regenerate the antioxidant GSH. This may contribute to magnified oxidative stress within mitochondria. Mitochondrial scavengers for ONOOH and its derived radicals such as $CO_3^{2-}$ and $NO_2^-$ include cytochrome $c$ oxidase, GSH and ubiquinol. These scavenging systems may diminish the reactions of these oxidants in mitochondria (125) but scavenging by GSH of ONOOH-derived radicals can result in thiol radical formation, which may in turn mediate secondary oxidation reactions (125).

1.6.3 Endoplasmic reticulum stress

In cells, the endoplasmic reticulum (ER) plays a key role in the synthesis and folding of proteins. Within these organelles, multiple protein post-translational modifications are executed and intracellular calcium is stored (219). The chaperones and foldases of the ER catalyse folding of newly synthesised secretory and transmembrane proteins into their native conformation and contribute to disulphide bond formation. Disruption of ER function can therefore result in the accumulation of unfolded or misfolded proteins, a condition known as ER stress. ER stress activates the unfolded protein response (UPR) to reduce protein synthesis and prevent the accumulation of unfolded proteins in cells (220). In the presence of ER stress, physiological concentrations of ONOOH maintain cellular homeostasis by activating the unfolded protein response (221). In contrast, excessive ONOOH has been shown to induce ER stress by causing calcium depletion in vascular endothelial cells (222). Proteins containing 3NT colocalised with ER stress markers such as GRP78 and GRP94 (chaperones). This led to enhanced cytosolic calcium levels and programmed cell death. This study indicates that ONOOH promotes ER stress in vascular endothelial cells and may promote atherosclerosis.
1.6.4 Induction of autophagy

Autophagy is a self-degradative cellular process that recycles proteins and organelles using lysosomes. This process removes misfolded, damaged and aggregated proteins and eliminates damaged organelles e.g. mitochondria, endoplasmic reticulum and peroxisomes. Autophagy also destroys intracellular pathogens (223). It has been shown that excess ONOOH formation increases nitrosative stress in endothelial cells during ischemic stress and that this process is associated with the induction of the autophagy-lysosome pathway (224). It has been proposed that the regulation of excessive activation of the autophagy-lysosome pathway by ONOOH might represent a pathophysiological cellular mechanism that results in enhancement of ischemic stress (224).

1.6.5 Cell death

When ONOOH-mediated cellular damage supercedes cellular repair mechanisms, cells undergo either apoptotic or necrotic pathways. Apoptosis has been shown to be triggered by low concentrations of ONOOH, whereas high concentrations of ONOOH compromise the apoptotic machinery causing necrosis (Figure 1.5). In cells ONOOH exposure conditions and cellular energetics (ATP and NAD) act as switches between these two modes of cell death (69, 225, 226).

ONOOH has been shown to induce apoptosis in multiple cell types such as thymocytes (188), HL-60 cells (227), PC12 cells (211), fibroblasts (228), SH-SY5Y neuroblastoma cells (229), primary neurons (230), astrocytes (231), oligodendrocytes (232), endothelial cells (15), beta islet cells (233), neutrophils (234), chondrocytes (235), cardiomyocytes (236) and renal tubular cells (237). ONOOH has also been reported to induce necrosis in pulmonary (238) and intestinal epithelial cells (239), vascular endothelial and smooth muscle cells (195, 240), fibroblasts (241), macrophages (242) and cardiomyocytes (243) amongst others.
1.6.5.1 Apoptosis

Apoptosis is a form of programmed cell death which is generally characterised by cell shrinkage, membrane blebbing and chromatin condensation. ONOOH can initiate apoptosis through the modulation of different receptors and apoptotic pathways including activation of the FAS receptor, a “death receptor” (244). Other essential apoptotic proteins that are activated by ONOOH oxidation include the caspases, protein-cutting enzymes that are referred to as “executioner proteins” (245), and Bax a protein that promotes apoptosis (246).

ONOOH-mediated nitration of tyrosine residues can also mediate apoptosis. For example, 3NT formation has been proposed to stimulate apoptosis by inducing DNA damage and eliciting endothelial dysfunction and neurodegenerative disorders (154, 247). ONOOH-mediated tyrosine nitration of PI-3 kinase leads to inactivation of vascular endothelial growth factor (VEGF) (248), a potent pro-survival/anti-apoptotic factor that induces migration and proliferation of endothelial cells, increases vascular permeability and regulates thrombogenicity (249). Inactivated VEGF loses its capability to maintain cell survival in the presence of high glucose or oxidative stress and this process accelerates
apoptosis. Tyrosine phosphorylation is also inhibited when ONOOH-mediated oxidation of tyrosine occurs and this event may trigger apoptosis (250).

Protective mechanisms against ONOOH-mediated apoptosis of endothelial cells include the up-regulation of anti-apoptosis proteins. Exposure of endothelial cells to ONOOH causes increased expression of haem oxygenase -1, an important inducible protein that is up regulated in the presence of oxidative stress and protects cells against apoptosis (251). In endothelial cells, prolonged ONOOH-induced ER stress can lead to apoptosis through the depletion of ER-Ca$^{2+}$ and increased cytosolic Ca$^{2+}$ (222). Endothelial cells also increase the expression of the ER chaperone GRP78 and GRP94 in response to ONOOH, which may protect cells from apoptosis (252, 253).

1.6.5.2 Necrosis

Necrosis is a biochemically-unregulated form of passive cell death that is not energy dependent. Necrosis is distinguished by cytoplasmic swelling, reduced cell membrane integrity and excessive inflammation (14). The fate of oxidatively injured cells can be regulated by poly(ADP-ribose) polymerase-1 (PARP-1) activation. DNA strand breakage elicits PARP-1, which catalyses the cleavage of NAD$^+$ into nicotinamide and ADP-ribose. The later species is used to generate branched nucleic acid-like polymers, which may induce recruitment of DNA repair enzymes to DNA injury sites. PARP-1 is degenerated by poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosyl protein lyase (194).

The combined action of PARP-1 and PARG preserves a highly elevated ADP-ribose turnover in cells exposed to ONOOH. Consequently cellular NAD$^+$ levels are diminished causing defective glycolysis, decreased Krebs cycle activity, reduced mitochondrial electron transport and ultimately ATP depletion (191). Cells typically attempt to regenerate NAD$^+$ by using stored ATP and nicotinamide and this action can causes substantial declines in cellular ATP levels. Since apoptosis is dependent on ATP, apoptosis is incapacitated and necrosis is favoured (254).

In addition to this, ONOOH-induced PARP-1 mediated necrosis is also associated with mitochondrial changes such as mitochondrial membrane damage, break down of mitochondrial membrane potential, excessive O$_2$$^{.\cdot }$ generation and calcium mobilisation (225). It has been shown that intracellular calcium chelation eradicated ONOOH-mediated DNA breakage, disrupted ONOOH-induced PARP activation and protected the
cell from necrosis. However in a cell free environment, calcium chelators did not decrease ONOOH-mediated DNA strand breakage (255). These findings indicate that PARP-1 plays an important role in ONOOH-mediated mitochondrial alterations, and PARP-1 activation may direct the death pathway towards necrosis and away from apoptosis (the default death pathway) (2).

1.6.6 Extracellular matrix modification

Normal blood vessel walls consist of endothelial cells, smooth muscle cells (SMC), various other cells and the extracellular matrix (ECM), which differs in composition in different layers of the vessel walls. The endothelial cells lie on the basement membrane. The basement membrane in arteries is characterised as a thin and continuous physical barrier against cellular migration, such that it separates the endothelial cells from the SMC (256). ECM is a complex structural meshwork of tightly cross-linked proteins (e.g. collagen and elastin), glycosaminoglycans (GAGs e.g. hyaluronan, heparan sulphate, chondroitin sulphate and keratan sulphate), proteoglycans, which are proteins decorated with GAG chains e.g. heparan sulphate proteoglycans (perlecan, agrin) and chondroitin sulphate proteoglycans (versican). Other critical ECM proteins include keratan sulphate-decorated species (e.g. lumican) and glycoproteins (e.g. fibronectin and laminin) (178, 256).

The ECM is continuously changing due to biological cues to and from the surrounding cells. The ECM plays an important role in controlling cell behaviour by regulating adhesion, migration and proliferation and provides mechanical strength, elasticity (important in artery walls) and binding sites for cells (256). Intracellular sites are believed to be more protected from oxidation due to the presence of high concentrations of antioxidants and repair enzymes whereas extracellular components appear to be less well protected from oxidation. In atherosclerotic lesions, most of the oxidative damage to proteins has been detected on the ECM rather than intracellular proteins (257). Extracellular components are therefore exposed to higher oxidative stress and significant changes in redox balance. These damaged materials are also thought to accumulate to high levels due to the long lifetime of matrix proteins, slow turnover and poor repair mechanisms (258, 259).
Oxidation can modulate ECM properties and functions. This has been linked to inflammatory diseases such as atherosclerosis, kidney disease, rheumatoid arthritis and osteoarthritis (260, 261). It is thought that modification of the ECM within the basement membrane plays an important role in the development of atherosclerosis (262). Weakening of the ECM structure may enhance rupture of atherosclerotic lesions, which leads to both heart attacks and strokes (263). ECM damage can arise from protein unfolding, elevated susceptibility to proteolytic cleavage, functional deactivation of ligand binding sites and elevated ECM permeability (261). Studies have demonstrated that high concentrations of oxidants such as ONOOH can induce the release of ECM fragments (181, 257) as well as crosslinking and aggregation of ECM (264). Endothelial cells have been reported to adhere and proliferate on native ECM to a greater extent than on oxidised ECM (265). Oxidation can also make the endothelial cell layer more permeable (266) and disturb endothelial cell adhesion.

Perlecan is a major heparan sulphate proteoglycan found in the basement membrane and the pericellular environment. Perlecan plays an important role in vascular homeostasis by stabilising and organising the ECM and controlling the adhesion, differentiation and proliferation of vascular cells. These roles are conducted by interaction of the protein core and heparan sulphate chains of perlecan with numerous ECM molecules, growth factors and adhesion molecules (267). Exposure of isolated perlecan to ONOOH has been shown to result in structural and functional changes to this protein (264). Antibodies against the protein core and heparan sulphate chains lose their recognition on exposure of the protein to ONOOH and dose-dependent 3NT formation occurs. ONOOH exposure also induces aggregate formation, consistent with protein crosslinking. Exposure to ONOOH also alters the functional properties of perlecan, which depend on both the protein core (reduced binding of human coronary artery endothelial cells) and the heparan sulphate chains (reduced binding of fibroblast growth factor 2 to the heparan sulphate chains) (264).

Immunofluorescence of advanced human atherosclerotic lesions showed the presence of perlecan and 3NT in the intimal regions. Co-localisation of 3NT epitopes, perlecan and macrophages was also detected (Figure 1.6) (264). Since perlecan lies underneath the endothelial cells, it is unknown whether endothelial dysfunction is a cause or consequence of the damage induced by ONOOH (detected by 3NT formation) to various
components of the ECM. It is also unclear how this damage is associated with the development and progression of atherosclerosis.

Figure 1.6 Immunofluorescence showing perlecann (right) and 3NT (middle) epitopes in advanced human atherosclerotic lesion. 3NT colocalised with perlecann (left) [taken from (264)].

1.6.7 LDL oxidation and nitration

Lipoproteins are composed of both proteins and lipids (cholesterol, cholesteryl esters, phospholipids and fatty acids) and this enables fats to be transported into and out of cells. Examples of lipoproteins include low density lipoprotein (LDL) and high-density lipoprotein (HDL). LDL is one of the five major groups of lipoproteins and is often described as ‘bad cholesterol’. LDL transports fat molecules such as cholesterol to cells via the bloodstream. LDL is made of phospholipids, unesterified cholesterol and a single large protein apolipoprotein B-100.

LDL can be modified by many factors such that it is recognised by macrophage scavenger receptors. Some of these factors include acetylation (268), acetoacetylation (269), malondialdehyde treatment (270), hypochlorite treatment (271), oxidation by copper (272) and ONOOH (273). LDL exposed to ONOOH (274) or simultaneous generation of NO‘ and O2’ can result in the formation of oxLDL, which have an increased atherogenic properties (152, 273). Nitrated LDL has been detected in atherosclerotic plaques and serum of patients suffering from atherosclerosis (152). In atherosclerotic lesions, oxidation of lipoproteins is anticipated to be critical in the generation of foam cells from macrophages (275), with ONOOH-modified LDL binding with high affinity to macrophage receptors causing accumulation of cholesteryl esters,
which participate in fatty streak formation (276), a key feature of atherosclerosis. Therefore lipoprotein modification by ONOOH may be a key process in the initiation of atherosclerosis (277).

When macrophages take up excessive oxidised LDL, foam cells are generated. Foam cells are lipid loaded macrophages. Foam cells are involved in inflammatory responses and tissue remodelling (278). Native LDL does not induce formation of lipid laden macrophages because the cellular accumulation of cholesteryl esters is inhibited by a sterol-dependent down-regulation of the native LDL receptor (279). In contrast, oxidised LDL has alterations to its biologic properties that may have pathogenic importance such as increased uptake by macrophages, cytotoxicity and chemotactic activity for monocytes (280).

LDL oxidative modification induces compositional and structural changes which includes elevated electrophoretic mobility, increased density, apolipoprotein B fragmentation, phosphatidylcholine hydrolysis, lysine residue derivatisation and fluorescent adduct formation due to covalent binding of lipid oxidation products to apolipoprotein B. Accumulating evidence suggests that oxidised LDL is a modified form of LDL which contributes to foam cell formation in vivo (280). Ingestion of lipids by macrophages is the process by which foam cells form.

Lipid oxidation can be induced by many factors such as free and protein-bound metal ions, thiols, ROS, lipoxygenase, ONOOH and myeloperoxidase (18). In LDL, NO’ diffuses and concentrates in the hydrophobic core and acts as a potent inhibitor of lipid oxidation processes whereas ONOOH is a mediator of oxidative modifications. ONOOH has been reported to cause initiation and sustain propagation of lipid oxidation including peroxidation of the lipid component of LDL (276, 281, 282).

ONOOH can oxidise or nitrate both the lipid and protein components of LDL. LDL oxidation by ONOOH is a complex process, which is controlled by ONOOH generation, LDL availability and the presence of NO’, ascorbate and α–tocopherol (α–TOH) (283). Cysteine, methionine and tryptophan residues are the main targets of ONOOH in apolipoprotein B-100 (283), with fragmentation and nitration also reported (274). Protection against ONOOH induced LDL oxidation is provided by urate, ascorbate, α–
TOH and NO’ due to their radical scavenging abilities, which includes inactivation of lipid peroxyl radicals (LOO’), NO₂⁻ and CO₃⁻, and their ability to reduce high oxidation state metal ion centres (283).

1.6.8 HDL oxidation and nitration

High density lipoproteins (HDL) are the smallest and the densest lipoproteins that transport lipids such as cholesterol and triglycerides in blood. HDL is anti-inflammatory and is often termed ‘good cholesterol’ which plays a key role in cholesterol reverse transport whereby excess cholesterol is removed from peripheral tissues and carried to liver for excretion or reutilisation. Thus HDL protects the artery wall from atherosclerosis by removing cholesterol from arterial macrophages (284, 285), blocking of LDL oxidation by cells (286, 287), decreasing lipid hydroperoxides and transporting oxidised lipids to liver for excretion (288). HDL also blocks the cytotoxic effects of oxidised LDL on cultured cells (289).

Interestingly, in vitro studies have indicated that HDL is more prone to oxidation than LDL, and modification can turn HDL into a pro-atherogenic and cytotoxic particle (290, 291). It has been shown that oxidised HDL is present in atherosclerotic plaques and attaches to human vascular endothelial cells (292). This indicates that oxidatively-modified HDL may be important in the development of atherosclerosis.

Exposure of HDL particles to ONOOH induces nitration of tyrosine residues, intermolecular cross-linking of the surface apolipoproteins and generation of aggregates (277). The viability of human vascular endothelial cells exposed to either nitrated HDL or nitrated LDL has been shown to significantly decrease with nitrated HDL being more cytotoxic than nitrated LDL (277). Interestingly, neither nitrated HDL or nitrated LDL interfered with NO’ or O₂⁻ production from enzymes in endothelial cells. Nitrated HDL reduced the activity of catalase, an antioxidant enzyme, while nitrated LDL decreased the expression and activity of Cu²⁺/Zn²⁺ SOD (277). HDL is believed to be more prone to nitration than LDL as a result of the composition and localisation of apolipoproteins in the particles. It has also been concluded that oxygen radicals generated from nitrated lipoproteins are involved in nitrated lipoprotein-induced endothelial dysfunction (277).
Pennathur and coworkers have measured 3NT levels in HDL within the atherosclerotic intima and blood of patients with established coronary artery disease in order to explore the role of RNS in atherosclerosis. The average level of 3NT in HDL isolated from the intima of patients was 6-fold higher than the circulating HDL (293). In this study the level of 3NT detected in lesion HDL was similar to the previously reported level of 3NT on lesion LDL (152) implying that the extent of nitration of these lipoproteins is similar in the human artery wall. Twice as much 3NT was detected in HDL from plasma of patients with the disease compared to the healthy subjects (152). This suggests that nitrated HDL may be an important marker for vascular disease. However the exact mechanism of RNS generation was not identified with nitration potentially mediated by either ONOOH or myeloperoxidase. Therefore the exact pathway of RNS generation remains to be elucidated. Despite this, this study indicates that nitrated HDL may represent a link between nitrosative stress induced by inflammation and atherogenesis (293).

### 1.6.9 Plasma damage

Activated phagocytes and endothelial cells generate NO and O₂⁻ and hence ONOOH may be released into plasma. The production of these species is upregulated under inflammatory conditions. In plasma, ONOOH induces oxidative damage to both proteins and lipids by generating modified side chains on proteins and forming lipid hydroperoxides. ONOOH also depletes antioxidants such as ascorbic acid, bilirubin, uric acid and plasma thiols (71). Electron paramagnetic resonance studies have shown that in plasma, ONOOH induces the generation of ascorbyl and urate radicals along with thiyl (294), tryptophanyl (105) and tyrosyl (104) protein-bound radicals; these reactive radicals can propagate the damage initially induced by ONOOH (99).

Human serum albumin (HSA) is the most abundant plasma protein. HSA is a globular 66 kDa protein that consists of 18 tyrosine, 6 methionine, 1 tryptophan, 17 disulphide bridges and 1 free cysteine (Cys 34). HSA maintains colloid pressure and binds and transports multiple ligands such as fatty acids and hormones (295, 296). HSA has also been proposed to act as an antioxidant. The thiol group in Cys 34 is mainly responsible for the antioxidant properties of this protein. The thiol group accounts for about 80 % of the net free thiols in plasma and reacts preferentially with ROS and RNS. It has been reported that the reaction of HSA with ONOOH results in the formation of sulphenic...
acid intermediates, which can react further with free thiols to mediate disulphide formation (297). This could be a protective mechanism towards ROS and RNS, as generation of sulphenic acids on HSA could potentially restrict plasma thiol oxidation to reversible states, as sulphenic acids are readily reduced. Therefore this pathway could be protecting HSA from being inactivated by ONOOH (297).

Bilirubin is a bile pigment and an end-product of haem catabolism. Bile is also an antioxidant which plays a major physiological role in the protection against oxidation (298). A study investigated the effect of ONOOH on the antioxidant properties of bilirubin in human plasma (99). The major oxidation product formed was biliverdin. When present at micromolar concentrations, bilirubin significantly protected against tryptophan oxidation and tyrosine nitration by ONOOH. A striking observation of this study was that bilirubin protected plasma proteins against ONOOH oxidation even when it was added 5 seconds after ONOOH. This indicates that in the plasma some ONOOH-derived species persist longer than the anticipated lifetime of ONOOH. In the presence of HCO₃⁻, ONOOH-mediated oxidation of bilirubin was reduced suggesting that the reactive species produced from ONOOCO₂⁻ do not react rapidly with bilirubin (99). Bilirubin therefore seems to be a versatile antioxidant for ONOOH-mediated damage in human plasma.

1.7 ONOOH in diseases

There is considerable and accumulating evidence for the in vivo formation of ONOOH in human diseases such as atherosclerosis, vascular diseases, circulatory shock, local inflammation, cancer, stroke, neurodegenerative disorders diabetes, human acute lung injury and chronic inflammation (2, 299). An overview of how ONOOH may be involved in development of atherosclerosis follows.

1.7.1 ONOOH in atherosclerosis

Cardiovascular diseases are the main source of morbidity and mortality in developed countries. The economic costs are enormous; for instance in the USA, the medical care and indirect costs of cardiovascular disease was about $450 billion per year in 2010 and this cost is expected to increase to over $1 trillion per year by 2030 (300). Atherosclerosis is the main cause of cardiovascular diseases. In Australia atherosclerosis
is responsible for about 40% of all deaths and the medical cost is greater than $4 billion per year. Therefore there is a great need for prevention and reversal of this disease and its consequent huge economic burden.

The exact cause of this disease is unknown, however three hypotheses have been postulated to explain disease initiation and progression. The ‘response to retention’ hypothesis postulates that accumulation of LDL is critical in initiation of atherosclerosis. According to this hypothesis a key initiation event in early atherosclerosis is the retention of cholesterol rich atherogenic lipoproteins. These retained lipoproteins stimulate a cascade of responses that result in lesion formation (301). The ‘response-to-injury’ hypothesis postulates that atherosclerosis is an inflammatory disease within which various aspects of wound healing are involved. According to this hypothesis, endothelial dysfunction, lymphocytes and monocytes migration into the intima, smooth muscle cell proliferation and excessive secretion of matrix molecules are important events in the generation and development of atherosclerosis (302).

The ‘oxidative modification’ hypothesis postulates that LDL oxidation occurs at an early stage of atherosclerosis and it is this oxidised LDL that contributes to atherogenesis (Figure 1.7) (303). According to this hypothesis, in the early stages of atherosclerosis, the native LDL is oxidised by various oxidants, which may include ONOOH. These oxidants can be produced by endothelial cells and activated phagocytes such as macrophages, monocytes and neutrophils. Activated endothelial cells can induce monocyte adhesion and migration into the subendothelium.

Endothelial dysfunction is an early and important event in the pathogenesis of atherosclerosis and is believed to contribute to plaque initiation and progression. Endothelial dysfunction is a functional and reversible or irreversible alteration of endothelial cells. Possible causes of endothelial dysfunction include a reduction in the bioavailability of NO’, increased and modified LDL, elevated oxidative and nitrosative stress, ongoing inflammation, cigarette smoking, hypertension, diabetes mellitus, genetic alterations, increased concentrations of plasma homocysteine, infectious microorganisms (e.g. herpes viruses or Chlamydia pneumonia) and a combination of these factors (7, 76, 304-307).
Endothelial dysfunction changes the normal homeostatic properties of the endothelium. An injury can occur via different forms and results in enhanced endothelial permeability to lipoproteins. This process may be mediated by NO$, prostacyclin, platelet-derived growth factor, angiotension II and/or endothelin. During this process leukocyte adhesion molecules such as L-selectin, integrins and platelet-endothelial-cell adhesion molecule 1 are upregulated (308) as well as endothelial cell adhesion molecules including E-selectin, P-selectin, intercellular adhesion molecule 1 and vascular-cell adhesion molecule 1 (309). The adhesiveness of leukocytes and platelets to the endothelium is also upregulated. Injury can cause procoagulant instead of anticoagulant properties of the endothelium and produce vasoactive molecules, cytokines and growth factors. These processes initiate migration and proliferation of smooth muscle cells to the area of inflammation to form an intermediate lesion. If this process is not prevented or reversed, the artery wall can thicken (308, 310).

Continued inflammation increases monocyte, macrophage and lymphocyte cell populations in the artery wall with these cells either migrating from the blood or multiplying in the subintimal space of the blood vessel (Figure 1.8) (148). Migration of these leukocytes can be mediated by oxidised LDL, monocyte chemotactic protein 1, interleukin-8, platelet-derived growth factor, macrophage colony-stimulating factor and osteopontin. Activation of these leukocytes results in the generation and release of
hydrolytic enzymes, cytokines, chemokines and growth factors that can promote lesion development (308).

The failure of monocytes and macrophages to remove modified LDL may result in progression of the atherosclerotic lesion. Lipids accumulate within the innermost layer of arterial wall causing fatty streak formation (268, 311, 312). Fatty streaks consist of white blood cells loaded with lipids, which have infiltrated the arterial wall. Cycles of accumulation of oxidised LDL, monocytes, macrophages and lymphocytes along with migration and proliferation of smooth muscle cells and generation of fibrous tissue causes further enlargement and remodelling of the lesion. At this stage, the lesion consists of a core of lipid and necrotic tissue. The lesion then becomes an advanced and complicated lesion, which is covered by a fibrous cap (262, 308).

The existence of activated monocytes and macrophages which can generate reactive oxidants in atherosclerotic lesions suggests that oxidative damage may be associated with the onset and progression of atherosclerosis (308). Oxidised lipids, sterols and proteins have been detected in early, intermediate and advanced human atherosclerotic lesions at significantly higher levels than in control tissue, and reduced levels of antioxidants have also been detected (303, 313, 314). Oxidised amino acids have also been detected on proteins extracted from human atherosclerotic lesions at significantly elevated levels.
Although oxidation or nitration is known to be present, the role of ONOOH in modifying human coronary artery endothelial cells is not well established.

1.8 Hypothesis

The main hypothesis behind the studies reported in this Thesis is that ONOOH, which is formed at sites of inflammation in the artery wall, reacts with targets in human coronary artery endothelial cells (HCAEC) and that endothelial cell proteins are major target of damage. Furthermore it is proposed that damage will occur predominantly at sulphur-containing amino acids (both free and on protein) and that this will occur at multiple locations within cells particularly on cytosolic and membrane proteins due to the sites of O$_2^•$ and NO’ formation inside and external to cells.

1.9 Aims

This hypothesis will be examined by the following specific aims:

1. Investigate the extent and order of amino acid damage induced by ONOOH and ONOOOCO$_2^-$ on isolated peptides and protein.

2. Investigate the mechanisms and consequences of reaction of ONOOH and ONOOOCO$_2^-$, added both as bolus or generated slowly from SIN-1, with endothelial cell protein both cytosolic and membrane-derived.

3. Investigate the protein targets of ONOOH in HCAEC using LC/MS/MS.

ONOOH-mediated damage will be assessed by measuring thiol oxidation (loss) via ThioGlo assay, 3NT formation via Western blotting (using anti-3NT antibodies), protein changes via silver staining of SDS-PAGE gels and amino acid analysis by UPLC. The effect of ONOOOCO$_2^-$ on thiol oxidation, 3NT formation, protein modifications and amino acids will also be examined. Cytosolic and membrane proteins will be separated via fractional centrifugation experiments in order to identify whether cytosolic and/or membrane proteins are mainly targeted by ONOOH. Using mass spectroscopy (LC/MS/MS), we will aim to identify which proteins and specific residues are modified by ONOOH. We will also aim to enrich the sample for nitrated proteins via immunoprecipitation and subsequently perform LC/MS/MS in order to determine which HCAEC proteins or tyrosine residues are site-specifically nitrated by ONOOH.
Chapter 2: Materials and Methods
2.1 General Information

This Chapter contains information relating to the Materials and Methods used during this Thesis. The concentrations described are final concentrations after addition of reagents unless otherwise stated.

2.2 Materials

All commercial chemicals and solutions were of high purity and used as supplied, unless otherwise stated. All buffers and aqueous solutions were prepared using Nanopure water (npH2O). npH2O is purified water obtained from a four-stage Milli Q System (Milipore-Water, Lane Cove, NSW, Australia).

Table 2.1 Suppliers of reagents

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2.3 Methods

2.3.1 Buffers, pH calibration and centrifugation

0.1 M Sodium phosphate buffer was prepared by adding 0.1 M monobasic sodium phosphate (chelexed) to 0.1 M dibasic sodium phosphate (chelexed) and the pH was adjusted to 7.4. 0.1 M sodium phosphate buffer containing 25 mM sodium bicarbonate (NaHCO₃) was also prepared and the pH of this solution was adjusted to 7.4. Both buffers were filtered through a 0.33 µm pore sized filter (Corning Incorporated) and stored at 4 °C.

Commercial Hanks Balanced Salts Solution (HBSS) containing 1.26 mM calcium chloride (anhydrous), 0.81 mM magnesium sulphate (anhydrous), 5.37 mM potassium chloride, 0.44 mM potassium phosphate monobasic (anhydrous), 136.89 mM sodium chloride, 0.34 mM sodium phosphate dibasic (anhydrous) and 5.55 mM D-glucose was purchased from Sigma Aldrich. HBSS containing 25 mM NaHCO₃ was also prepared. Prior to every experiment, the pH of HBSS (± 25 mM NaHCO₃) was adjusted to 7.4 and the solution was filtered through a 0.33 µm pore sized filter (Corning Incorporated).

The pH of solutions was measured using a Radiometer Analytical PHM220 pH meter (Radiometer Analytical, France). The pH meter was calibrated using pH 4, 7 and 10 standards (Radiometer Analytical). Unless otherwise stated, samples were centrifuged in Microcentrifuge 5415R (Eppendorf).

2.3.2 Quantification and preparation of oxidants

2.3.2.1 Peroxynitrous acid synthesis

Peroxynitrous acid (ONOOH) was synthesised by diluting 8.8 mL of 30 % hydrogen peroxide in 11.2 mL npH₂O and this solution was cooled to 4 °C on an ice/water mixture. 16 mL of 5 M sodium hydroxide (NaOH) was gently added. 2 mL of 0.04 M diethylenetriaminepentaacetic acid (prepared in 0.05 M NaOH) was also gently added. 2 mL of npH₂O was added and the pH was checked and found to be around 12. This solution was vigorously mixed with 10.8 mL of isoamyl nitrite at 21 °C overnight. Using a separating funnel, 600 mL of dichloromethane was washed with 2 volumes of npH₂O.
The hydrogen peroxide and isoamyl nitrite solution was washed 6 times with 2 volumes of washed dichloromethane.

A 1.5 x 10 cm column was filled with 25 g granular manganese dioxide and washed with 20 mL of npH2O followed by 20 mL of 0.5 M NaOH. Unreacted hydrogen peroxide was removed by filtering the washed hydrogen peroxide and isoamyl nitrite solution through the manganese dioxide column. The initial eluent from the column was discarded until the yellow solution of ONOOH started eluting from the column (318). The ONOOH solution was flushed with nitrogen and stored in 100 µL aliquots at –80 °C.

2.3.2.2 ONOOH quantification
Prior to every experiment, frozen concentrated stock solutions of ONOOH (stored at -80 °C) were thawed and diluted 1:500 and 1:1000 in 20 mM NaOH solution in order to quantify their concentrations. The absorbance was then measured using a UV-Vis spectrophotometer (Shimadzu) at 302 nm, the concentration of the stock solutions were determined using the molar absorption coefficient (ε) of 1670 M⁻¹cm⁻¹ (319). About 800 mM ONOOH concentration was detected in the frozen concentrate stock solutions. The stock solutions were then diluted in 20 mM NaOH to the desired ONOOH concentrations to perform experiments.

20 mM NaOH solution (chelexed) was prepared on a weekly basis. This solution was filtered through a 0.33 µm pore sized filter (Corning Incorporated) and stored at 4 °C. For all experiments in this Thesis, a new frozen aliquot of ONOOH was thawed and used. The thawed and diluted ONOOH solutions were kept on ice. At the end of each experiment, the thawed stock and the diluted solutions were discarded.

2.3.2.3 ONOOH donor: SIN-1
3-Morpholinosydnonimine-N-ethylcarbamide (SIN-1) was purchased from Cayman Chemicals. Fresh SIN-1 stock solutions were prepared to desired concentrations in npH2O immediately prior to each experiment. In solution, SIN-1 has a half-life of about 30 min (58, 59). At the end of each experiment all the SIN-1 solutions were discarded.
2.3.3 Oxidant treatment of isolated bovine serum albumin and glutathione

Fresh solutions of 15 µM bovine serum albumin (BSA) were prepared in 0.1 M sodium phosphate buffer (± 25 mM NaHCO₃) (pH 7.4) and treated with 0 – 1000 µM ONOOH for 5 min at 21 °C before analysis. 15 µM glutathione (GSH) was also freshly prepared in 0.1 M sodium phosphate buffer (± 25 mM NaHCO₃) (pH 7.4) and treated with 0 – 100 µM ONOOH for 5 min at 21 °C.

2.3.4 Tissue culture: human coronary artery endothelial cells

All cell experiments in this Thesis were performed using human coronary artery endothelial cells (HCAEC) purchased from Cell Applications (Catalogue number 212-500). HCAEC are primary cells obtained from normal human coronary arteries. For all experiments in this Thesis, 3 different HCAEC donors were used to get representative results. These HCAEC donors include 2135 (19 year old Hispanic male), 2286 (48 year old black male) and 2366 (60 year old Caucasian male). HCAEC at passages 3 and/or 4 were used for all experiments.

2.3.4.1 Culture

Cells were cultured in MesoEndo cell growth media (does not contain growth factors) in 175 cm² flasks under sterile conditions in an atmosphere that contained humidified 5 % CO₂ at 37 °C. On every second day, cells were washed twice with phosphate-buffered saline (PBS) and new media was added. Once cells reached confluency, cells were washed with PBS and treated with 10 mL of trypsin (0.05 % w/v) in ethylenediaminetetraacetic acid (EDTA) (0.02 % w/v) solution for 5 min at 37 °C. This treatment lifted cells off the flask. The action of trypsin-EDTA was neutralised by addition of fresh media and cells were centrifuged in an Allegra X-15R centrifuge (Beckman Coulter) at 524 g and 22 °C for 5 min to form a pellet. The pellet was resuspended in 10 mL of fresh media, 5 mL of cell suspension was transferred to a new flask and the final volume was adjusted to 20 mL with media. Cells reached confluency about 7 days after passaging. Media, PBS and trypsin EDTA solutions were warmed to 37 °C prior to use.
2.3.4.2 Seeding
Once cells reached confluency, cells were washed with PBS and lifted off the flask as described above. The cell pellet was then resuspended in 10 mL of fresh media, and the cells were counted in the presence of trypan blue (to determine the percentage of cell viability) using a haemocytometer slide and a microscope. Cells were resuspended in media to the desired concentration before seeding. Cells were seeded in either 6-, 12- or 24-well tissue culture plates at a seeding density of 4-, 1.6- and 0.8 x 10^5 cells per well respectively. The tissue culture plates were made of polystyrene (Corning Incorporated) and had flat clear bottoms. Cells were left overnight in a 37 °C incubator to adhere to culture plates before experiments.

2.3.5 Oxidant treatment of human coronary artery endothelial cells

2.3.5.1 Treatment of intact cells with oxidant
Cells were seeded in a 6-well plate at a seeding density of 4 x 10^5 cells per well as described in Section 2.3.4.2. On the following day cells were washed twice with 750 µL of warm PBS and incubated in 725 µL of HBSS (+ 25 mM NaHCO₃). Cells were then treated with 25 µL of either 0 - 1000 µM ONOOH for 5 min or 0 - 1000 µM SIN-1 for 4 h at 37 °C and pH 7.4. After the required incubation period, the excess oxidant solution was removed and cells were washed with 700 µL of warm PBS. 400 µL of cold (4 °C) npH₂O was added to lyse cells and the cells were scraped using a plunger from a 1 mL syringe. Cells were left at 4 °C for 10 min to ensure complete lysis. Cellular debris were removed by centrifugation at 16,100 g and 4 °C for 10 min. These samples were then subjected to analysis such as the ThioGlo assay, Western blotting or silver staining as described below.

2.3.5.2 Lysate treatment with oxidant
For HCAEC lysate experiments, cells were seeded in a 6-well plate at a seeding density of 4 x 10^5 cells per well as described in Section 2.3.4.2. On the following day, cells were washed with PBS, exposed to cold (4 °C) npH₂O and scraped using a plunger to lyse cells. Cells were left at 4 °C for 10 min to ensure complete lysis. Lysates were collected and centrifuged at 16,100 g and 4 °C for 10 min to remove cellular debris. The 290 µL of lysates were then treated with 10 µL of either 0 - 1000 µM ONOOH (+ 25 mM NaHCO₃)
for 5 min or 0 - 1000 µM SIN-1 (± 25 mM NaHCO₃) for 4 h at 37 °C and pH 7.4. The treated lysates were subjected to analysis as described below.

### 2.3.6 Sub-cellular fractional centrifugation

To detect whether cytosolic or membrane proteins of HCAEC were damaged by oxidants, fractional centrifugation was employed. Fractional centrifugation was employed to separate the cytosolic and membrane fraction of cells, using a commercial membrane extraction kit, the ProteoExtract native membrane protein extraction kit (320). This kit is designed for a high yield membrane protein extraction (3 - 5 fold enrichment) without employing denaturing conditions.

The kit contains Extraction Buffer I and II, a cocktail of protease inhibitor and a wash buffer. The exact content of these reagents was not disclosed in the protocol issued by the manufacturer. Intact cells were treated with ONOOH or SIN-1 (± 25 mM NaHCO₃) and the subcellular fractions were separated as described in Section 2.3.6.1. Alternatively, the subcellular fractions of untreated cells were separated before both fractions are treated with ONOOH or SIN-1 (± 25 mM NaHCO₃) separately as described in Section 2.3.6.2.

#### 2.3.6.1 Intact cells

HCAEC were seeded at a cell density of 4 x 10⁵ cells per well in a 6 well plate as described in 2.3.4.2. Cells were washed twice with 750 µL of warm PBS and incubated in 725 µL of HBSS (± 25 mM NaHCO₃). Cells were then treated with 25 µL of either 0 - 1000 µM ONOOH for 5 min or 0 - 1000 µM SIN-1 for 4 h at 37 °C and pH 7.4. Cells were washed twice with cold (4 °C) wash buffer. 800 µL of cold (4 °C) Extraction Buffer I and 4 µL of Protease Inhibitor Cocktail were added. The plate was swirled gently and incubated at 4 °C for 10 min under gentle agitation. The supernatant that contained the cytosolic fraction was removed and stored on ice. Immediately 400 µL of cold (4 °C) Extraction Buffer II and 4 µL of Protease Inhibitor Cocktail were added to cells in the plate. The plate was swirled gently and incubated at 4 °C for 30 min under gentle agitation. The supernatant that contained membrane fraction was removed and stored on ice. All samples were centrifuged at 16,100 g and 4 °C for 10 min and stored on ice.
The ThioGlo assay was performed to detect loss of thiols in both fractions using 150 µL of each sample. The amount of protein was equalised in all samples, subsequently Western blotting and silver staining were performed to detect 3-nitrotyrosine formation and protein modifications respectively. 60 µL of each sample was used to perform Western blotting and silver staining. ABC-G1 and VEGF are membrane and cytosolic proteins respectively. Anti-ABC-G1 and anti-VEGF antibodies were used to check the efficiency of the subcellular fractions separation.

2.3.6.2 Lysates

Purified subcellular fractions of untreated cells were separated as described in Section 2.3.6.1 and kept on ice. The ThioGlo assay was immediately performed using fractions that contained equal amount of thiols as described in Section 2.3.8. The thiol concentrations in the cytosolic and membrane fractions were equalised to equivalent concentrations. 186 µL of each fraction was mixed with 7 µL of HBSS (± 25 mM NaHCO₃). This solution was treated with 7 µL of either 0 - 1000 µM ONOOH for 5 min or 0 - 1000 µM SIN-1 for 4 h at 37 °C and pH 7.4. 150 µL of these samples (treated and control) were used to perform the ThioGlo assay again to detect oxidant-mediated thiol loss. Oxidant-mediated thiol loss was established by comparing the treated samples to the non-treated controls.

Western blotting and silver staining was performed using fractions that contained equal amounts of protein. The BCA assay was performed to normalise the protein concentrations in both fractions. 186 µL of fractions were mixed with 7 µL of HBSS (± 25 mM NaHCO₃). The resulting solution was then exposed to 7 µL of either 0 - 1000 µM ONOOH for 5 min or 0 - 1000 µM SIN-1 for 4 h at 37 °C and pH 7.4. Using 60 µL of these samples, gel electrophoresis was performed as described in Section 2.3.11. Subsequently Western blotting and silver staining were performed as described in Sections 2.3.12 and 2.3.14 respectively. Western blotting and silver staining detected 3-nitrotyrosine formation on proteins and protein modifications respectively. Oxidant-mediated 3-nitrotyrosine formation or protein modifications were established by comparing the treated samples to the non-treated controls. Anti-ABC-G1 and anti-VEGF antibodies were used to confirm the purity of each fraction.
2.3.7 Cell viability assay: MTT Assay

Cellular viability after oxidant treatment was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Metabolically active cells take up MTT and convert it to formazan crystals, which have a purple color (321). These formazan crystals can be solubilised by the addition of a solvent such as DMSO.

A commercial CellTiter 96 Non-radioactive Cell Proliferation assay kit was used to perform the MTT assay according to the manufacturers instructions. The kit contained the MTT solution (a dye) and the solubilising solution. The exact composition of the MTT solution was not disclosed in the protocol issued by the manufacturer. However the solubilising solution contained 25 - 50 % (w/v) N,N-dimethylformamide, 15 – 20 % (w/v) sodium dodecyl sulphate (SDS) and less than 2.5 % (v/v) acetic acid.

The MTT assay was performed using HCAEC intact cells treated with ONOOH or SIN-1 (± 25 mM NaHCO₃). Cells were seeded in a 12-well plate at a seeding density of 1.6 x 10⁵ cells per well as described in Section 2.3.4.2. On the following day, cells were washed twice with 400 µL of warm PBS and incubated in 390 µL of HBSS (pH 7.4). Cells were treated with 10 µL of either 0 - 1000 µM ONOOH (± 25 mM NaHCO₃) for 5 min or 0 - 1000 µM SIN-1 (± 25 mM NaHCO₃) for 4 h at 37 °C and pH 7.4.

After the required incubation period, the excess oxidant solution was removed and cells were washed with PBS. Cells were then incubated in 340 µL of warm media containing 60 µL of MTT solution for 4 h at 37 °C. During the 4 h period, metabolically active cells formed formazan crystals. 400 µL of solubilising solution was added to dissolve the formazan crystals. The absorbance of each sample was measured at 570 nm in a Tecan Sunrise plate reader with a reference at 630 nm. Cellular viability was assessed by comparing the treated samples with the non-treated controls.

2.3.8 Thiol measurements: ThioGlo 1™ assay

ThioGlo 1™ is a maleimide-based fluorescence dye used to detect and determine thiols on the side chain of cysteine residues. The ThioGlo 1™ (non-fluorescent) selectively binds to reduced thiols resulting in the formation of highly fluorescent adducts (322).
ThioGlo assay was performed using 15 μM BSA, 15 μM GSH and HCAEC samples with or without exposure to ONOOH or SIN-1 (± 25 mM NaHCO₃). HCAEC samples included intact cells, lysates or subcellular fractions. For all HCAEC samples, cells were seeded at a cell density of 4 x 10⁵ cells per well as described in Section 2.3.4.2.

GSH standards (2-10 μM) were prepared prior to each experiment. 2.6 mM stock solution of ThioGlo 1™ in acetonitrile was prepared and stored in the dark at 4 °C. The stock solution was diluted 1:100 in PBS (pH 7.4) to form ThioGlo 1™ working reagent immediately before use. 50 μL of ThioGlo 1™ working reagent was added to 50 μL of sample and standards in a 96 well plate in triplicates. The plate was incubated in the dark at 21 °C for 5 min. The concentration of thiols was measured by fluorescence spectroscopy using a SpectraMax M2® plate reader at λ_excitation = 384 nm and λ_emission = 513 nm at 21 °C. A GSH standard curve was prepared and the thiol concentration in each sample was established by comparison to the GSH standard curve (323).

The ThioGlo™ 3 and 5 reagents are analogues of ThioGlo™ 1 (324, 325). The protocol for performing ThioGlo assay with these reagents is same as that described above, however the excitation and emission wavelengths are different compared to those used for the ThioGlo™ 1. The ThioGlo™ 3 has λ_excitation = 365 nm and λ_emission = 445 nm while the ThioGlo™ 5 has λ_excitation = 365 nm and λ_emission = 536 nm.

2.3.9 Protein assay: BCA assay

The bicinchoninic acid (BCA) assay is a colorimetric assay used to quantify protein concentrations, in which the protein reduces Cu²⁺ to Cu¹⁺. BCA reacts with Cu¹⁺ at high pH to form BCA - Cu¹⁺ complex that has an intense purple color, which is quantified by measuring the absorbance at 562 nm. The amount of Cu²⁺ reduced is directly proportional to the amount of protein present in sample (326).

0.1-0.5 mg mL⁻¹ BSA standards were freshly prepared prior to each experiment. Commercial Pierce BCA Protein Assay Reagent A contained sodium carbonate, sodium bicarbonate, Pierce BCA detection reagent and sodium tartrate in 0.1 M sodium hydroxide. This reagent was mixed with 4 % (w/v) copper sulphate in 50:1 ratio to form the BCA working reagent. 200 μL of the BCA working reagent was added to 25 μL of
sample or BSA standard in a 96 well plate in triplicate. The samples included BSA or HCAEC (intact cells, lysates or subcellular fractions) with or without exposure to ONOOH and SIN-1. The plate was incubated at 60 °C for 30 min. The absorbance was measured at 562 nm using a Tecan Sunrise plate reader. The unknown protein concentration in each sample was established by comparison to the BSA standard curve.

2.3.10 Ultra performance liquid chromatography

Ultra performance liquid chromatography (UPLC) was performed to detect loss of specific amino acids and product formation in samples containing BSA modified by ONOOH (± 25 mM NaHCO₃). 15 µM BSA was treated with 0 – 10 mM ONOOH (± 25 mM NaHCO₃) for 5 min at 21 °C and pH 7.4. Proteins were delipidated and precipitated by the addition of 25 µL of 0.3 % (w/v) deoxycholic acid and 50 µL of 50 % (w/v) trichloroacetic acid respectively, followed by centrifugation for 2 min at 7,500 g and 5 °C to form a pellet. The pellet was washed twice with 200 µL of ice-cold (4 °C) acetone and dried with a gentle stream of nitrogen gas. The pellet was resuspended in 150 µL of 4 M methanesulfonic acid containing 0.2 % (w/v) tryptamine (sealed in ampoules) and transferred to PicoTag hydrolysis vessels (323).

The PicoTag hydrolysis vessels were placed under vacuum and back flushed 3 times with nitrogen to ensure complete removal of oxygen prior to incubating overnight at 110 °C. After cooling to 21 °C, the samples were neutralised by addition of 150 µL of fresh 4 M NaOH and filtered through a 0.22 µm centrifugal filtration device (Millipore) with centrifugation at 10,000 g and 5 °C for 2 min. Samples were then diluted 10-fold, transferred to UPLC vials and subsequently the vials were transferred to the UPLC auto injector. Amino acid and methionine sulfoxide standards (0-50 pmol) were prepared in npH₂O. 40 µL of samples and standards were transferred to glass vial tubes. The injection volume of the samples and standards was 6 µL. The amino acid derivatives were separated using the gradient described in Table 2.2.
1 mL of incomplete o-phthalaldehyde (OPA) was activated by addition of 5 µL of 2-mercaptoethanol. 20 µL of OPA was added to 40 µL of sample using an autosampler immediately before injecting 6 µL. The OPA tag is fluorescent; consequently amino acids were quantified by fluorescence ($\lambda_{\text{excitation}}$ 340 nm and $\lambda_{\text{emission}}$ 440 nm). The concentration of individual amino acid was determined from the standards of known concentration. A sample trace showing separation of multiple amino acids with their retention times is shown in Figure 2.1.

1 M sodium acetate pH 5.3 was prepared by adding 1 M acetic acid to 1 M sodium acetate until the pH was 5.3. Buffer A consisted of 10 \% (v/v) 1 M sodium acetate (pH 5.3), 2.5 \% (v/v) tetrahydrofuran and 20 \% (v/v) methanol. Buffer B consisted of 10 \% (v/v) 1 M sodium acetate (pH 5.3), 2.5 \% (v/v) tetrahydrofuran and 80 \% (v/v) methanol. The pH of Buffer A and B were adjusted to 5.3. Prior to use both Buffer A and B were filtered through a 0.2 µm filter (Pall). The UPLC was performed using a Shimadzu Nexera system and the column used was a Shimpack C18 100 mm x 4.6 mm UPLC column, which had a 2.2 µm pore size. This column was connected to a Phenomenex Krud Katcher guard column. The flow rate was set at 1.2 mL min$^{-1}$ and the column was equilibrated and incubated at 40 °C.

### Table 2.2 Gradient for the separation of multiple amino acid derivatives.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% of Buffer B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>0.01</td>
<td>1.2</td>
</tr>
<tr>
<td>6</td>
<td>25</td>
</tr>
<tr>
<td>7</td>
<td>25</td>
</tr>
<tr>
<td>7.5</td>
<td>62</td>
</tr>
<tr>
<td>10</td>
<td>62</td>
</tr>
<tr>
<td>12</td>
<td>100</td>
</tr>
<tr>
<td>13</td>
<td>100</td>
</tr>
<tr>
<td>13.5</td>
<td>0</td>
</tr>
</tbody>
</table>
2.3.11 1D gel electrophoresis

BSA, lysates or intact cells that had been either treated or non-treated with ONOOH or SIN-1 were used to perform 1-dimensional gel electrophoresis. 20 µL of each sample was reduced by adding 4 µL of NuPAGE reducing agent and 10 µL of NuPAGE lithium dodecyl sulfate (LDS) sample buffer. The sample buffer contains lithium dodecyl sulfate (pH 8.4) and a high concentration of glycerol that helps to denature the proteins before reduction of the disulphide bonds. The reducing agent was 500 mM dithiothreitol (DTT) that maintains the protein in a reduced state by preventing disulphide bond formation between cysteine residues of proteins. The inclusion of 500 mM DTT in the sample buffer could reverse some of the oxidative effects of ONOOH or SIN-1 treatment. However this is not a concern at this stage.

The resulting solutions were heated to 70 °C for 10 min. After cooling to 21 °C, 20 µL of the reduced sample (containing about 10 µg of protein) was loaded onto each well of the NuPAGE Novex 4-12 % Bis-Tris gel. 3 µL of Precision Plus Protein marker (BioRad) were also loaded onto one of the lanes in each gel. SDS-PAGE was conducted using the NuPAGE MOPS SDS running buffer for 1 h at 150 V.
2.3.12 Western blotting

Following 1D gel electrophoresis as described above, proteins were transferred from the gel to iBlot nitrocellulose membranes using an iBlot transfer device (Life Technologies). The iBlot bottom membrane [Invitrogen iBlot Nitrocellulose Anode stack bottom membrane regular] was placed into the iBlot and a small volume of MQ water was squeezed onto the membrane. The gel was then placed directly onto the bottom membrane. The iBlot filter paper (regular) was then wetted and placed on top of the gel. The gel was then rolled several times to remove air bubbles. The top cathode [Invitrogen iBlot cathode stack top regular] was then placed on top of the gel with the jelly side facing the gel. A sponge [iBlot disposable sponge] was placed on the lid of the iBlot. The iBlot was then placed in P3 mode for 7 min to transfer the proteins onto the membrane. This was confirmed by an efficient transfer of the Precision Plus Protein marker (BioRad) onto the membrane.

All membranes were blocked with 5 % (w/v) skim milk prepared in PBS containing Tween-20 (0.1 %) (v/v) (PBST) for 1 h, before incubating each with primary antibody at 4 °C overnight. Following the removal of primary antibodies, membranes were washed for 3 x 5 min in PBST and then incubated with the respective secondary antibodies for 1 h. All primary and secondary antibodies were diluted in the blocking solution (Table 2.3). After the removal of secondary antibodies, all membranes were washed for 6 x 10 min in PBST and then for 3 x 5 min in PBS.

Immunodetection was conducted with Western Lightning Plus ECL, an enhanced chemiluminescence HRP substrate that is commonly used for Western blotting. This substrate is highly sensitive (detects down to picogram levels of immobilized proteins on a membrane), easy to use, fast, non radioactive and provides low background. This substrate was purchased as a kit, which contains the Enhance Luminal Reagent and Oxidising Reagent. Prior to use, 1 mL of the Enhance Luminal Reagent was mixed with 1 mL of Oxidising Reagent and poured over the membrane. The membrane was then transferred to the transilluminator sample area of the ChemiDoc (BioRad).
Table 2.3 Primary and secondary antibodies used in Western blot analysis to detect individual proteins.

<table>
<thead>
<tr>
<th>Detection</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-nitrotyrosine</td>
<td>Anti-3NT</td>
<td>Anti-mouse HRP</td>
</tr>
<tr>
<td></td>
<td>1:1000</td>
<td>1:2000</td>
</tr>
<tr>
<td>β-actin</td>
<td>Anti-β actin</td>
<td>Anti-mouse HRP</td>
</tr>
<tr>
<td></td>
<td>1:1000</td>
<td>1:2000</td>
</tr>
<tr>
<td>β-tubulin</td>
<td>Anti-β tubulin</td>
<td>Anti-rabbit HRP</td>
</tr>
<tr>
<td></td>
<td>1:500</td>
<td>1:2000</td>
</tr>
<tr>
<td>ABC-G1</td>
<td>Anti-ABCG1</td>
<td>Anti-rabbit HRP</td>
</tr>
<tr>
<td></td>
<td>1:500</td>
<td>1:1000</td>
</tr>
<tr>
<td>VEGF</td>
<td>Anti-VEGF</td>
<td>Anti-rabbit HRP</td>
</tr>
<tr>
<td></td>
<td>1:500</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

Images of proteins of interest were captured using Image Lab software at regular intervals until a saturation point was reached. For every membrane, an image of the marker protein lane was also captured. The best image with low background, no signal saturation and high band intensity was used for further analysis. Protein band analysis was performed using Image J software. The pixel density of protein bands of interest was quantified using this software. ONOOH induced protein modifications were assessed by comparing the treated samples with the non-treated controls.

2.3.13 Coomassie staining

After performing gel electrophoresis as described in Section 2.3.11, the gel was incubated in 100 mL of Coomassie stain solution and heated in the microwave for 20 s. Coomassie stain solution contained 0.1 % (w/v) Coomassie R-250, 40 % (v/v) ethanol, and 10 % (v/v) acetic acid. The gel was left on a rotator for 15 min at 21 °C. The gel was then washed once with npH2O and incubated in 100 mL of destain solution. Destain solution contained 10 % (v/v) ethanol and 7.5 % (v/v) acetic acid. The gel and the destain
solution were heated in microwave for 20 s and left on the rotator at 21 °C. Once protein bands were clearly visible and the desired background was achieved, the gel was washed with npH$_2$O. Images of the gel were captured using a wet scanner. The gel was stored in 1 % (v/v) acetic acid at 4 °C until subjected to in-gel digestion for mass spectroscopy (LC/MS/MS) analysis.

2.3.14 Silver staining

After performing 1D gel electrophoresis as stated in Section 2.3.11, the gel was incubated in 50 % (v/v) methanol and 10 % (v/v) acetic acid solution for 30 min. The gel was then incubated in 5 % methanol for 15 min and washed with npH$_2$O. Subsequently the gel was incubated in freshly prepared 1.26 mM sodium thiosulfate for 2 min and then washed in npH$_2$O. The gel was incubated in cold (4 °C) 11.77 mM silver nitrate for 25 min and then washed in npH$_2$O. Protein bands were visualised using a freshly prepared developer solution [283.05 mM sodium carbonate, 0.05 % (v/v) formaldehyde and 0.03 mM sodium thiosulfate]. 47.91 mM EDTA stop solution was added to chelate silver ions and prevent protein bands from becoming over exposed. Images of gels were captured using a wet scanner and the band intensities of proteins were analysed using Image J software. ONOOH-mediated protein modifications were assessed by comparing the treated samples with the non-treated controls.

2.3.15 Densitometric analysis

After Western blotting and silver staining, densitometric analysis was performed using Image J software. Boxes were constructed around either a lane of proteins or individual protein bands of all controls and samples treated with ONOOH/SIN-1. The pixel density of all controls and treated samples were measured. ONOOH/SIN-1-mediated modifications were calculated by comparing to the respective control.

2.3.16 Immunoprecipitation

A commercially available Dynabeads Protein G Immunoprecipitation Kit was employed to perform small-scale immunoprecipitation (IP). The kit contains Dynabeads Protein G (magnetic beads with recombinant Protein G covalently attached to the surface) and buffers such as the washing buffer and elution buffer. The magnetic separation is fast,
gentle and easy way to separate proteins of interest from cell lysates. This technique dramatically reduces background caused by non-specific binding and induces minimal physical stress to proteins. Due to these advantages, this kit was used to separate nitrated proteins from intact HCAEC exposed to 0 or 500 µM ONOOH.

2.3.16.1 Sample preparation
Cells were seeded at a cell density of $4 \times 10^5$ cells per well in 2 x 6-well plates as described in Section 2.3.4.2. Cells in both plates were washed once with 800 µL of HBSS (pH 7.4) and incubated with 725 µL of HBSS. Cells in one plate were treated with 0 µM ONOOH while cells in the other plate were treated with 500 µM ONOOH. Both plates were incubated for 5 min at 37 °C. The oxidant was removed by aspiration of the solution from the well via vacuum suction. Cells were then washed once with HBSS. 500 µL of lysis buffer was added to all wells. Lysis buffer consisted of 35 mM HEPES, 0.1 % Triton X-100 and 1X Roche complete protease inhibitor. A plunger was used to lyse cells and plates were incubated at 4 °C for 30 min to ensure complete lysis. Lysates from each plates were pooled together and stored on ice.

2.3.16.2 Sample cleanup
The Dynabeads were vortexed and 50 µL were transferred to clean Eppendorf tubes. The tubes were placed on a DynaMag Spin magnet (Life Technologies) and the supernatant was removed. The tubes were removed from the magnet. 750 µL of samples (prepared as described in the above Section) were incubated with the Dynabeads for 1 h at 21 °C in the PTR-25 360° vertical mini rotator (Grant-Bio). Proteins or antibodies within the sample that bind non-specifically to the Dynabeads and block the binding of the antibodies or proteins of interest, will bind to the Dynabeads. The tube was placed on the magnet and the supernatant (containing clean sample) was removed and stored on ice. The Dynabeads containing contaminants were discarded.

2.3.16.3 Antibody complexing
50 µL of unused Dynabeads were transferred to new clean tube. The tube was placed on a magnet and the supernatant was removed. The tube was removed from the magnet. 0.025 µg µL⁻¹ 3-nitrotyrosine antibody (Ab) was prepared in PBST. 200 µL of the 3-nitrotyrosine in PBST was added to the Dynabeads to form Dynabeads-Ab complex and placed in the mini rotator for 10 min at 21 °C. The tube was placed on a magnet and the supernatant was removed. The tube was then removed from the magnet. The Dynabeads-
Ab complex was resuspended in 200 µL PBST to eliminate the excess antibody. The tube was again placed on the magnet and the supernatant was removed. The tube was removed from the magnet.

2.3.16.4 Cross-linking reagent
The light- and heavy-chains of antibodies immobilised on the Dynabeads often co-elute with the proteins of interest and this may interfere with the downstream analysis. This can be avoided by using a cross-linking reagent such as Bis(sulfosuccinimidyl)suberate (BS³). BS³ is a water-soluble crosslinker, which forms irreversible amine-to-amine bonds at physiological pH. The Dynabeads-Ab complex prepared in the above Section was incubated with 200 µL of conjugation buffer. The conjugation buffer contained 20 mM sodium phosphate, 0.15 M sodium chloride and the pH was between 7 and 9. The tube was placed on a magnet, supernatant was removed and the tube was removed from the magnet.

Prior to use, fresh 5 mM BS³ was prepared in conjugation buffer. The Dynabeads were resuspended in 250 µL of 5 mM BS³ to form Dynabeads-Ab-BS³ complex. The tubes were rotated in the mini rotator for 30 min at 21 °C. This cross-linking reaction was quenched by adding 12.5 µL of quenching buffer and left in the mini rotator for 15 min at 21 °C. The quenching buffer contained 1 M Tris hydrochloride and the pH was adjusted to 7.5. The tube was placed on a magnet and the supernatant was removed. The cross-linked Dynabeads were washed 3 times with 200 µL PBST. Between each wash the tubes were placed on a magnet, the supernatant was removed and the tubes were removed from the magnet.

2.3.16.5 Target antigen (nitrated proteins)
750 µL of cleaned up sample from Section 2.3.15.2 was added to the Dynabeads-Ab-BS³ complex, gently resuspended and incubated for 10 min at 21 °C in the mini rotator. This resulted in the formation of a Dynabeads-Ab-BS³-antigen complex. The tube was placed on the magnet and the supernatant was removed. The Dynabeads-Ab-BS³-antigen complex was washed 3 times using 200 µL washing buffer. Between each wash the tubes were placed on a magnet, the supernatant was removed and the tubes were removed from the magnet. The Dynabeads-Ab-BS³-antigen complex was resuspended in 100 µL of washing buffer and transferred to a clean new tube to prevent the contamination of
proteins attached to the tube wall. The tube was placed on a magnet, the supernatant was removed and discarded.

2.3.16.6 Elution

20 µL of elution buffer was added to 10 µL of premixed NuPAGE LDS sample buffer (4X) and NuPAGE reducing agent (10X) as per the manufacturer’s instructions. The resulting solution was added to the Dynabeads-Ab-BS₃-antigen complex (prepared in the above Section) and heated for 10 min at 70 °C to release the antigen (nitrated proteins). The samples were then cooled to 21 °C. The tube was placed on a magnet and the supernatant was removed. All supernatant from treated and non-treated samples were pooled together and gel electrophoresis was performed as described in Section 2.3.11. The resulting gel was subjected to Coomassie staining as described in Section 2.3.13 and then subjected to in-gel digestion for mass spec (LC/MS/MS) analysis.

2.3.17 Protein excision and in-gel digestion

The Coomassie blue stained gel lane was cut into 10 slices using a sterile scalpel. Each lane was then further sliced into many 1 mm² cubes. The gel slices were destained by washing twice with the destain solution for 10 min with vortexing. The destain solution contained 50 % (v/v) acetonitrile (ACN) and 50 mM ammonium bicarbonate (NH₄HCO₃) (pH 9). The gel slices were dehydrated twice with 200 µL of 100 % (v/v) ACN for 10 min with vortexing. The gels were then reduced and alkylated by incubating in 200 µL of 5 mM tributylphosphine and 20 mM acrylamide solution in 100 mM NH₄HCO₃ for 1 h at 21 °C. The gel slices were washed 3 - 4 times with 50 % (v/v) ACN and 50 mM NH₄HCO₃ solution for 10 min with vortexing until the smell was eliminated.

The gel slices were then dehydrated with 100 % (v/v) ACN for 10 min and rehydrated in 50 µL of 100 mM NH₄HCO₃ (pH 9) containing 12.5 ng µL⁻¹ of trypsin (Promega). After incubating at 4 °C for 30 min, 50 µL of 100 mM NH₄HCO₃ was added and the samples were incubated overnight at 37 °C. The digest solution was transferred to a new tube. 50 µL of 50 % ACN (v/v) in 0.2 % (v/v) trifluoroacetic acid was added and incubated for 10 min in a sonicating water bath at full power. The solution was removed and pooled with the digest solution and then placed in a vacuum concentrator to reduce the volume to 15 µL. The samples were stored at 4 °C or transferred to an autosampler vial.
2.3.18 Mass spectroscopy: LC/MS/MS

Using an Eksigent AS-1 autosampler connected to a Tempo nanoLC system (Eksigent, USA), 10 µL of the sample was loaded at 20 µL min⁻¹ with MS buffer A (2 % ACN + 0.2 % trifluoroacetic acid) onto a C8 trap column (Michrom, USA). After washing the trap for 3 min, the peptides were washed off the trap at 300 nL min⁻¹ onto a PicoFrit column (75 µmID x 150 mm; New Objective, Woburn, MA) packed with Magic C18AQ resin (Michrom Bioreources, Auburn, CA). Peptides were eluted from the column and into the source of a QSTAR Elite hybrid quadrupole-time-of-flight mass spectrometer (AB Sciex) using the following program: 5 - 30 % MS buffer B (98 % ACN + 0.2 % trifluoroacetic acid) over 30 min, 30 - 80% MS buffer B over 3 min, 80 % MS buffer B for 2 min, 80 - 5 % for 3 min.

The eluting peptides were ionised at 2300 V. An Intelligent Data Acquisition (IDA) experiment was performed, with a mass range of 350-1500 Da continuously scanned for peptides of charge state 2+ - 5+ with an intensity of more than 30 counts s⁻¹. Selected peptides were fragmented and the product ion fragment masses measured over a mass range of 50-1500 Da. The mass of the precursor peptide was then excluded for 30 s.

The MS/MS data files produced by the QSTAR were searched using Mascot (version 2.4; Perkins, D.N. 1999; provided by the Walter and Eliza Hall Institute) against the LudwigNR database (comprised of the UniProt, plasmoDB and Ensembl databases, vQ214. 32,769,512 sequences) with the following parameter settings. Taxonomy: human. Fixed modifications: none. Variable modifications: propionamide, tyrosine and tryptophan nitration, cysteine, histidine and methionine oxidation. Enzyme: semi-trypsin. Number of allowed missed cleavages: 3. Peptide mass tolerance: 100 ppm. MS/MS mass tolerance: 0.2 Da. Charge state: 2+, 3+ and 4+. The results of the search were then filtered by including only protein hits with at least one unique peptide (Bold Red) and excluding peptide hits with a p-value greater than 0.05.

Peptides identified by Mascot were further validated using Scaffold (v4.0, Proteome Software, Portland, OR) and by manual inspection of the MS/MS spectra for the peptide to ensure the b- and y-ion series were sufficiently extensive for an accurate identification. Peptide identifications were accepted if they could be established at greater than 95.0 % probability as specified by the Peptide Prophet algorithm (327). Protein identifications
were accepted if they could be established at greater than 95.0 % probability assigned by the Protein Prophet algorithm (328). Data was also searched using Peaks Studio (v7, Bioinformatics Solutions, Waterloo, Ontario) employing the Peaks PTM algorithm.

2.3.19 Statistical analysis

After performing an experiment 3 different times, the averages and standard deviations of each treatment were calculated. Using the averages and standard deviations, statistical analysis was performed using Prism software. One-way ANOVA was performed using the Dunnett’s post-hoc test to compare ONOOH/SIN-1 treated samples with their respective controls. 2-Way ANOVA was performed using Bonferroni post-hoc to investigate the effect of 25 mM NaHCO₃ on ONOOH or SIN-1 mediated damage. When the p-value of a modification was determined to be less than 0.05, that modification was considered significant.
Chapter 3: Damage Induced by Peroxynitrous Acid (ONOOH)

to isolated peptides and proteins
3.1 Introduction

Peroxyynitrous acid (ONOOh) modifies antioxidants, nucleic acids, proteins, lipids and carbohydrates (63). About 70% of the dry mass of cells is made of proteins. As a consequence, proteins are an important target of ONOOH due to their abundance and high reactivity (323). ONOOH oxidises amino acids such as cysteine, methionine, tyrosine, tryptophan, histidine and phenylalanine (63, 163). ONOOH also nitrates certain amino acids such as tyrosine and tryptophan (155). The most crucial protein modifications reported in the literature for ONOOH include thiol oxidation and tyrosine nitration (117).

Glutathione (GSH) is a ubiquitous water-soluble tripeptide that consists of the amino acids glutamate, cysteine and glycine. GSH is a highly abundant peptide thiol found in every cell type. In some tissues, GSH can reach up to millimolar concentrations (329). GSH is a major endogenous antioxidant that detoxifies many electrophilic species and peroxides through its role in acting as a cofactor for many cytoplasmic enzymes such as glutathione S-transferases and glutathione peroxidases (109). GSH may also play a role in mediating important post-translational modifications of numerous proteins. GSH is also crucial for other cellular reactions such as the glyoxalase system, reduction of ribonucleotides to deoxyribonucleotides and modulation of protein and gene expression through thiol to disulphide exchange reactions (330).

The thiol group of the cysteine residue in GSH is a potent reducing agent and acts as a nucleophile during reaction with both exogenous and endogenous electrophilic species. Consequently ROS and RNS can be removed by GSH via both spontaneous and catalytic reactions (330). GSH oxidation has been used as a quantitative marker of oxidative stress and forms multiple products such as glutathione disulphide (GSSG), mixed disulphides with other intracellular thiols, glutathione sulphonamide and oxy acids (e.g. sulphenic acid RSOH, sulphinic acid RSO2H and sulphonic acid RSO3H) (323, 331). Maintaining an optimal GSH:GSSG ratio is crucial for cell survival, with a deficiency in GSH making cells susceptible to oxidative damage (330).

Thiols are believed to be a key target for ONOOH. ONOOH has been reported to oxidise the thiol group of GSH to form GSSG via an oxygen-consuming pathway with a rate constant of 1350 M⁻¹s⁻¹ at pH 7.4 and 37 °C (62). Although this rate constant is not very
high, the high intracellular GSH concentrations make GSH an efficient scavenger of ONOOH (110).

Oxidation of protein-bound cysteine residues is commonly used as a non-specific measure of protein modification. This reaction occurs with multiple oxidants such as hydrogen peroxide and other peroxides (332), ONOOH (333), nitric oxide (334), singlet oxygen (335) and hypochlorous acid (336). The products include cystine (disulphide), mixed disulphides (such as protein-glutathione species), nitroso adducts and oxy acids (e.g. sulphenic acid RSOH, sulphinic acid RSO\(_2\)H and sulphonlic acid RSO\(_3\)H) (337). Since multiple enzymes have a cysteine at their active site, which is crucial for their activity, thiol oxidation or modification can induce loss of enzyme function (323).

It has been shown that ONOOH modifies BSA by targeting its amino acids (118, 119, 338). BSA has 35 cysteine residues but 34 of these are present in 17 disulphide bonds. The free single cysteine residue (Cys 34) has been proposed to be a major target of ONOOH (118). In a previous study 60.6 µM BSA was treated with ONOOH at 10:1, 1:1, 1:5 and 1:10 ratio of protein to ONOOH at pH 7.4 and 25 °C. Exposure of the protein to ONOOH at a ratio of 1:1, 1:5 and 1:10 caused oxidation of 41, 74 and 77 % of the cysteine residue respectively (118). The second order rate constant for the reaction of ONOOH with Cys 34 of BSA has been reported to be 2,700 M\(^{-1}\)s\(^{-1}\) at pH 7.4 and 37 °C (61).

The 21 tyrosine residues on BSA (339) are also reported to be important targets of ONOOH. ONOOH reacts indirectly with tyrosine residues to form 3-nitrotyrosine (3NT) (140). 3NT formation has been detected in BSA exposed to ONOOH at physiological pH (106, 119). Reaction of protein with ONOOH at a ratio of 1:1, 1:5 and 1:10, results in the nitration of 0.12, 1.4 and 3.2 % of the tyrosine residues (118). The reaction of free tryptophan with ONOOH is known to form 6-nitrotryptophan (the major product) and minor products such as 4-, 5- and 7-nitrotryptophan (158). The 3 tryptophan residues of BSA (339) are also a target of ONOOH. Treatment of BSA with 1:1, 1:5 and 1:10 ratio of protein to ONOOH caused oxidation of 12, 29 and 45 % of the tryptophan residues respectively.
With a protein to \textit{ONOOH} ratio of 1:1 or greater, a dose-dependent increase in protein carbonyl formation was also detected (118). Exposure of BSA to \textit{ONOOH} has also been reported to cause protein fragmentation with an increase in band intensity of species with molecular mass less than that of BSA. In the absence of CO$_2$, low molecular mass molecules/antioxidants, such as uric acid, ascorbate and sulphhydrolys, inhibit 3NT formation (106). However in the presence of CO$_2$, 3NT formation increased in a concentration dependent manner.

From the above data it is clear that BSA can be modified by \textit{ONOOH}. However the extent and order in which amino acids of BSA are modified by \textit{ONOOH} is currently unclear. Therefore the initial studies of this project tested different methods of detecting thiol loss using HCAEC lysates as a model and then investigated the efficacy of \textit{ONOOH} and ONOOCO$_2^-$-mediated modification of the thiol residue of GSH, a simple tripeptide. Subsequent studies were carried out using \textit{ONOOH} and ONOOCO$_2^-$-mediated modification of BSA amino acids, a more complicated system. We aimed to identify the order and efficacy at which \textit{ONOOH} and ONOOCO$_2^-$-modify specific BSA residues.

\subsection*{3.2 Aims}

The aim of the experiments reported in this Chapter was first to compare different assays for measuring thiol loss using HCAEC lysates, and to investigate the damage induced by \textit{ONOOH} to the amino acids of GSH and BSA. Importantly this Chapter aimed to identify the order and efficacy with which \textit{ONOOH} modifies the amino acids of BSA. The effect of 25 mM NaHCO$_3$ (which yields physiological levels of CO$_2$) on \textit{ONOOH}-mediated damaged were also examined. The damage induced by \textit{ONOOH} to BSA and GSH was assessed by measuring thiol loss via ThioGlo assay. Loss of other BSA amino acids such as histidine, methionine, phenylalanine, tryptophan and tyrosine were detected via Ultra Performance Liquid Chromatography. 3-nitrotyrosine formation was detected via Western blotting and \textit{ONOOH}-mediated protein modifications were detected via silver staining.
3.3 Method Development and Results

3.3.1 Assessment of thiol Loss

3.3.1.1 Methods for measuring thiol loss

Initial studies focussed on examining thiol modification induced by ONOOH using HCAEC lysates, as damage to these cells was a primary aim of this project. Protein and peptide-bound thiols can be detected by multiple methods including spectrophotometric (e.g. 5,5’-dithiobis(2-nitrobenzoic acid), commonly known as DTNB) (340), fluorometric (e.g. ThioGlo™ 1,3,5) (323) or mass spectrometry (341) amongst others. The DTNB and ThioGlo assays are often preferred over other techniques because they are easy, highly sensitive and require small amounts of sample, and so represent a cost effective way to measure large numbers of samples containing thiols (323).

In initial studies a comparison was carried out between the fluorimetric methods using ThioGlo 1, 3 and 5, and DTNB. ThioGlo™ 1 (10-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)-9-methoxy-3-oxo-3H-naphthol[2,1-b]pyran-2 carboxylic acid methyl ester) is a maleimide derivative of a naphthopyranone fluorophore that forms a fluorescent adduct upon reaction with reduced thiols at neutral pH (Figure 3.1). The adduct can be detected fluorimetrically (λ_{excitation} = 384 nm and λ_{emission} = 513 nm) (323). ThioGlo™ 1 has a detection limit of about 10 nM for most thiols (120).

![Figure 3.1 Reaction of ThioGlo™ 1 reagent with thiols.](image)

Reaction of ThioGlo™ 1 reagent with thiol containing compounds forms a fluorescent adduct. The fluorescent adduct can be detected at λ_{excitation} = 384 nm and λ_{emission} = 513 nm. This figure has been taken from (323).
ThioGlo™ 3 (9-acetoxy-2-(4-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)pyenyl)-3-oxo-3H-naphtho[2,1-b]pyran) is an analogue of ThioGlo™ 1. Like ThioGlo™ 1, ThioGlo™ 3 is a maleimide reagent that is commercially available. ThioGlo™ 3 is non-fluorescent compound which reacts rapidly with reduced thiols at neutral pH to form a fluorescent adduct (Figure 3.2). The adduct can be detected fluorimetrically (\(\lambda_{\text{excitation}} = 365\) nm and \(\lambda_{\text{emission}} = 445\) nm) (325).

![Figure 3.2 Reaction of ThioGlo™ 3 reagent with thiols.](image)

**Figure 3.2 Reaction of ThioGlo™ 3 reagent with thiols.**

Reaction of ThioGlo™ 3 reagent with thiol containing compounds forms a fluorescent adduct. The fluorescent adduct can be detected at \(\lambda_{\text{excitation}} = 365\) nm and \(\lambda_{\text{emission}} = 445\) nm. This figure has been taken from (325).

ThioGlo™ 5 (methyl 9-maleimido-8-methoxy-2-oxo-2H-naphtho[2,3-b]pyran-3-carboxylate) is a related non-fluorescent commercially available maleimide reagent that reacts rapidly with reduced thiols at neutral pH to form a fluorescent adduct (Figure 3.3) which can be detected fluorimetrically (\(\lambda_{\text{excitation}} = 365\) nm and \(\lambda_{\text{emission}} = 536\) nm).
Apart from ThioGlo™ 1, 3 and 5, the DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) assay can also be used to measure thiols. DTNB reacts with reduced thiols to form 5-thio-2-nitrobenzoic acid (TNB) and a mixed disulphide (Figure 3.4). TNB can be detected by UV/Vis spectrophotometry at 412 nm (323).
3.3.1.2 Comparison of different methods for measuring thiol loss

As damage to human coronary artery endothelial cells (HCAEC) is a primary focus of this project, initial studies aim to identify the best reagent for quantifying thiols using HCAEC lysates, which will also be applied to BSA and GSH. It was expected that in the absence of HCAEC cell membrane, a dramatic thiol loss would occur when the intracellular thiols were exposed to increasing concentrations of ONOOH.

HCAEC were seeded at a cell density of 4 \times 10^5 cells per well in a 6 well plate. Cells were washed and lysed in cold (4 °C) npH2O. ONOOH was prepared in 20 mM NaOH. The lysates were treated with varying concentrations of ONOOH (0-100 µM) for 5 min at pH 7.4 and 21 °C. At physiological pH, ONOOH has a half-life of about 10 ms (68). As a result, a 5 min incubation time was chosen as the reaction time. The lysates were also treated with decomposed 100 µM ONOOH to examine whether there was any other thiol modifying agent present in the decomposed samples. The thiol concentrations remaining after ONOOH exposure were quantified using the ThioGlo assay (ThioGlo™ 1, 3 and 5 reagents) and DTNB assay. The protein concentrations were determined via the BCA assay. The results were expressed as thiol concentrations relative to the protein concentrations.

Initially thiol concentrations were measured using ThioGlo™ 1. In the untreated lysates (HCAEC lysates only; HO) and lysates treated with 0 µM ONOOH (i.e. 20 mM NaOH in which ONOOH was dissolved), about 75 and 74 µmoles/g of thiols relative to protein were detected respectively (Figure 3.5A). No significant thiol loss was detected when the untreated lysates (HO) were compared to 0 µM ONOOH, indicating that 20 mM NaOH did not oxidise thiol residues of lysates. No significant thiol loss was also detected when lysates were treated with decomposed 100 µM ONOOH (De), which indicated that in the absence of active ONOOH no other thiol modifying species were present. Treatment of lysates with 5 µM ONOOH oxidised approximately 20 µmoles/g of thiols relative to protein concentration. A greater extent of thiol loss was detected when lysates were treated with 10 µM or higher concentrations of ONOOH. Treatment of lysates with 75 µM ONOOH oxidised almost all of the thiol residues.

ThioGlo™ 3 detected 9 µmoles/g of thiols relative to protein in the HO sample (Figure 3.5B). No significant thiol loss was detected when lysates were treated with De (100 µM) and 0 µM ONOOH compared to the HO control. Treatment of lysates with 5 and 10 µM
ONOOh caused no significant thiol loss. In contrast exposure to 20 μM ONOOH induced a significant thiol loss (50 %) and more thiols were lost with increasing ONOOH concentrations. Although the overall trend seen with ThioGlo™ 3 is similar to that seen with ThioGlo™ 1 (Figure 3.5A), the overall thiol concentrations relative to protein detected with ThioGlo™ 3 reagent were significantly lower than those detected with ThioGlo™ 1 reagent (9 and 75 μmoles/g of thiols relative to protein concentration respectively).

ThioGlo™ 5 detected about 82 μmoles/g of thiols relative to protein concentration in the HO control (Figure 3.5C). No significant thiol loss was detected when lysates were treated with De (100 μM), 0 and 5 μM ONOOH compared to the HO control. Treatment of lysates with 10 μM ONOOH oxidised about 40 μmoles/g of thiols relative to protein concentration. More thiols were oxidised by ONOOH in a dose-dependent manner. Exposure to 75 μM ONOOH oxidised almost all thiol residues.

The thiol concentrations detected with ThioGlo™ 5 were close to those detected with the ThioGlo™ 1 reagent. For instance in the HO control, ThioGlo™ 1 and 5 reagents detected about 74 and 82 μmoles/g of thiols relative to protein concentration. Both of these values are significantly higher than those detected with the ThioGlo™ 3 reagent (Figure 3.5B). The similar molecular structures of ThioGlo™ 1 and 5 reagents may be responsible for the detection of similar thiol concentrations.

The DTNB assay is another technique used to measure thiols. Reaction of reduced thiols with DTNB generates free TNB and a mixed disulphide (Figure 3.4). When this method was used to examine the HCAEC lysates in a similar manner to the experiments described above, the DTNB assay detected significantly higher thiol values relative to protein concentrations (Figure 3.6) compared to those achieved with the ThioGlo assay (Figure 3.5). For instance, the DTNB assay and ThioGlo assay (using ThioGlo™ 1 reagent) detected about 124 and 75 μmoles/g of thiols relative to protein concentrations in the HO control sample respectively.
Figure 3.5 ThioGlo assay detected ONOOH-mediated thiol loss in HCAEC lysates. The ThioGlo assay employed ThioGlo™ 1, 3 and 5 reagents.

HCAEC lysates were treated with ONOOH (0-100 µM) for 5 min at 37 °C and pH 7.4 followed by determination of thiols using the ThioGlo assay. ThioGlo assay was performed using either ThioGlo™ 1 (green), 3 (blue) or 5 (yellow) reagents. Results are expressed as concentrations of thiols relative to the protein concentration. HO represents untreated lysates. De represents lysates treated with decomposed 100 µM ONOOH. * represents significant difference (p < 0.05) in thiol concentrations relative to those obtained with 0 µM ONOOH, as determined by one-way ANOVA, with Dunnett’s post-hoc test. Values are means ± standard deviation and represent results obtained from 3 independent experiments using 3 different HCAEC donors.
No significant thiol loss was detected using the DTNB assay when lysates were treated with De (100 µM) and 0 µM ONOOH compared to the HO control. Surprisingly, no significant thiol loss was detected when lysates were exposed to up to 100 µM ONOOH though there was a clear trend towards a decrease but also a large variability in the data. The thiol values did not go down to zero even with high ONOOH concentrations. This indicates that use of this assay with HCAEC lysates gives rise to a high background absorbance. This could be due to formation of other product such as 3NT that absorb at the wavelengths employed in the assay. Like TNB (Figure 3.4), 3NT has a yellow colour and could be responsible for the high background absorbance.

![Figure 3.6 DTNB assay detected thiol loss when lysates were treated with ONOOH.](image)
HCAEC lysates were treated with ONOOH (0-100 µM) for 5 min at 37 °C and pH 7.4 followed by determination of thiols using the DTNB assay. Results are expressed as concentrations of thiols over protein. HO represents untreated lysates. De represents lysates treated with decomposed 100 µM ONOOH. Values are means ± standard deviation and represent results obtained from 3 independent experiments using 3 different HCAEC donors.

A comparison was made between the DTNB and ThioGlo assay (ThioGlo™ 1, 3 & 5 reagents) to determine which is the best assay to detect thiol loss. A suitable assay would be affordable, sensitive and not suffer from significant background interference particularly in complex samples (e.g. cell samples) containing low levels of thiols.

ThioGlo™ 1 reagent did not non-specifically bind to other amino acids in HCAEC lysates and this was detected using N-Ethylmaleimide. No significant difference was detected between the absolute thiol concentrations detectable using ThioGlo™ 1 and 5 reagents (Figure 3.7). Due to their similar structures (Figure 3.1 & 3.3), both of these reagents may be binding to thiols with similar affinities. Although ThioGlo™ 5 is more
expensive than ThioGlo™ 1, the former reagent seems to be a good alternative to ThioGlo™ 1, though the variability in the data obtained with ThioGlo 5 was higher than with ThioGlo 1 (see data in Figure 3.5). In contrast, significant differences in the thiol concentrations were detected between ThioGlo™ 1 and 3 (Figure 3.7). Compared to ThioGlo™ 1, the thiol concentrations detected using ThioGlo™ 3 were significantly lower. This is most probably due to differences in the structure or reactivity of these reagents. The thiol concentrations detected using the DTNB assay were significantly higher compared to those detected with ThioGlo™ 1, however large error bars were detected in the DTNB assay and the thiol values do not go to zero when lysates are exposed to up to 100 µM ONOOH. As a result, quantification of thiols using DTNB assay does not seem to be a good way of measuring an overall loss of thiols in this system.

These data suggest that under these experimental conditions, the ThioGlo assay gives better results than DTNB assay. The ThioGlo assay appears to be more sensitive than the DTNB assay, does not suffer from significant background interference and requires lower amounts of protein; this is consistent with a previous report that ThioGlo™ 1 reagent is 40 times more sensitive than DTNB (120). ThioGlo™ 1 was therefore preferred over ThioGlo™ 3 and 5 and DTNB for further experiments.

3.3.2 Thiol oxidation

Having examined the relative efficiencies and sensitivities of the various tested methods for determining thiol levels, studies were carried out to examine ONOOH-mediated thiol oxidation in GSH and BSA.

3.3.2.1 GSH oxidation

7.5 µM GSH was treated with varying concentrations of ONOOH (0-100 µM) for 5 min at 21 °C and pH 7.4. The thiol concentrations in GSH remaining after exposure to ONOOH were detected using the ThioGlo™ 1 reagent. 7.5 µM of thiol residues were detected in the non-treated controls, which were prepared using 7.5 µM GSH. A significant loss (approximately 60 % loss) of thiols was detected when GSH was treated with 10 µM ONOOH compared to the non-treated control (Figure 3.8). About 90 % of thiols were oxidised when GSH was exposed to 20 µM ONOOH. Approximately 30 µM ONOOH completely oxidised all the thiols present.
Figure 3.7 Comparison of different methods to detect ONOOH-mediated thiol loss. These methods included ThioGlo assay (using ThioGlo™ 1, 3 and 5 reagents) and DTNB Assay.

HCAEC lysates were treated with ONOOH (0-100 µM) for 5 min at 37 °C and pH 7.4 followed by determination of thiols using DTNB (pink), ThioGlo™ 1 (green), 3 (blue) or 5 (yellow) reagents. Results are expressed as concentrations of thiols over protein. HO represents untreated lysates. De represents lysates treated with decomposed 100 µM ONOOH. * and # represents significant difference (p < 0.05) in thiol concentrations relative to those obtained with ThioGlo™ 1 reagent, as determined by repeated measures two way ANOVA, with Bonferroni post-hoc test. Values are means ± standard deviation and represent results obtained from 3 independent experiments using 3 different HCAEC donors.

As CO₂ is known to modulate the reactions of ONOOH, the effect of 25 mM NaHCO₃ (which gives a near physiological level of CO₂ of 1.3 mM (99)) on GSH thiol loss induced by ONOOH was investigated. In the presence of 25 mM NaHCO₃, a significant loss (approximately 30 %) of thiols was detected when GSH was treated with 10 µM ONOOH compared to the non-treated control. More thiols were oxidised as the ONOOH concentrations used were increased. About 50 µM ONOOH was required to oxidise all the thiol residues of GSH in the presence of 25 mM NaHCO₃. The percentage of thiol loss in the presence of CO₂ was statistically different to the system in the absence of CO₂. These results suggest that the presence of CO₂ (generated from 25 mM NaHCO₃) is protecting against thiol oxidation mediated by ONOOH. This is believed to be due to differences in the reactivity of ONOOCO₂⁻ and ONOOH.
Figure 3.8 Loss of thiols in GSH treated with ONOOH.

7.5 µM GSH was incubated with ONOOH (0-100 µM) for 5 min at 21 °C and pH 7.4 followed by determination of thiols using ThioGlo™ 1 reagent. The red and black bars represent loss of thiols either in the presence or absence of 25 mM NaHCO$_3$ respectively. Results are expressed as percentage of thiols relative to the untreated control. * and # represents significant loss (p < 0.0001 and 0.001 respectively) of thiols relative to the untreated control, as determined by one way ANOVA, with Dunnett’s post-hoc test. ^ represents significant difference between the absence and presence of 25 mM NaHCO$_3$, as determined by repeated measures two way ANOVA, with Bonferroni post-hoc test. Thiols were lost to a greater extent in the absence of 25 mM NaHCO$_3$. Small error bars were detected on the black bars while large error bars were detected on the red bars. Values are means ± standard deviation and represent results obtained from 3 independent experiments.

3.3.2.2 BSA thiol oxidation

BSA has 35 cysteine residues but 34 of these exist as disulphide bonds leaving a single free cysteine residue (Cys 34). Commercial BSA is known to be partially oxidised at the single Cys34 residue and it was therefore important to know the initial thiol concentration. Using ThioGlo 1™ reagent, ~ 2.1 µM of thiol residues were detected in the untreated samples which contained 7.5 µM BSA. 7.5 µM BSA was subsequently treated with varying concentrations of ONOOH (0-100 µM) for 5 min at 21 °C and pH 7.4, followed by detection of thiols using ThioGlo 1™ reagent.

A significant loss (approximately 40 %) of thiols was detected when BSA was exposed to 10 µM ONOOH (Figure 3.9). A greater extent of thiol loss was seen as the ONOOH concentration was increased. 50 µM ONOOH oxidised almost all thiol residues of BSA. Overall a 6-fold excess of ONOOH was needed to oxidise all the BSA thiol residues. When compared to the data obtained with GSH, a higher ONOOH concentration
appeared to be required to oxidise all thiol residues of BSA. These data are consistent with ONOOH reacting also at other sites on this protein. The effect of 25 mM NaHCO₃ (and hence CO₂) on thiol loss induced by ONOOH was also investigated. In the presence of 25 mM NaHCO₃, a significant loss (approximately 40 %) of thiols was detected when BSA was treated with 20 µM ONOOH. The percentage of thiol loss in the presence of CO₂ was statistically lower than that for the system where CO₂ was absent.

Overall these data indicate that ONOOH and to a lesser extent ONOOCO₂⁻ rapidly and effectively oxidise the single free cysteine residues of BSA and GSH. The reaction is efficient and near stoichiometric with GSH. However more ONOOH was required to induce the same extent of thiol loss in BSA. This result is consistent with damage to other residues of BSA in addition to cysteine, which is examined in subsequent experiments.

![Figure 3.9 Loss of thiols in BSA treated with ONOOH.](image)

7.5 µM BSA was incubated with ONOOH (0-100 µM) for 5 min at 21 °C and pH 7.4 followed by determination of thiols using ThioGlo™ 1 reagent. Under these experimental conditions, untreated 7.5 µM BSA contained about ~ 2.1 µM thiols. The red and black bars represent loss of thiols either in the presence or absence of 25 mM NaHCO₃ respectively. Results are expressed as percentage of thiols relative to the untreated control. * and #represents significant loss (p < 0.0001 and 0.001 respectively) of thiols relative to the untreated control, as determined by one way ANOVA, with Dunnett’s post-hoc test. ^ represents significant difference between the absence and presence of 25 mM NaHCO₃, as determined by repeated measures two way ANOVA, with Bonferroni post-hoc test. Thiols were lost to a greater extent in the absence of 25 mM NaHCO₃. Values are means ± standard deviation and represent results obtained from 3 independent experiments.
3.3.3 Modification of other amino acid residues of BSA

In the light of the above data, ONOOH-mediated modifications of various amino acids in BSA were investigated using total amino acid analysis by Ultra Peformance Liquid Chromatography (UPLC). In this method, protein(s) are hydrolysed to their constituent free amino acids, which are then derivatised with o-phthalaldehyde (OPA) and later separated via UPLC. OPA is a fluorescent tag, which in the derivatisation step reacts with the amine groups of amino acids to form adducts that can be detected and quantified via fluorescence relative to amino acid standards (323). This method also enables the quantification of both parent amino acids (such as alanine, arginine, aspartate, glutamate, glycine, isoleucine, histidine, leucine, lysine methionine, phenylalanine, serine, threonine, tryptophan, tyrosine and valine) and some oxidation and modification products such as methionine sulphoxide (342) and advanced glycation end products such as S-(carboxymethyl) cysteine (343), but not cysteine or cysteine, which are destroyed by the acidic conditions, or proline, which does not react with OPA rapidly.

Methanesulphonic acid (MSA) was used for the protein hydrolysis because MSA does not destroy methionine and tryptophan residues (as occurs with 6 M hydrochloric acid) (323) which are potentially important targets for ONOOH (63). During MSA hydrolysis, asparagine and glutamine are converted to aspartic acid and glutamic acid respectively (323). The hydrolysis was conducted under vacuum to minimise artifactual oxidation (323). As isoleucine is known to be unreactive with ONOOH (63), the recovery of all amino acids has been normalised to this amino acid.

7.5 µM BSA was exposed to varying concentrations of ONOOH (0 – 10 mM) for 5 min (due to short half life of ONOOH) at pH 7.4 and 21 °C and then subjected to analysis. The following subsections discuss the data obtained for histidine, methionine, phenylalanine, tryptophan and tyrosine as these species have all been previously reported to be targets for ONOOH (63). BSA contains 17 histidine, 5 methionine, 30 phenylalanine, 3 tryptophan and 21 tyrosine residues (339). The other amino acids present in BSA are not known targets of ONOOH (63).

3.3.3.1 Percentage recovery of amino acids

7.5 µM BSA was treated with 0 – 10 mM ONOOH for 5 min at pH 7.4 and 21 °C. 2.14 pmoles of untreated protein was injected into the UPLC after MSA hydrolysis. When
isolated proteins are subjected to MSA hydrolysis, generally 80-90 % of all amino acids are recovered after the hydrolysis except for methionine and tryptophan (323), where typical values are 60-80 %. The percentage recoveries of multiple BSA (untreated) amino acids detected in these experiments are shown in Table 3.1. The percentage recoveries of most of the amino acids were within their expected range except for glycine, tryptophan and tyrosine. The chromatogram showed that peaks for all amino acids in the control and treated samples were normal except for tyrosine and tryptophan.

Unexpectedly, the percentage recovery of tyrosine in the control sample was $\sim 228 \pm 116 \%$. A chromatogram showing the tyrosine peak is shown in Figure 3.10A. The tyrosine peaks in all control samples appeared normal, and lacked shoulders, tails or any distortion. All the tyrosine peaks had the same retention times in all control samples. The high percentage recovery is most probably due to a coeluting peak that significantly increased the peak area. A coeluting peak may also be responsible for the high percentage recovery ($117 \pm 37 \%$) of glycine. Due to large variations in the percentage recoveries of tyrosine and glycine between replicates, larger standard deviations were detected. Due to such high standard deviation (37 %), the percentage recovery for glycine (117 %) is not significantly different to 100 %.

The percentage recovery of tryptophan was $97 \pm 68 \%$. A chromatogram showing the tryptophan peak in a control sample is shown in Figure 3.10B. Unlike the tyrosine peak, the tryptophan peak appears distorted with a slight shoulder at the beginning of the peak. This most probably increased the peak area and resulted in a higher than expected percentage recovery of tryptophan compared to the typical values of 60-80 %. In this situation, it was difficult to quantify the exact peak area because integration of the distorted peak is likely to either under- or over-estimate the true peak area.

Methionine residues are readily oxidised and one of the major products is methionine sulfoxide though this can be further oxidised to methionine sulphone (167). In this control sample $\sim 30 \%$ of methionine residues were recovered, which indicated that the majority of the methionine residues were oxidised prior or during hydrolysis. 41 % of methionine sulfoxide was detected, which indicated that despite efforts to prevent artifactual generation; some methionine sulfoxide was already present in the control samples. This product might be formed either during protein isolation, storage or in vivo.
All of the subsequent data are expressed relative to the control samples therefore the low recovery of some of the amino acids is not a major problem. This should not alter the data (at least in most cases), unless the recovery values vary with oxidation.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Percentage Recovery (%)</th>
<th>Standard Deviation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>61</td>
<td>8</td>
</tr>
<tr>
<td>Arginine</td>
<td>77</td>
<td>9</td>
</tr>
<tr>
<td>Aspartate</td>
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<td>11</td>
</tr>
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<td>Glutamate</td>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
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<td>9</td>
</tr>
<tr>
<td>Leucine</td>
<td>65</td>
<td>6</td>
</tr>
<tr>
<td>Lysine</td>
<td>68</td>
<td>3</td>
</tr>
<tr>
<td>Methionine</td>
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</tr>
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<td>54</td>
</tr>
<tr>
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<td>4</td>
</tr>
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<td>Serine</td>
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</tr>
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<td>116</td>
</tr>
<tr>
<td>Valine</td>
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<td>6</td>
</tr>
</tbody>
</table>

Table 3.1 Percentage recoveries of untreated BSA amino acids or product (methionine sulphoxide) after MSA hydrolysis and UPLC.

The percentage recoveries and their respective standard deviations of amino acids and product (methionine sulphoxide) were calculated using BSA control sample (untreated) after the protein was subjected to MSA hydrolysis and UPLC. Values are means ± standard deviation and represent results obtained from 3 independent experiments.
Figure 3.10 UPLC chromatogram showing tyrosine (A) and tryptophan (B) peaks in the control samples.

In this UPLC experiment, tyrosine (A) peak appeared normal while the tryptophan (B) peak had a slight shoulder in the control samples (untreated BSA). Similar results were detected in all replicates. Integration of tryptophan peak can either over- or under-estimate the true peak area.
3.3.3.2 Aromatic amino acids

Low ONOOH concentrations (≤ 1000 µM) did not induce any changes to the tyrosine peak. Unexpectedly, the chromatogram of the tyrosine peak in the BSA sample treated with 10 mM ONOOH showed the presence of a tail (Figure 3.11A). The tail of this peak was removed by splitting the peak and then the area under the peak was estimated. Evidence was obtained for about 34% loss of tyrosine residues when BSA was treated with 10 mM ONOOH compared to its respective control (Figure 3.12A). A similar result was detected in the presence of 25 mM NaHCO₃, suggesting that ONOOH and ONOOCO₂⁻ modified tyrosine residues to a similar extent.

With low concentrations of ONOOH (≤ 1000 µM), no change in tryptophan peak area was detected. However, the chromatograms of the samples treated with 10 mM ONOOH also showed distorted tryptophan peaks (Figure 3.11B). A shoulder appears at the beginning of the tryptophan peak, which would increase the peak area and result in inaccurate tryptophan quantification. This peak was split and the area of the peak was estimated. A 24 and 45% loss of tryptophan residues were detected when BSA was treated with 1 and 10 mM ONOOH respectively (Figure 3.12B). Similar data were obtained when these experiments were performed in the presence of 25 mM NaHCO₃, suggesting that the presence of ONOOCO₂⁻ made no significant difference to the loss of tryptophan residues under these conditions.

No significant loss of histidine residues was detected when BSA was treated with up to 10 mM ONOOH compared to its respective control (Figure 3.12C). In the presence of 25 mM NaHCO₃, approximately 20% of histidine residues were lost when BSA was treated with 10 mM ONOOH compared to the control. No loss was detected at lower oxidant levels. These data indicate that ONOOCO₂⁻ oxidised histidine residues to a greater extent than ONOOH particularly at high concentrations.

In contrast, no significant loss of phenylalanine was detected when BSA was treated with up to 10 mM ONOOH (Figure 3.12D). Similar data were obtained in the presence of 25 mM NaHCO₃. These data indicate that under these experimental conditions neither ONOOH nor ONOOCO₂⁻ gave rise to significant modification of phenylalanine.
Figure 3.11 UPLC chromatogram showing tyrosine (A) and tryptophan (B) peaks in the sample treated with 10 mM ONOOH.

In this UPLC experiment, tyrosine (A) peak showed evidence of tailing while the tryptophan (B) peak had a slight shoulder in the samples treated with 10 mM ONOOH. Similar results were detected in all replicates. Integration of tyrosine and tryptophan peaks can either over- or under-estimate the true peak area. Consequently it was difficult to accurately calculate the area under these peaks and to quantify the concentration of tyrosine and tryptophan in these samples.
Figure 3.12 ONOOH-mediated modification of aromatic amino acids of BSA.

7.5 µM BSA was incubated with ONOOH (0-10 mM) for 5 min at 21 °C and pH 7.4 followed by determination of loss of (A) tyrosine, (B) tryptophan, (C) histidine and (D) phenylalanine using total amino acid analysis by Ultra Performance Liquid Chromatography. The red and black bars represent loss of amino acids either in the presence or absence of 25 mM NaHCO₃ respectively. Results are expressed as percentage of amino acids relative to 0 µM ONOOH. * and # represents significant loss of amino acids relative to 0 µM ONOOH, as determined by repeated measures ANOVA, with Bonferroni post-hoc test. ^ represents significant difference between the absence and presence of 25 mM NaHCO₃. Values are means ± standard deviation and represent results obtained from 3 independent experiments.
3.3.3.3 Methionine

Methionine and its oxidation product, methionine sulphoxide can be detected via total amino acid analysis by UPLC. A significant loss of methionine residues (Figure 3.13A) and a corresponding increase in methionine sulphoxide (Figure 3.13B) formation were detected when the BSA was exposed to increasing concentrations of ONOOH. In the presence of 25 mM NaHCO₃, a lesser extent of methionine loss (Figure 3.13A) and methionine sulphoxide (Figure 3.13B) formation were detected. Overall the presence of 25 mM NaHCO₃ in the reaction mixtures seems to protect methionine residues from being oxidised by ONOOH.

![Graph A: Methionine](image)

![Graph B: Methionine Sulphoxide](image)

**Figure 3.13 ONOOH-mediated modification of methionine of BSA.**

7.5 µM BSA was incubated with ONOOH (0-10 mM) for 5 min at 21°C and pH 7.4 followed by determination of loss of methionine and formation of methionine sulphoxide using total amino acid analysis by UPLC. The red and black bars represent loss of amino acids either in the presence or absence of 25 mM NaHCO₃ respectively. Results are expressed as percentage of amino acid relative to 0 µM ONOOH. * and # represents significant loss of amino acids relative to 0 µM ONOOH, as determined by repeated measures ANOVA, with Bonferroni post-hoc test. ^ represents significant difference between the absence and presence of 25 mM NaHCO₃. Values are means ± standard deviation and represent results obtained from 3 independent experiments.

The results of the above experiments indicate that multiple amino acids including tyrosine, tryptophan, histidine and methionine from BSA are lost at the highest dose of ONOOH and ONOOCO₂⁻. The other amino acids did not appear to be modified by these oxidants. A dose-dependent generation of methionine sulphoxide (a methionine oxidation product) was also detected. Tyrosine is one of the major amino acids modified by ONOOH, with this giving rise to the stable product 3-nitrotyrosine (3NT) (140). Subsequent experiments therefore aimed to detect 3NT formation on BSA modified by exposure of the protein to varying concentrations of ONOOH via Western blotting after separation on a gel.

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3.3.4 3-Nitrotyrosine formation and protein modification

3-Nitrotyrosine (3NT) is generated when tyrosine residues are nitrated by ONOOH (140). 3NT can be detected via antibody based methods (e.g. immunohistochemistry (148, 344), ELISA (181, 345) and Western blotting (264, 346)), gas chromatography (347), high performance liquid chromatography (coupled with either ultraviolet (348), fluorescence (349) or electrochemical (350) detection) or mass spectroscopy detection (after separation by gas chromatography (351) or liquid chromatography (352)). In this project, Western blotting was chosen to detect 3NT formation as it is a sensitive method and the use of gels also allowed protein aggregation and fragmentation to be examined simultaneously.

7.5 µM BSA was exposed to 0-500 µM ONOOH for 5 min at 21 °C and pH 7.4, in either the absence or presence of 25 mM NaHCO₃. Following treatment with the oxidant, BSA was separated by SDS-PAGE. Western blotting was performed, after transferring the proteins onto a nitrocellulose membrane, using an anti-3NT monoclonal antibody to detect 3NT formation. The anti-3NT monoclonal antibody is specific for nitrotyrosine present on nitrated proteins and free amino acids. Silver staining of parallel gels was also performed to examine potential changes in BSA mass (i.e. aggregation or fragmentation) on exposure to ONOOH.

Significant 3NT formation was detected when BSA was treated with 50 µM or higher concentrations of ONOOH (Figure 3.14A), with the levels of this product increasing with increasing concentrations of ONOOH as evidenced by the increasing pixel density of the BSA band. 3NT was not detected in the control sample where ONOOH was absent. This latter result indicates that in the absence of ONOOH, no other nitrating species were present and that no significant 3NT formation had occurred in vivo or during BSA isolation and processing.

Silver staining showed that native BSA (69 kDa) along with other lower and higher molecular mass material was present in these commercial samples (Figure 3.14B). Exposure of BSA to 100 µM or higher concentrations of ONOOH appears to induce BSA fragmentation as evidenced by an increase in the intensity of bands (presumed to have come from BSA) at lower molecular mass. These fragments were also nitrated when BSA was treated with 150 µM or higher concentrations of ONOOH (Figure 3.14A).
BSA dimers were also detected via silver staining (Figure 3.14B). Exposure of BSA to 500 µM ONOOH induced significant protein changes to the dimer protein band with this becoming significantly less intense compared to the control sample (BSA only). Western blotting analysis showed that these dimers were also nitrated by 50 µM or higher concentrations of ONOOH (Figure 3.14A) with the pixel density of these bands becoming more intense when exposed to 75 µM or higher concentrations of ONOOH. Other higher molecular mass species were also detected by silver staining (Figure 3.14B), which could be BSA trimers or tetramers. These higher molecular mass proteins were also nitrated when exposed to 100 µM or greater concentrations of ONOOH (Figure 3.14A).

In order to examine the effect of CO₂ on these reactions, 7.5 µM BSA was treated with 0-500 µM ONOOH in the presence of 25 mM NaHCO₃. Significant 3NT formation was detected by Western blotting on both the monomer and higher molecular mass species when BSA was treated with 50 µM or higher concentrations of ONOOH in the presence of 25 mM NaHCO₃ (Figures 3.15A and B). The pixel density of nitrated BSA bands increased with higher concentrations of ONOOCO₂⁻. 3NT formation was not detected in the control samples (BSA only and 0 µM ONOOH) indicating that in the absence of ONOOH, no other nitrating species were present. In addition, 20 mM NaOH (used as a vehicle for ONOOH) did not induce 3NT formation. Silver staining after SDS-PAGE showed the presence of the BSA monomer (69 kDa), together with other lower and higher molecular mass proteins in these samples when NaHCO₃ was present (Figure 3.15B).

These data indicate that ONOOH-mediated BSA nitration occurs in both the absence and presence of 25 mM NaHCO₃ with a similar dose-dependent generation of 3NT. However in the presence of 25 mM NaHCO₃, BSA fragmentation induced by ONOOH did not appear to occur to the same extent as in the absence of NaHCO₃. Thus BSA fragments were not readily seen in the silver stained gel in the presence of 25 mM NaHCO₃ (Figure 3.15B) compared to the absence of 25 mM NaHCO₃ (Figure 3.14B) and nitrated BSA fragments were also not detected in the presence of 25 mM NaHCO₃ (Figure 3.15A).
Figure 3.14 3-nitrotyrosine (3NT) formation on BSA treated with ONOOH as detected by SDS-PAGE and subsequent Western blotting (A). Silver staining of the SDS-PAGE gels shows ONOOH-mediated protein modifications (B).

7.5 µM BSA was incubated with ONOOH (0-500 µM) for 5 min at 21 °C and pH 7.4. Subsequently Western blotting was performed to detect 3NT formation using an anti-3NT antibody (A) and silver staining was performed to detect ONOOH-mediated protein modifications to BSA (B). Western blotting showed significant increase in 3NT formation in BSA exposed to ONOOH. BSA dimers and fragment nitration was also detected. Silver staining showed that BSA was present along with higher molecular mass proteins (potentially BSA dimers and tetramers). Exposure to high concentrations of ONOOH induced BSA fragmentation. The presence of dimers in the control samples suggests that nitration of higher molecular weight BSA is not causally related to the polymerisation process; instead these additional targets are being nitrated directly. Western blotting and silver staining were performed in 3 independent experiments.
Figure 3.15 3-nitrotyrosine (3NT) formation on BSA treated with ONOOH in the presence of 25 mM NaHCO₃ as detected by SDS-PAGE and subsequent Western blotting (A). Silver staining of the SDS-PAGE gels shows ONOOH-mediated protein modifications in the presence of 25 mM NaHCO₃ (B).

7.5 µM BSA was incubated with ONOOH (0-500 µM) in the presence of 25 mM NaHCO₃ for 5 min at 21 °C and pH 7.4. Subsequently Western blotting was performed to detect 3NT formation using an anti-3NT antibody (A) and silver staining was performed to detect ONOOH-mediated protein modifications to BSA (B). Western blotting showed significant increase in 3NT formation in BSA exposed to ONOOH. BSA dimers and fragment nitration was also detected. Silver staining showed that BSA was present along with higher molecular mass proteins (potentially BSA dimers and tetramers). Exposure to high concentrations of ONOOH induced BSA fragmentation. Western blotting and silver staining were performed in 3 independent experiments.
In the protein samples modified by ONOOH, nitrated BSA and possibly other proteins were detected via Western blotting. Densitometric analysis was performed to quantify 3NT formation in BSA exposed to ONOOH and to investigate the effect of 25 mM NaHCO$_3$ on 3NT formation. Densitometric analysis was conducted on the entire lane using Image J software. The results showed that significant 3NT formation occurs when 7.5 µM BSA was treated with 75 µM ONOOH both in the presence or absence of 25 mM NaHCO$_3$ (Figure 3.14). Significantly greater 3NT formation appears to occur in the absence of 25 mM NaHCO$_3$ than its presence. These data indicate that under these experimental conditions, the species generated directly from ONOOH are more effective nitrating agents than those generated from ONOOCO$_2$.

Figure 3.16 Densitometric analysis of 3NT formation on BSA treated with ONOOH as detected by Western blotting after separation by SDS-PAGE.

7.5 µM BSA was incubated with ONOOH (0-500 µM) both in the presence and absence of 25 mM NaHCO$_3$, for 5 min at 21 °C and pH 7.4. Western blotting was performed to detect 3NT formation using an anti-3NT antibody and subsequently densitometric analysis was performed on the entire lane using Image J software. The red and black bars represent 3NT formation either in the presence or absence of 25 mM NaHCO$_3$ respectively. * and # represents significant 3NT formation (p < 0.05), as determined by repeated measures ANOVA, with Bonferroni post-hoc test. ^ represents significant difference (p < 0.01) between the absence and presence of 25 mM NaHCO$_3$. Values are means ± standard deviation and represent results obtained from 3 independent experiments.
3.4 Discussion

The aim of the experiments reported in this Chapter was to compare different assays for measuring thiol loss using HCAEC lysates and to subsequently examine ONOOH-mediated damage to GSH and BSA. Peroxynitrite is often used to refer to the sum of ONOO⁻ and ONOOH. ONOOH can both react directly with targets via 2-electron reactions and also undergo homolysis to form hydroxyl radicals, nitrogen dioxide radicals and nitrate anions (62). ONOO⁻ reacts with CO₂ to form the nitrosoperoxycarbonate anion adduct (ONOOCO₂⁻), which can induce direct oxidation but also decomposes to nitrogen dioxide radicals, carbonate radicals and other reactive derivatives (100). It is however still unclear as to which species is mostly responsible for ONOOH-mediated damage in intact biological systems.

In the absence of CO₂, the damaging species can be either ONOOH itself or radicals generated from it. It is well established that different amino acids react with ONOOH with different rate constants (63). For instance, the rate constants for reaction with cysteine, methionine and tryptophan are 4.5 x 10³, 3.64 x 10² and 37 M⁻¹s⁻¹ respectively at 37 °C and pH 7.4 (61, 66, 353). It is evident from these rate constants that these amino acids when present on proteins are likely to react with ONOOH to different extents.

ONOOH is known to readily oxidise the thiol group of cysteine residues, and these are believed to be a major target of ONOOH (61). Initially a comparison was carried out between the fluorimetric methods using ThioGlo assay (using either ThioGlo™ 1, 3 or 5) and DTNB assay to determine which was the best assay to measure thiols. In 7.5 µM BSA treated with ONOOH, ThioGlo™ 1 and 5 reagents detected similar amount of thiols relative to protein concentrations, however ThioGlo™ 5 is more expensive than ThioGlo™ 1 and gave more variable data, hence the latter was employed in preference to ThioGlo™ 5. ThioGlo™ 1 did not non-specifically bind to other proteins in the sample. ThioGlo™ 3 gave much lower values and hence was not considered an appropriate reagent. The DTNB assay detected high (apparent) thiol concentrations but these measurements were associated with large error bars (when compared to ThioGlo™ 1) and high background absorbances were also detected. ThioGlo™ 1 was therefore used in preference to DTNB.
ONOOH-mediated thiol oxidation was investigated using GSH. 7.5 µM GSH (i.e. 7.5 µM thiols) was treated with 0-100 µM ONOOH for 5 min at 21 °C and pH 7.4. It was shown that about 30 µM ONOOH completely oxidised all the thiol residues. In the presence of 25 mM NaHCO₃, less GSH thiol residues were oxidised by an equivalent amount of oxidant. These data indicate that NaHCO₃ is protecting GSH thiols against ONOOH-mediated damage presumably as a result of the formation of the less reactive species ONOOCO₂⁻. A similar effect has been reported in a previous study where CO₂ partially inhibited thiol oxidation of human serum albumin (HSA) when treated with ONOOH (353).

BSA has 35 cysteine residues, 17 of these participate in disulphide bonds and the other is a free cysteine residue (Cys 34). The latter is a known target of ONOOH (118). However as this protein also contains multiple other reactive sites, it was of interest to determine the extent of damage at Cys versus these other targets. 7.5 µM BSA was exposed to increasing concentrations of ONOOH (0-100 µM) before quantification of thiol loss using the ThioGlo™ 1 assay. 7.5 µM of native BSA contained about 2.1 µM of thiol residues and 50 µM ONOOH oxidised almost all these thiol residues. Higher concentrations of ONOOH were required to see significant loss of thiols in BSA when compared to GSH with this likely to be due to steric restrictions and the hindered location of the single thiol group in BSA. As with GSH, the presence of NaHCO₃ reduced the loss of this species.

Overall these data suggest that ONOOH (or the radicals generated from ONOOH homolysis) are more efficient thiol oxidising species than ONOOCO₂⁻ (or species generated by its homolysis). HCO₃⁻ might also be scavenging radicals generated from ONOOH before they react with the thiol residues. However given the very high rate constants for reaction of hydroxyl radicals with RSH (k ~ 10¹⁰ M⁻¹s⁻¹) (354), this is not the most likely cause of this inhibition. The changing pH is a factor for this reaction and both reactants are diffusion controlled. Overall NaHCO₃ appears to have a protective effect against ONOOH-mediated thiol oxidation in GSH and BSA.

Apart from thiols, BSA has other amino acids such as histidine, methionine, phenylalanine, tryptophan and tyrosine that may also be significant targets of ONOOH (63). Investigation of the extent to which ONOOH modifies each of these amino acids
was examined using amino acid analysis. After MSA hydrolysis of native BSA, the percentage recovery of histidine, methionine, phenylalanine, tryptophan and tyrosine were 75, 30, 64, 97 and 228 % respectively. The percentage recoveries for histidine and phenylalanine are close to their expected values. A low percentage recovery was detected for methionine and this was most probably due to oxidation occurring in vivo or during protein isolation, storage and processing. Unexpectedly the percentage recovery of tryptophan and tyrosine were high. This is most likely due to the presence of a coeluting peak that increases the peak area and hence the percentage recovery. The fact that the % recovery values for tyrosine and glycine were > 100 % indicates that the assay is detecting additional chemical moieties which may have completely different oxidation/nitration profiles to tyrosine and therefore skew any estimates of Km values for ONOOH ONOOCO$_2^-$ and tyrosine residues. This is a limitation of this assay.

When 7.5 µM BSA was treated with 10 mM ONOOH a significant loss (34 %) of tyrosine residues was detected. No significant loss was detected at lower oxidant concentrations. A similar result was detected in the presence of 25 mM NaHCO$_3$. 3NT formation was detected by Western blotting on BSA exposed to 50 µM or higher concentrations of ONOOH as evidenced by the increasing pixel density of the BSA band. Less intense nitrated bands were detected when BSA was treated with ONOOH in the presence of 25 mM NaHCO$_3$. Densitometric analysis indicated that more 3NT formation occurred in the absence than in the presence of 25 mM NaHCO$_3$. This difference was not detected for loss of the parent amino acid by UPLC possibly because of the high levels of parent amino acids and a low conversion to products. Analogously, nitrated BSA dimers and trimers/tetramers were detected and the pixel density of these nitrated higher molecular mass species became more intense as the ONOOH concentrations increased. These data are consistent with either aggregate formation and subsequent nitration, or aggregation of these nitrated proteins.

Under these experimental conditions, ONOOH seems to be inducing 3NT formation to a greater extent than ONOOCO$_2^-$. This is in contrast to a previous study in which it was reported that NaHCO$_3$ acts as a catalyst in ONOOH-mediated protein tyrosine nitration (106) and that NaHCO$_3$ increased nitration yield when HSA was treated with ONOOH (353). The reason for this difference is not known.
No significant loss of phenylalanine residues was detected even at high concentrations of ONOOH. A similar result was detected in the presence of 25 mM NaHCO₃. Thus under these experimental conditions, both ONOOH and ONOOCO₂⁻ seem to be non-reactive towards phenylalanine. It has been shown previously that ONOOH reacts with phenylalanine to form a small amount of p-, m- and o-tyrosine and some nitrated products of phenylalanine (such as 4-nitrophenylalanine) and that this resulted in a ONOOH dependent reduction in phenylalanine concentration. The current experimental data do not agree with this study because no dose-dependent loss of phenylalanine residues was detected under these experimental conditions. This is possibly because of the high availability of other more reactive targets such as cysteine, methionine, tyrosine and tryptophan. The phenylalanine residues are usually buried deeper in the hydrophobic region, thus accessibility of the oxidant to the residue may also play a bearing on the oxidation reaction.

A significant loss (45 %) of tryptophan residues was detected when BSA was treated with high concentrations of ONOOH. 25 mM NaHCO₃ made no significant difference to the loss of tryptophan residues. Overall these data suggest that ONOOH and ONOOCO₂⁻ may be modifying tryptophan residues to a similar extent, even at high concentrations. Tryptophan residues are known to be a major target of ONOOH, and can be both nitrated and oxidised by ONOOH. Nitration of tryptophan by ONOOH forms 6-nitrotryptophan (the major product) and lower levels of 4-, 5- and 7-nitrotryptophan. ONOOH-mediated oxidation of tryptophan residues forms multiple products including hydroxytryptophan, oxindol-3-alanine, hydroxyproloindole and N-formylkynurenine. It has been reported that ONOOH-mediated tryptophan loss occurs when human recombinant copper- and zinc-superoxide dismutase (CuZnSOD) is exposed to ONOOH and this resulted in partial inactivation of this enzyme. The current experimental results are consistent with this earlier study.

A significant loss (77 %) of methionine residues and substantial methionine sulfoxide formation (47 %) was detected when BSA was exposed to 10 mM ONOOH. ONOOH is known to oxidise methionine to generate methionine sulfoxide, which gets further oxidised to methionine sulphone. In the presence of 25 mM NaHCO₃, a lower percentage of methionine residues were lost and a smaller yield of methionine sulfoxide was detected. This oxidation may be a direct oxidation process and the differences detected between the oxidants are due to differences in the reactivity of ONOOH and
ONOOCO$_2^-$从而导致更严重的甲硫氨酸损失。它有以前报道说，ONOOH 诱导的甲硫氨酸氧化在谷氨酰胺合成酶从 *Escherichia coli* 而且这导致了酶活性的抑制 (169)。我们的实验结果与这项研究的结果一致。

没有检测到显著的组氨酸残基的损失，即使当 BSA 被暴露于最高 10 mM ONOOH。然而，当 BSA 被处理与 10 mM ONOOH 在存在 25 mM NaHCO$_3$。这些数据表明 ONOOCO$_2^-$ 修饰组氨酸残基的程度高于 ONOOH。组氨酸残基是被修改的是由 ONOOH 通过一个自由基机制形成组氨酸自由基 (164) 并且这个机制可能对 ONOOH-介导的活性抑制人类 CuZnSOD (164) 负责。在先前的研究中，将 5 µM CuZnSOD 露于大约 100 µM ONOOH 诱导 50% 酶活性的损失，尽管不确定这个活性的损失直接等同于组氨酸氧化。

ONOOH 已被之前报道为结构上改变蛋白质，这可以导致蛋白质聚集体形成，片段化和交联 (63, 118, 181, 264)。银染结果显示，暴露于 BSA 100 µM 或更高浓度的 ONOOH 似乎诱导蛋白质聚集体形成。这个观察没有在存在 25 mM NaHCO$_3$，表明 ONOOH 诱导蛋白质聚集体形成的程度高于 ONOOCO$_2^-$。这些片段被硝化当暴露于 150 µM 或更高浓度的 ONOOH 或 ONOOCO$_2^-$。显著的增加在硝化发生在当 BSA 被暴露于 75 µM 或更高浓度的 ONOOH 蛋白质聚集体当这个蛋白质被暴露于 100 µM 或更高浓度的 ONOOH。在这些实验条件下，无论是硝化可以导致片段化或者片段化可以导致硝化。我们的实验数据不能检测到哪个过程首先发生。

在 ThioGlo 试验中，低浓度 (10 µM) 的 ONOOH 被要求来检测显著的半胱氨酸残基的损失。Western 转印结果表明 75 µM 或更高浓度的 ONOOH 诱导显著的 3NT 形成在 BSA 蛋白质。银染结果表明 100 µM 或更高浓度的 ONOOH 导致显著的蛋白质片段化。数据来自 UPLC 研究表明
very high concentrations (1 and 10 mM) of ONOOH were needed to detect loss of tyrosine, tryptophan, methionine and histidine. The UPLC data also indicated a corresponding increase in methionine sulphoxide formation when exposed to high oxidant concentrations. Each of these amino acids are known targets of ONOOH, but these residues are most probably buried within the protein structure. Consequently high concentrations of ONOOH may be needed to modify these amino acids and these modifications may denature and unfold the protein.

Overall these data suggest that ONOOH and ONOOCO$_2^-$ have a significant effect on amino acids of BSA. Under the experimental conditions used, ONOOH and ONOOCO$_2^-$ modified multiple amino acids such as cysteine (thiol group), tyrosine, methionine, and histidine. ONOOH and ONOOCO$_2^-$ mediated formation of products such as 3NT and methionine sulfoxide has also been detected. The results obtained indicate that ONOOH induced cysteine (thiol) and methionine oxidation to a greater extent than ONOOCO$_2^-$. ONOOH also induced greater formation of methionine sulfoxide and 3-nitrotyrosine as well as protein fragmentation than ONOOCO$_2^-$. In contrast ONOOCO$_2^-$ induced histidine oxidation to a greater extent than ONOOH. ONOOH and ONOOCO$_2^-$ modified tryptophan and tyrosine residues to a similar extent while both of these oxidants did not have any significant effect on phenylalanine.

It has been reported previously that CO$_2$ inhibited cysteine and methionine oxidation (169, 353). In the current study, CO$_2$ also inhibited cysteine and methionine oxidation when BSA and GSH were exposed to ONOOH, therefore these results concur. It was also previously shown that CO$_2$ increased ONOOH-mediated nitration yields (106, 169, 353). Our experimental data do not agree with the latter studies, as CO$_2$ did not increase nitration yields when BSA was exposed to ONOOH. The extent of tyrosine nitration is known to be dependent on several factors, including the proximity of tyrosine residues to the nitrating agents, the abundance of tyrosine residues within proteins, the primary sequence of proteins and the local environment (356); these factors may therefore account for the discrepancies between these studies.

The results achieved in this Chapter give some indication of the order in which ONOOH modifies BSA amino acids. Firstly cysteine residues are targeted, followed by methionine, tryptophan, tyrosine, histidine and phenylalanine residues (Figure 3.17). Based on the rate constants for reaction between ONOOH and free amino acids it was
previously suggested that cysteine, methionine and tryptophan are the most reactive amino acids (353). Our experimental data agree with these kinetic data since under our experimental conditions, cysteine, methionine, tryptophan and tyrosine were the next most reactive amino acids on BSA.

Figure 3.17 ONOOH-mediated loss of multiple amino acids of BSA.

In this Chapter ONOOH-mediated modification of peptides and an isolated model protein was investigated with these data providing information as to the susceptibility of different amino acids to oxidation. In light of this information, subsequent studies investigated ONOOH-mediated modifications to HCAEC, a cell system which is more complex than isolated peptides and proteins. These data are reported in the next Chapter.
Chapter 4: Damage Induced by Peroxynitrous Acid (ONOOH)

to Human Coronary Artery Endothelial Cells
4.1 Introduction

Endothelial cells are versatile and multifunctional. Approximately $10^{14}$ endothelial cells form the lining of blood vessels and the heart. These cells attach to the basement membrane of the extracellular matrix and are the main cellular components on the lumen side of the internal elastic lamina. Endothelial cells create a physical and functional semi-permeable anticoagulant barrier between the flowing blood and the stroma of the arterial wall, and control the movement of small and large molecules from the blood into the artery wall (85).

Fully functional endothelial cells protect against atherosclerosis and thrombosis (312). Numerous cardiovascular risk factors such as aging, hypercholesterolemia, hypertension, smoking, diabetes and a family history of coronary artery disease can induce endothelial dysfunction (357, 358). Endothelial dysfunction is a key and defining feature of early atherosclerosis. Endothelial dysfunction is a reversible disorder, which can be detected prior to the identification of structural changes to the blood vessel wall (359).

The exact mechanism(s) that underlie endothelial dysfunction remain to be elucidated. However accumulating evidence suggests that oxidative stress can lead to the development and progression of atherosclerosis by stimulating endothelial dysfunction, inflammation and reduced NO$^\cdot$ availability (360, 361). Endothelial dysfunction is characterised by damage to eNOS, which causes a decrease in NO$^\cdot$ formation and reduces smooth muscle relaxation. Animal models of endothelial dysfunction and clinical data have provided evidence that NO$^\cdot$ bioavailability is reduced by elevated oxidant production in the vessel wall during the progression of atherosclerosis (362, 363). This may be due to direct effects of oxidants on eNOS or alternatively damage to endothelial cells, which then produce less NO$^\cdot$ from eNOS.

Normal blood vessels are made of endothelial cells, smooth muscle cells, other cell types and the extracellular matrix. Previous studies have reported that ONOOH damages the extracellular matrix (181, 257, 264). To date, no study has investigated how ONOOH damages human coronary artery endothelial cells (HCAEC). HCAEC can be extracted from the coronary arteries. These primary cells play vital roles in modulation of coronary blood flow and cardiac functions. As a result, these cells are a possible model for studying endothelial cell dysfunction in the early stages of atherosclerosis and
hypertension. Currently it is unknown how ONOOH, a powerful oxidant, induces endothelial dysfunction in HCAEC. This Chapter investigates how ONOOH induces endothelial damage to HCAEC. In light of the data presented in the previous Chapter, the potential routes may be via protein and peptide oxidation and particularly damage to thiol and tyrosine residues.

4.2 Aims

The aim of the experiments reported in this Chapter was to investigate the damage induced by ONOOH to HCAEC. ONOOH-mediated damage was assessed by thiol oxidation (loss), 3NT formation and other protein modifications. Prior to such analysis, it was crucial to investigate ONOOH-mediated cytotoxicity to HCAEC. As a consequence, initial experiments examined the effect of ONOOH on HCAEC cell viability using the MTT assay.

Subsequently intact HCAEC were exposed to varying concentrations of ONOOH. Loss of thiols and 3NT formation were detected via use of the ThioGlo assay and Western Blotting respectively. Other protein modifications were detected via silver staining after SDS-PAGE. Lysates were also exposed to ONOOH in order to investigate whether the cell membrane has any protective effect against the damage induced by ONOOH.

Fractional centrifugation was employed to examine the nature and extent of ONOOH-mediated damage to cytosolic and membrane proteins of HCAEC. Intact cells were treated with ONOOH and then the cytosolic and membrane fractions were separated followed by detection of loss of thiols, 3NT formation and other protein modifications. Additionally, the cytosolic and membrane fractions were separated and then both fractions were treated with ONOOH. Subsequently thiol loss, 3NT formation and other protein modifications were detected.

In all the experiments mentioned above, 25 mM NaHCO₃ was also added to give physiological levels of CO₂, which would react with ONOOH to form ONOOCO₂⁻. These experiments therefore allowed a comparison to be made between the effect of ONOOH and ONOOCO₂⁻ and their respective secondary radicals on HCAEC viability, thiol levels, 3NT formation and other protein modifications.
4.3 Results

4.3.1 Cell viability

The effect of ONOOH on HCAEC cell viability was investigated using the compound MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) in a colorimetric assay. Metabolically active cells take up MTT and convert it to formazan crystals, which have a purple colour (321). After 4 hours of incubation, the formazan crystals within the cells are dissolved in DMSO and quantified via their absorbance. Cellular viability was assessed by measuring the absorbance at 570 nm with a reference at 630 nm relative to non treated control cells. ONOOH-mediated toxicity was evaluated because in the subsequent experiments sublethal concentrations of ONOOH were used.

ONOOH is only stable as its anion in basic solutions, hence ONOOH was prepared in sodium hydroxide (NaOH). High concentrations of NaOH are toxic to cells. It was therefore important to investigate what concentration of NaOH was not toxic to HCAEC. HCAEC were seeded at a density of 4 x 10⁵ cells per well in a 6 well plate overnight at 37 °C. Cells were incubated in HBSS (pH 7.4) and treated with varying concentrations (0–100 mM) of NaOH for 5 min at 37 °C. Exposure to NaOH concentrations greater than 20 mM was toxic to HCAEC (data not shown). ONOOH was stable in 20 mM NaOH. Consequently in all experiments, ONOOH was prepared in 20 mM NaOH.

Cells were incubated in HBSS (pH 7.4) and treated with varying concentrations of ONOOH (0-1250 µM) for 5 min at 37 °C and pH 7.4. Subsequently the MTT assay was performed. In order to investigate the effect of CO₂ on ONOOH mediated cytotoxicity, this experiment was also performed on cells exposed to ONOOH in the presence of 25 mM NaHCO₃. No significant loss of cell viability was detected when cells were treated with 500 µM or lower concentrations of ONOOH for a short, 5 min incubation period (Figure 4.1). HCAEC cell viability was reduced by 15 % when cells were treated with 1250 µM ONOOH.

No significant loss of cell viability was detected when cells were exposed to 25 mM NaHCO₃ alone for 5 min (Figure 4.1). This result suggests that 25 mM NaHCO₃ is not toxic to cells. About a 10 % loss of cell viability was detected when cells were treated with 1250 µM ONOOH in the presence of 25 mM NaHCO₃. Overall, the presence of 25
mM NaHCO$_3$ made no significant difference to the loss of HCAEC viability induced by ONOOH. In the subsequent experiments, sublethal concentrations of ONOOH were used to investigate ONOOH mediated cellular damage.

![Figure 4.1 Loss of cell viability in HCAEC treated with ONOOH.](image)

HCAEC (4 x 10$^5$ cells) were present in HBSS (pH 7.4) and treated with 0–1250 µM ONOOH for 5 min at 37 °C and pH 7.4 followed by determination of cell viability using the MTT Assay. The red and black bars represent the cell viability either in the presence or absence of 25 mM NaHCO$_3$ respectively. Results are expressed as percentage of viable cells relative to the untreated control. * and # represent significant loss (p < 0.05) of cell viability relative to the untreated control, as determined by one way ANOVA, with Dunnett’s post-hoc test. Significant loss of cell viability was detected when HCAEC were treated with 1250 µM ONOOH. A similar result was detected in the presence of 25 mM NaHCO$_3$. The effect of 25 mM NaHCO$_3$ was analysed using repeated measures ANOVA, with Bonferroni post-hoc test; 25 mM NaHCO$_3$ made no significant difference to loss of HCAEC viability. Values are means ± standard deviation and represent results obtained using 3 different HCAEC donors in 3 independent experiments.

### 4.3.2 Thiol oxidation

#### 4.3.2.1 Intact cells

These experiments aimed to mimic the effect of in vivo extracellular ONOOH formation on HCAEC thiols, and to identify which ONOOH concentrations induced significant thiol oxidation if this powerful oxidant was generated in excess extracellularly. HCAEC were seeded at a cell density of 4 x 10$^5$ cells per well in a 6-well plate at 37 °C overnight. Intact cells were incubated in HBSS (pH 7.4) and treated with 0-1250 µM ONOOH as
well as decomposed (De) 1250 µM ONOOH for 5 min at 37 °C and pH 7.4. The non-
treated and treated cells were lysed in cold (4 °C) npH2O and the residual thiol levels
were immediately quantified using the ThioGlo™ 1 reagent.

HCAEC only (HO) represents cells that were not exposed to ONOOH or NaOH. De
(1250) represents cells that were treated with decomposed 1250 µM ONOOH. In order to
investigate if other thiol oxidising species were present in the ONOOH stocks, intact
HCAEC were treated with decomposed (1250) µM ONOOH which was incubated at 60
°C overnight before use. No significant loss of thiols was detected when cells were
treated with the decomposed 1250 µM ONOOH compared to the HO control (Figure 4.2).
This indicates that apart from ONOOH, no other thiol oxidising species were present. 0
µM ONOOH represents cells treated with 20 mM NaOH, the vehicle for ONOOH. No
significant thiol loss was detected when intact HCAEC were treated with 20 mM NaOH
compared to the HO control. This suggests that 20 mM NaOH alone was not a thiol
oxidising species.

In the HO control, there was an unexpected significant difference in concentration of
thiols in the presence and absence of 25 mM NaHCO3. In the absence of 25 mM
NaHCO3 40 µmoles/g of thiols relative to protein was present in the HCAEC only
control. In contrast, about 65 µmoles/g of thiols relative to protein was present in the
HCAEC only control in the presence of 25 mM NaHCO3. The exact reason for these
differences in the initial thiol concentrations is unknown. This is possibly due to effects
of HCO3-/CO2 on an endogenous oxidation process.

No significant loss of thiols was detected when cells were treated with 500 µM or lower
concentrations of ONOOH compared to the 0 µM ONOOH condition. A significant loss
of thiols (about 20 %) was detected when cells were treated with 1250 µM ONOOH. A
similar result was detected in the presence of 25 mM NaHCO3. These data indicate that
when HCAEC were exposed to 1250 µM ONOOH (present extracellularly) for 5 min, a
significant amount of thiol oxidation occurs. High concentrations of ONOOH may be
required to detect a significant loss of thiols because the cell membrane is protecting the
cells against ONOOH-mediated intracellular thiol loss.
Figure 4.2 Loss of thiols in intact HCAEC treated with ONOOH.

Intact HCAEC (4 x 10^5 cells) were treated with 0-1250 µM ONOOH and decomposed 1250 µM ONOOH for 5 min at 37 °C followed by determination of residual thiols using the ThioGlo™ 1 reagent. ONOOH was prepared in 20 mM NaOH. HO represents HCAEC only sample that was not treated with ONOOH or NaOH. De (1250) represents intact HCAEC treated with decomposed 1250 µM ONOOH. The red and black bars represent thiols either in the presence or absence of 25 mM NaHCO₃ respectively. Results are expressed as concentration of thiols over their respective protein concentration. * and # represent significant loss (p < 0.05) of thiols relative to 0 µM ONOOH, as determined by one way ANOVA, with Dunnett’s post-hoc test. Significant thiol loss was detected when cells were treated with 1250 µM ONOOH both in the absence and presence of 25 mM NaHCO₃. ^ represent significant difference (p < 0.05) between the absence and presence of 25 mM NaHCO₃, as determined by repeated measures ANOVA, with Bonferroni post-hoc test. Values are means ± standard deviation and represent results obtained using 3 different HCAEC donors in 3 independent experiments.

4.3.2.2 HCAEC lysates

A significant loss of thiols was detected when intact HCAEC were exposed to 1250 µM ONOOH for 5 min at 37 °C and pH 7.4 (Figure 4.2). A high concentration of ONOOH may be required to detect significant loss of thiols because the cell membrane is a physical barrier, which is protecting against intracellular thiol oxidation induced by ONOOH. It was therefore felt to be important to investigate whether the cell membrane has any protective effect against the damage induced by ONOOH; this was investigated by examining thiol loss in cell lysates. The protective role of membrane thiols will be tested by comparing the loss of thiols in cell lysates vs intact cells.
HCAEC were seeded at a cell density of $4 \times 10^5$ cells per well in a 6-well plate at 37 °C overnight. Intact cells were lysed in cold (4 °C) npH2O and then lysates (containing about 75 µmoles of thiols per gram of protein) were treated with varying concentrations of ONOOH (0-100 µM) for 5 min at 21 °C. In these lysate samples, the cell membrane was disrupted and all intracellular components were directly exposed to ONOOH.

In the HCAEC only controls, no significant difference in thiol concentrations was detected either in the presence or absence of 25 mM NaHCO3 (Figure 4.3). This suggests that the presence of 25 mM NaHCO3 alone does not affect thiol levels. No significant difference was also seen between the thiol concentrations of the HCAEC lysates alone and those treated with 0 µM ONOOH (20 mM NaOH). A similar result was detected in the presence of 25 mM NaHCO3. This indicates that 20 mM NaOH alone did not oxidise thiol residues.

Under these experimental conditions, a significant loss of thiol residues was detected when lysates were treated with 5 µM or greater concentrations of ONOOH compared to the non-treated control (Figure 4.3). Exposure to 5 µM ONOOH oxidised about 25 % (20 µmoles/g) of thiols and about 55 µmoles/g of thiols remained. Exposure of HCAEC lysates to 75 µM ONOOH completely oxidised all the thiol residues. These results indicate that low levels of ONOOH can have a significant effect on thiols when there is more unrestricted access of ONOOH to intracellular components.

A greater proportion of the thiols were lost in the presence of 25 mM NaHCO3 compared to its absence. For instance, in the absence of 25 mM NaHCO3, treatment with 5 µM ONOOH oxidised about 20 µmoles/g (25 %) of thiols whereas in the presence of 25 mM NaHCO3, about 35 µmoles of thiols (50 %) were oxidised. This suggests that in the absence of the cell membrane of HCAEC, ONOOCO$_2^-$ or the radicals generated via ONOOCO$_2^-$ decomposition, oxidise more thiols than are consumed by ONOOH or secondary radicals derived from ONOOH homolysis in contrast to data obtained with isolated GSH and BSA outlined in Chapter 3.
HCAEC lysates were incubated with 0-100 µM ONOOH for 5 min at 21 °C followed by determination of residual thiols using the ThioGlo Assay. The red and black bars represent loss of thiols in the presence and absence of 25 mM NaHCO$_3$ respectively. HO represents HCAEC only sample that was not treated with ONOOH or NaOH. Results are expressed as concentration of thiols over the respective protein concentration. * and # represent significant loss (p < 0.0001) of thiols relative to 0 µM ONOOH, as determined by one way ANOVA, with Dunnett’s post-hoc test. Significant thiol loss was detected when lysates were treated with 5 µM or greater concentrations of ONOOH. ^ represent significant difference (p < 0.05) between the absence and presence of 25 mM NaHCO$_3$, as determined by repeated measures ANOVA, with Bonferroni post-hoc test. Values are means ± standard deviation and represent results obtained using 3 different HCAEC donors in 3 independent experiments.

4.3.2.3 Optimization of cellular fractionation

In intact HCAEC and lysates a significant loss of thiol residues was detected when the cells or lysates were treated with 1250 (Figure 4.2) and 5 µM (Figure 4.3) ONOOH respectively. The cell membrane is most likely preventing intracellular thiol oxidation induced by ONOOH. It was therefore of interest to investigate whether the thiol residues of cytosol or membrane proteins of HCAEC are being targeted by ONOOH. Experiments were carried out to examine the effect of extracellular ONOOH on cytosolic and membrane thiols of intact HCAEC, with the aim of identifying whether cytosolic and/or membrane thiols are mainly targeted by ONOOH when this oxidant was generated in excess extracellularly.
Membrane proteins are low in abundance in cell lysates, are typically large in size and often hydrophobic in nature. Due to these characteristics, it is difficult to isolate and purify membrane-bound proteins. There are various commercial kits available which can separate the subcellular fractions. Previously a comparison was made between 5 commercial kits in order to identify the most efficient kit (320). Out of the 5 kits tested, ProteoExtract Native Membrane Protein Extraction Kit was determined to be the best in separating the subcellular fractions such as the cytosolic and membrane fractions; these fractions contained the cytosolic and membrane proteins respectively.

Initially the efficiency of the ProteoExtract Native Membrane Protein Extraction kit in separating the subcellular fractions of untreated HCAEC was tested. We also aimed to identify the best cytosolic and membrane proteins that can be employed as markers of efficient separation. Cytosolic and membrane control proteins should only be detected in the cytosolic and membrane fractions respectively and not vice versa.

HCAEC were seeded at a cell density of 4 x 10^5 cells per well in a 6-well plate at 37 °C and pH 7.4 overnight. The untreated HCAEC were then washed with washing buffer. Subsequently cells were incubated in Extraction Buffer I and protease inhibitor cocktail for 10 min at 4 °C. The protease inhibitor cocktail avoids potential oxidation and degradation prior to analysis. The cytosolic fraction was extracted and stored on ice. Then the cells were incubated in Extraction Buffer II and protease inhibitor cocktail for 30 min at 4 °C. The membrane fraction was extracted and stored on ice. The protein concentrations were adjusted so that both fractions contained equal amounts of protein. Alternatively intact HCAEC were lysed in cold (4 °C) npH2O to obtain total cell lysates, which contained both cytosolic and membrane fractions.

Western blotting was performed after SDS-PAGE using total cell lysates and the separated subcellular fractions, with the membranes probed using various cytosolic and membrane antibodies. The cytosolic protein antibodies employed include β-actin, β-tubulin, P65 and VEGF while the membrane protein antibodies include ABC-G1 and HO-1. However HO-1 is not exclusive to plasma membrane since this protein is also found in the endoplasmic reticulum (364) and mitochondrial membranes (365). ABC-G1 was detected in total cell lysates and to a greater extent in the membrane fraction, while HO-1 was detected only in the membrane fraction (Figure 4.4). VEGF was detected in
both total cell lysates and cytosolic fraction and to a very minor extent in the membrane fraction. P65, β-actin and β-tubulin were detected in the total cell lysates and the cytosolic fraction, but a significant amount of these proteins were also detected in the membrane fraction, which indicated that these proteins had not been separated well or were naturally present in both fractions.

**Figure 4.4 Detection of various cytosolic and membrane proteins in the total lysates (T), membrane fraction (M) or cytosolic fraction (C).**

HCAEC were seeded at a cell density of 4 x 10⁵ cells per well in a 6-well plate overnight. Cells were washed with wash buffer and incubated in Extraction Buffer I and protease inhibitor for 10 min at 4 °C. The supernatant containing cytosolic fraction was extracted and stored on ice. Extraction Buffer II and protease inhibitor was added to the cells and incubated for 30 min at 4 °C. The supernatant containing membrane fraction was then removed and stored on ice. Alternatively cells were scraped using a plunger and lysed in cold (4 °C) npH₂O for 10 min to obtain total lysates. Using the total lysates and separated fractions Western blotting was performed after SDS-PAGE. The membranes were blotted with the appropriate antibodies.

Under these experimental conditions, ABC-G1 and VEGF appear to be the best control proteins for indicating efficient separation of the subcellular fractions of HCAEC. These data also indicated that the kit is quite efficient since the membrane proteins ABC-G1 and HO-1 were only detected in the membrane fraction and not in the cytosolic fraction.
VEGF was only detected in the total cell lysates and cytosolic fraction and only to a very small extent in the membrane fraction.

4.3.2.4 Cellular fractions of intact cells

We aimed to identify whether the cytosol or membrane-bound thiols of HCAEC were mainly oxidised by ONOOH if this oxidant was generated in excess extracellularly. HCAEC were seeded at a cell density of 4 x 10^5 cells per well in a 6-well plate at 37 °C and pH 7.4 overnight. Cells were treated with 0-1000 µM ONOOH as well as decomposed 1000 µM ONOOH for 5 min at 37 °C and pH 7.4 followed by separation of the cytosolic and membrane fractions, which contained the cytosolic and membrane proteins respectively. Residual thiol levels were immediately quantified using the ThioGlo assay.

In the untreated intact HCAEC, the initial thiol concentrations of the cytosolic and membrane fractions were 36.57 and 4.69 µmoles/g relative to the protein concentrations respectively. In both fractions no significant difference was detected between the HCAEC only and intact cells treated with 0 µM ONOOH (20 mM NaOH) (Figure 4.5). This indicated that NaOH alone did not oxidise thiol residues. Additionally, no significant loss of thiols was detected when intact cells were treated with decomposed 1000 µM ONOOH. This suggests that no thiol oxidising species were present in the absence of active ONOOH.

Exposure of the intact cells with up to 250 µM ONOOH induced no significant loss of the cytosolic thiols (Figure 4.5A). Treatment of cells with 500 and 750 µM ONOOH induced 10 and 17 % reduction in the cytosolic thiols respectively compared to the control. 10 and 17 % of thiols corresponds to 3.66 and 6.12 µmoles/g of thiols relative to the protein concentrations respectively. Exposure of intact cells to up to 500 µM ONOOH induced no significant loss of membrane-bound thiols (Figure 4.5B). Treatment of cells with 750 µM ONOOH caused about 30 % (1.41 µmoles/g) reduction in the membrane-bound thiols compared to the control. More cytosol and membrane-bound thiols were oxidised as the ONOOH concentrations were increased. These data suggest that ONOOH can pass through the cell membrane and oxidise the cytosolic thiols of HCAEC. On the basis of these data, it appears that the membrane-bound thiols are more susceptible to ONOOH mediated oxidation compared to cytosolic thiols.
The presence of 25 mM NaHCO$_3$ made no significant difference to the loss of thiols in the cytosolic and membrane fractions of intact cells. In the presence of 25 mM NaHCO$_3$, the initial thiol concentrations in the cytosolic and membrane fractions were 36.63 and 4.85 µmoles/g relative to the protein concentrations respectively. These data indicate that similar initial thiol concentrations were detected in the presence and absence of 25 mM NaHCO$_3$. 25 mM NaHCO$_3$ alone did not oxidise thiol residues of cytosolic and membrane proteins.

These data overall suggest that in intact HCAEC, the membrane-bound thiols were more susceptible towards ONOOH mediated oxidation than those present in the cytosol. Therefore if excessive ONOOH formation occurs extracellularly, the membrane-bound thiols will be targeted to a greater extent than the cytosolic thiols. The cell membrane of HCAEC therefore appears to play a role in directing ONOOH-mediated damage. It was therefore of interest to investigate the damage induced by ONOOH to the cytosolic and membrane thiols in the absence of the cell membrane. In the next Section, ONOOH-mediated thiol oxidation was investigated in the absence of the HCAEC cell membrane by using cells lysed before exposure to ONOOH.

**4.3.2.5 Cell lysate experiments**

In these experiments, the aim was to identify whether the cytosolic and/or membrane thiols are mostly targeted by ONOOH if this oxidant was generated in excess intracellularly in the absence of a cell membrane. The thiol concentrations of both fractions were assessed by use of the ThioGlo assay. The concentration of membrane and cytosol thiols was then adjusted by dilution so that the thiol concentrations were equal in each fraction prior to exposure to ONOOH. Subsequently both fractions were directly exposed to 0-100 µM ONOOH as well as decomposed 100 µM ONOOH for 5 min at 21 °C and the thiol concentrations of both fractions were again quantified. Under these experimental conditions, the initial thiol concentrations of the untreated cytosolic and membrane fractions were 1.634 and 1.443 µM respectively. In both fractions no significant difference was detected between the HCAEC only and the sample treated with 0 µM ONOOH (20 mM NaOH) (Figure 4.6). This indicates that NaOH alone did not oxidise thiol residues. No significant loss of thiols was detected when both fractions were treated with decomposed 100 µM ONOOH. This indicated that no other thiol oxidising species were present in the absence of active ONOOH.
Figure 4.5 Loss of thiols in the cytosolic (A) and membrane (B) fractions of intact HCAEC treated with ONOOH.

Intact HCAEC (4 x 10^5 cells) were treated with 0-1000 µM ONOOH and decomposed 1000 µM ONOOH for 5 min at 37 °C and pH 7.4 followed by separation of cytosolic and membrane fractions. Immediately the residual thiols were quantified using ThioGlo assay and ThioGlo™ 1 reagent. The red and black bars represent loss of thiols in the presence or absence of 25 mM NaHCO₃ respectively. HO represents HCAEC only sample that was not treated with ONOOH or NaOH. De (1000) µM represents intact HCAEC treated with decomposed 1000 µM ONOOH. Results are expressed as thiol concentrations relative to protein concentrations. * and # represent significant loss (p < 0.05) of thiols relative to 0 µM ONOOH, as determined by one way ANOVA, with Dunnett’s post-hoc test. The effect of 25 mM NaHCO₃ was determined by repeated measures ANOVA, with Bonferroni post-hoc test. Values are means ± standard deviation and represent results obtained using 3 different HCAEC donors in 3 independent experiments.
Treatment of the cytosolic fraction with 10 µM ONOOH induced oxidation of 35% (0.572 µM) of thiols compared to the respective control (Figure 4.6A). In contrast, exposure of the membrane fraction to 10 µM ONOOH caused oxidation of about 25% (0.361 µM) of thiols compared to the respective control (Figure 4.6B). More thiols were oxidised in both fractions as ONOOH concentrations increased. Exposure to 100 µM ONOOH oxidised about 95 and 85% of the thiol residues of the cytosolic and membrane fraction respectively.

The initial thiol concentrations detected in the presence of 25 mM NaHCO₃ were similar to those detected in its absence. When the cytosolic fraction was treated with 5 µM ONOOH in the presence of 25 mM NaHCO₃, about 15% of cytosolic thiols were oxidised (Figure 4.6A). This significant loss was not detected when 25 mM NaHCO₃ was absent. However, overall the 2-way ANOVA statistical analysis suggested that the presence of 25 mM NaHCO₃ made no significant difference to the loss of thiols in both fractions (Figure 4.6A & B).

These data overall suggest that in the absence of cell membrane, the cytosolic protein thiols were more susceptible towards ONOOH mediated oxidation than those present on membrane proteins. Therefore if excessive ONOOH formation occurs intracellularly, the cytosolic protein thiols are likely to be targeted to a greater extent than membrane protein thiols. The data obtained in these experiments show that ONOOH can oxidise HCAEC cytosolic and membrane thiols. The increased susceptibility of membrane proteins to oxidation when cells are lysed versus intact indicates that the majority of thiol residues and other oxidation targets are present on the cytosolic face of such proteins. However whether this only applies to thiols is unclear so further studies were carried out, and reported in the next Section on other ONOOH-mediated protein modifications in intact HCAEC, lysates and subcellular fractions.
Figure 4.6 Loss of thiols in the HCAEC cytosolic (A) and membrane (B) fractions treated with ONOOH.

HCAEC cytosolic and membrane fractions were separated followed by equalisation of the thiol concentrations in both fractions using ThioGlo assay. The separated fractions were treated with 0-100 µM ONOOH and decomposed 100 µM ONOOH for 5 min at 21 °C and immediately the residual thiols were quantified. The red and black bars represent thiol concentrations relative to protein concentrations in the presence or absence of 25 mM NaHCO₃, respectively. HO represents HCAEC only sample that was not treated with ONOOH or NaOH. De (100) µM represents HCAEC fractions treated with decomposed 100 µM ONOOH. Results are expressed as thiols concentrations relative to 0 µM ONOOH. * and # represent significant loss (p < 0.05) of thiols relative to 0 µM ONOOH, as determined by one way ANOVA, with Dunnett’s post-hoc test. The effect of 25 mM NaHCO₃ was determined by repeated measures ANOVA, with Bonferroni post-hoc test. Values are means ± standard deviation and represent results obtained using 3 different HCAEC donors in 3 independent experiments.
4.3.3 Other protein modifications

4.3.3.1 Intact cells

Previous studies have demonstrated that ONOOH induces protein modifications such as fragmentation, aggregation and crosslinking (63, 118, 181, 264). It was therefore important to investigate if ONOOH induces such protein modifications in HCAEC. Silver staining was employed to detect protein modifications as it is a highly sensitive technique, which can detect proteins down to nanogram levels (366).

HCAEC were seeded at a cell density of 4 x 10^5 cells per well in a 6 well plate at 37 °C and pH 7.4 overnight. Cells were washed with PBS and incubated in HBSS (pH 7.4). Intact cells were then treated with 0-1250 µM and decomposed 1250 µM ONOOH for 5 min at 37 °C and pH 7.4. The excess oxidant was removed and cells were washed with PBS. Cells were then lysed in cold (4 °C) water and the proteins were isolated. The proteins were subsequently separated by SDS-PAGE and the gels were subjected to silver staining.

Silver staining detected a large number of HCAEC proteins in all treated and control samples (Figure 4.7). In the control (treatment with 0 µM ONOOH) no significant protein modifications were detected compared to the non-treated control (HO) (Figure 4.7A). A similar result was also obtained in the presence of 25 mM NaHCO_3 (Figure 4.7B). This indicated that in the absence of active ONOOH no other species were present which could modify HCAEC proteins in a manner apparent by silver staining after SDS-PAGE. These data also suggested that NaOH alone did not induce significant protein modification. Exposure of intact cells to decomposed 1250 µM ONOOH also caused no significant protein modifications compared to the non-treated control. This indicates that in the absence of active ONOOH, no other damaging species were present.

No significant protein modifications such as aggregation or fragmentation was detected when intact cells were treated with up to 1250 µM ONOOH for 5 min (Figure 4.7A). A similar result was also obtained in the presence of 25 mM NaHCO_3 (Figure 4.7B). These data suggest that during the 5 min incubation period, ONOOH and ONOOCO_2^- did not induce significant detectable protein fragmentation or aggregation in intact HCAEC.
Figure 4.7 ONOOH-mediated modification of intact HCAEC proteins.

Intact HCAEC (4 x 10⁵ cells) were incubated with 0-1250 µM ONOOH and decomposed 1250 µM ONOOH for 5 min at 37 °C and pH 7.4 either in the absence (A) or presence (B) of 25 mM NaHCO₃. Subsequently protein modifications were detected using silver staining after SDS-PAGE. HO represents cells that were not treated with ONOOH or NaOH. De represents cells that were treated with decomposed 1250 µM ONOOH. Gels are representative of 3 independent experiments with individual HCAEC donors.
4.3.3.2 HCAEC lysates

It was also of interest to investigate if ONOOH induces protein modifications to HCAEC when the cell membrane has been disrupted. HCAEC were seeded at a cell density of 4 x 10⁵ cells per well in a 6-well plate at 37 °C and pH 7.4 overnight. Cells were scraped with a plunger and lysed in cold (4 °C) water. The lysates were treated with 0-500 µM ONOOH as well as decomposed 500 µM ONOOH for 5 min at 21 °C. Silver staining was used to detect protein modifications induced by ONOOH.

Silver staining detected various HCAEC proteins in all treated and control samples (Figure 4.8). No detectable protein modifications were observed when lysates were exposed to decomposed 500 µM ONOOH (lane 3 in Figure 4.8A). These results indicate that in the absence of active ONOOH, no other damaging species were present which could modify HCAEC proteins in a manner apparent after SDS-PAGE. Exposure of lysates to 0 µM ONOOH did not induce significant protein modifications compared to the non-treated control (HCAEC only) (lane 4 in Figure 4.8A). These results suggested that 20 mM NaOH, the vehicle of ONOOH, alone did not induce protein modifications. Analogous results were detected in the presence of 25 mM NaHCO₃ (lanes 2-4 in Figure 4.8B).

No detectable protein modifications were detected when lysates were exposed to 25 µM ONOOH (lane 5 in Figure 4.8A). In contrast, protein smears were detected when lysates were exposed to 50–500 µM ONOOH concentrations (lanes 6-9) (Figure 4.8A). The presence of protein smears is consistent with protein modification by ONOOH. Smears could form due to protein aggregation and/or fragmentation. Interestingly in the presence of 25 mM NaHCO₃, protein smears were more obvious than in its absence (lanes 5-9 Figure 4.8B). Overall these data suggest that within 5 min of exposure, ONOOCO₂⁻ may induce some protein modifications to HCAEC proteins to a greater extent than ONOOH, in the absence of an intact cell membrane.
Figure 4.8 ONOOH-mediated modifications of HCAEC lysates proteins.

HCAEC lysates were treated with 0-500 µM ONOOH and decomposed 500 µM ONOOH for 5 min at 21 °C either in the absence (A) or presence (B) of 25 mM NaHCO$_3$. Subsequently protein modifications were detected using silver staining after SDS-PAGE. HO represents lysates that were not treated with ONOOH or NaOH. De represents lysates that were treated with decomposed 500 µM ONOOH. Gels are representative of 3 independent experiments with individual HCAEC donors.
4.3.3.3 Cellular fractions of intact cells

The results of the previous Section indicate that ONOOH modified HCAEC proteins in the absence of an intact cell membrane. It was of interest to investigate if ONOOH induces modifications preferentially to HCAEC cytosolic or membrane proteins if this oxidant was generated in excess extracellularly. HCAEC were seeded at a cell density of $4 \times 10^5$ cells per well in a 6-well plate at 37 °C and pH 7.4 overnight. Cells were treated with 0-1000 µM ONOOH as well as decomposed 1000 µM ONOOH for 5 min at 37 °C and pH 7.4 followed by separation of the cytosolic and membrane fractions. Cytosolic and membrane proteins were separated via SDS-PAGE and then the gels were subjected to silver staining.

Silver staining detected various cytosolic and membrane proteins (Figure 4.9 & 4.10). The overall protein band patterns seen in the cytosolic fraction were quite different to those seen with the membrane fraction. This suggests that different proteins were present in those fractions. In the controls (treatment with 0 µM) no detectable protein modifications were detected in both fractions compared to the non-treated control (HCAEC only) (Figure 4.9 & 4.10). A similar result was detected in the presence of 25 mM NaHCO$_3$. This suggested that 20 mM NaOH (0 µM ONOOH) alone did not induce protein modifications. No significant cytosolic and membrane protein modifications were detected when cells were exposed to decomposed 1000 µM ONOOH. This result was also apparent in the presence of 25 mM NaHCO$_3$. In the absence of active ONOOH, no other damaging species were present, which could modify HCAEC proteins.

No significant cytosolic- and membrane-protein modifications were detected when intact cells were exposed up to 1000 µM ONOOH (Figure 4.9 A & B). Similar results were also obtained in the presence of 25 mM NaHCO$_3$ (Figure 4.10A & B). These data collectively suggest that within 5 min of exposure to up to 1000 µM ONOOH / ONOOCO$_2^-$, no detectable protein fragmentation or aggregation was detected. This may be due to the short incubation period employed.
Figure 4.9 ONOOH-mediated modification of intact HCAEC cytosolic proteins.

Intact HCAEC (4 x 10⁵ cells) were incubated with 0-1000 µM ONOOH and decomposed 1000 µM ONOOH for 5 min at 37 °C and pH 7.4 both in the absence (A) and presence (B) of 25 mM NaHCO₃. Subsequently the cytosolic and membrane fractions were separated via a commercial kit. The protein concentrations were equalised in both fractions via the BCA Assay. Silver staining of the cytosolic fraction was performed after SDS-PAGE. HO represents cells that were not treated with ONOOH or NaOH. De represents cells that were treated with decomposed 1000 µM ONOOH. Gels are representative of 3 independent experiments with individual HCAEC donors.
Figure 4.10 ONOOH-mediated modification of intact HCAEC membrane proteins.

Intact HCAEC (4 x 10^5 cells) were incubated with 0-1000 µM ONOOH and decomposed 1000 µM ONOOH for 5 min at 37 °C and pH 7.4 both in the absence (A) and presence (B) of 25 mM NaHCO₃. Subsequently the cytosolic and membrane fractions were separated via a commercial kit. The protein concentrations were equalised in both fractions via the BCA Assay. Silver staining of the membrane fraction was performed after SDS-PAGE. HO represents cells that were not treated with ONOOH or NaOH. De represents cells that were treated with decomposed 1000 µM ONOOH. Gels are representative of 3 independent experiments with individual HCAEC donors.
4.3.3.4 Cell lysate experiments

A protein smear, which is indicative of aggregation and/or fragmentation, was detected when HCAEC lysates were exposed to 50 µM or higher concentrations of ONOOH for 5 min. Thus, it was of interest to investigate if ONOOH induces protein modifications preferentially to HCAEC cytosolic and membrane proteins in the absence of an intact cell membrane. HCAEC were seeded at a cell density of 4 x 10^5 cells per well in a 6-well plate at 37 °C and pH 7.4 overnight. The cytosolic and membrane fractions were separated using a commercial kit. The amount of proteins in both fractions were equalised via the BCA assay and the separated fractions were then treated with 0-1000 µM ONOOH as well as decomposed 1000 µM ONOOH for 5 min at 21 °C. The proteins were separated via SDS-PAGE and then silver staining was performed.

Silver staining detected various cytosolic (Figure 4.11A) and membrane (Figure 4.12A) proteins. The protein band patterns seen with the cytosolic fraction were quite different to those seen with the membrane fraction. This suggests that different proteins were present in those fractions. No detectable protein modifications were seen when either fraction was exposed to 0 µM ONOOH or decomposed 1000 µM ONOOH (Figure 4.11A & 4.12A). Similar results were obtained in the presence of 25 mM NaHCO₃ (Figure 4.11B & 4.12B) consistent with previous results.

A few protein bands in both the cytosolic and membrane fractions appear to become less well stained (lighter in colour) when exposed to high concentrations of ONOOH (indicated by arrows in Figure 4.11A & 4.12A). Similar results were detected in the presence of 25 mM NaHCO₃ (Figure 4.11B & 4.12B). Densitometric analysis of an unknown cytosolic protein (X) (Figure 4.13A) and unknown membrane protein (Y) (Figure 4.13B), which appeared to change in intensity on visual inspection, was also performed on these individual protein bands. The results indicated that no significant decrease in the pixel density of these proteins was detected. This could be due to the large error bars on the data arising from differences in protein expression of the three HCAEC donors used for this experiment. These results indicate that these proteins may be modified by ONOOH and ONOOCO₂⁻. However the exact nature and extent of ONOOH- and ONOOCO₂⁻-mediated HCAEC protein modifications could not be determined in greater detail.
Figure 4.11 ONOOH-mediated modification of HCAEC cytosolic proteins.

HCAEC cytosolic and membrane fractions were separated and the protein concentrations were equalised in both fractions via the BCA Assay. Both fractions were then treated with 0-1000 µM ONOOH and decomposed 1000 µM ONOOH for 5 min at 21 °C either in the absence (A) or presence (B) of 25 mM NaHCO₃. Silver staining of the cytosolic fraction was performed after SDS-PAGE. HO represents cytosolic fractions that were not treated with ONOOH or NaOH. De represents cytosolic fractions that were treated with decomposed 1000 µM ONOOH. Multiple unknown protein bands (marked with arrows) became lighter in colour as ONOOH concentrations increase. Densitometric analysis was performed on the individual bands of the unknown protein (marked X) to determine its pixel density when exposed to increasing concentrations of ONOOH. Gels are representative of 3 independent experiments with individual HCAEC donors.
HCAEC cytosolic and membrane fractions were separated and the protein concentrations were equalised in both fractions via the BCA Assay. Both fractions were then treated with 0-1000 µM ONOOH and decomposed 1000 µM ONOOH for 5 min at 21 °C either in the absence (A) or presence (B) of 25 mM NaHCO₃. Silver staining of the membrane fraction was performed after SDS-PAGE. HO represents membrane fractions that were not treated with ONOOH or NaOH. De represents membrane fractions that were treated with decomposed 1000 µM ONOOH. Multiple unknown protein bands (marked with arrows) became lighter in colour as ONOOH concentrations increase. Densitometric analysis was performed on individual bands of the unknown protein (marked Y) to determine its pixel density when exposed to increasing concentrations of ONOOH. Gels are representative of 3 independent experiments with individual HCAEC donors.
HCAEC cytosolic and membrane fractions were separated and the protein concentrations were equalised in both fractions via the BCA Assay. Both fractions were then treated with 0-1000 µM ONOOH and decomposed 1000 µM ONOOH for 5 min at 21 °C either in the absence (black bars) or presence (red bars) of 25 mM NaHCO$_3$. Silver staining of the fractions was performed after SDS-PAGE. HO represents fractions that were not treated with ONOOH or NaOH. De (1000) represents fractions that were treated with decomposed 1000 µM ONOOH. Densitometric analysis was performed on individual bands of the unknown cytosolic protein (X) and membrane protein (Y) to determine its pixel density when exposed to increasing concentrations of ONOOH. Similar results were obtained using 3 independent experiments with individual HCAEC donors.
4.3.4 3-Nitrotyrosine formation

4.3.4.1 Intact cells

3-Nitrotyrosine (3NT) has been used previously as a biomarker for ONOOH-mediated damage (140) and it was therefore of interest to examine 3NT formation in intact HCAEC exposed to ONOOH. These experiments aimed to mimic the effect of \textit{in vivo} extracellular ONOOH formation. HCAEC were seeded at a cell density of 4 x 10^5 cells per well in a 6-well plate at 37 °C and pH 7.4 overnight. Cells were washed with PBS and incubated in HBSS (pH 7.4). The cells were then treated with 0-1250 µM ONOOH as well as decomposed 1250 µM ONOOH for 5 min at 37 °C and pH 7.4. The excess oxidant was removed and cells were washed with PBS. Cells were scraped using a plunger and cold (4 °C) water was added to completely lyse the cells. Proteins were isolated and separated by SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and then the membrane was probed with an anti-3NT antibody to detect 3NT formation.

3NT formation was not detected in the controls (HCAEC only and 20 mM NaOH alone) (Figure 4.14A). This indicates that in these experiments in the absence of added ONOOH, no other nitrating species were present. Intact HCAEC were also treated with decomposed 1250 µM ONOOH; no 3NT formation was detected under these conditions. Analogous results were detected in the presence of 25 mM NaHCO$_3$.

Visual observations detected 3NT formation on multiple proteins when intact cells were treated with 100 µM or higher concentrations of ONOOH after a 5 min incubation period (Figure 4.14A). Many proteins were simultaneously affected by bolus ONOOH exposure. The band intensity of nitrated proteins increased with increasing concentrations of ONOOH. This clearly indicates that more 3NT was formed as the concentrations of ONOOH used were increased. Similar results were also detected in the presence of 25 mM NaHCO$_3$ (Figure 4.14B) suggesting that ONOOCO$_2^-$ can also dose-dependently and simultaneously nitrate multiple proteins. These results overall indicate that if ONOOH or ONOOCO$_2^-$ were generated extracellularly, these oxidants can nitrate various proteins. However the exact identities of these nitrated proteins are unknown at this stage. Also it is unknown whether these nitrated proteins are cytosolic or membrane proteins.
β-Actin (42 kDa) was used as a loading control to confirm that all lanes were loaded with equal amounts of protein. The membrane containing immobilised proteins was probed for β-actin using an anti-β-actin antibody. Direct observations suggest that β-actin protein seems to be modified by ONOOH especially in the presence of 25 mM NaHCO₃ shown by the band becoming progressively lighter with increasing ONOOH treatment.

Densitometric analysis of the entire lane of nitrated proteins and individual β-actin protein bands were performed using Image J software. Due to the high background reading detected even in the control lanes arising from the long exposure times needed to detect 3NT, this technology failed to show a significant increase in 3NT formation or modification of β-actin protein. Therefore it was difficult to determine what levels of ONOOH- or ONOOCO₂- mediated 3NT formation or modification to β-actin in intact HCAEC after a 5 min incubation period.

4.3.4.2 HCAEC lysates

In the experiments reported in the previous sections, 3NT formation was detected on various proteins of intact HCAEC treated with high concentrations of ONOOH. High concentrations of ONOOH were required to detect significant 3NT possibly because the cell membrane influences the extent of damage induced by ONOOH. It was therefore of interest to examine 3NT formation on HCAEC proteins exposed to ONOOH in the absence of an intact cell membrane. HCAEC were seeded at a cell density of 4 x 10⁵ cells per well in a 6-well plate at 37 °C and pH 7.4 overnight. Cells were washed with PBS and scraped using a plunger. Cold (4 °C) water was added to completely lyse the cells. HCAEC lysates were treated with 0-500 µM ONOOH as well as decomposed 500 µM ONOOH for 5 min at 21 °C and pH 7.4.

3NT formation was not detected in the controls (HCAEC only and 0 µM ONOOH) both in the absence (Figure 4.15A) and presence (Figure 4.15B) of 25 mM NaHCO₃. Similarly when HCAEC lysates were treated with decomposed 500 µM ONOOH, no 3NT formation was detected. A similar result was seen in the presence of 25 mM NaHCO₃. This indicates that apart from active ONOOH, no other nitrating agent was present in the ONOOH stock solution.
Figure 4.14 3NT formation on multiple proteins of intact HCAEC treated with ONOOH either in the absence (A) or presence (B) of 25 mM NaHCO₃.

Intact HCAEC (4 x 10⁵ cells) were treated with 0-1250 µM ONOOH and decomposed 1250 µM ONOOH for 5 min at 37 °C and pH 7.4 either in the absence (A) or presence (B) of 25 mM NaHCO₃. Subsequently 3NT formation was detected using an anti-3NT antibody and Western blotting. HO represents cells that were not treated with ONOOH or NaOH. De represents cells that were treated with decomposed 1250 µM ONOOH. β-Actin was employed as a loading control and this protein were detected using an anti-β-actin antibody and Western blotting. Similar results were obtained using 3 different HCAEC donors in 3 independent experiments.
3NT formation was detected on multiple proteins when lysates were treated with 50 µM and higher concentrations of ONOOH (Figure 4.15A). 3NT formation increased on proteins with increasing concentrations of ONOOH. Similar results were also obtained in the presence of 25 mM NaHCO₃ (Figure 4.15B). These results suggest that certain proteins are selectively nitrated by both ONOOH and ONOOCO₂⁻ after a 5 min incubation period.

Densitometric analysis of the entire lane of nitrated proteins indicated that significant 3NT formation was detected when lysates were exposed to 100 µM or higher concentrations of ONOOH (Figure 4.16A). The presence of 25 mM NaHCO₃ made no significant difference to the extent of ONOOH-mediated 3NT formation. These results indicate that in the absence of an intact cell membrane, low concentrations of ONOOH induce significant 3NT formation. However the exact identities of these nitrated proteins are unknown at this stage.

In the intact cell studies, β-actin (42 kDa) was employed as a loading control and this protein was also used as loading controls in these lysate experiments to confirm that all lanes were loaded with equal amounts of protein. The antibody for β-actin lost the ability to recognise this protein when the lysates were treated with increasing ONOOH concentrations (Figure 4.15A). Beta-tubulin (β-tubulin) (50 kDa) was also examined as an additional loading control. Interestingly, the antibody for the latter protein also lost its recognition when exposed to increasing concentrations of ONOOH (Figure 4.15A). Similar results were detected in the presence of 25 mM NaHCO₃ (Figure 4.15B).

Densitometric analysis of the individual bands of β-actin and β-tubulin proteins was performed using Image J software. A significant loss of β-actin protein was detected when lysates were treated with 250 µM or higher concentrations of ONOOH (Figure 4.16B). In the presence of 25 mM NaHCO₃, no significant loss of β-actin protein was detected. A significant loss of β-tubulin protein was also detected when lysates were exposed to 50 µM or higher concentrations of ONOOH (Figure 4.16C). In the presence of 25 mM NaHCO₃, loss of β-tubulin protein was detected to a greater extent when lysates were exposed to 50 µM of ONOOH. This may be associated with the ability of ONOOH to target these proteins, resulting in modification of the epitopes recognised by the antibodies in each case.
These results overall suggest that both of these proteins are being modified by ONOOH. ONOOH modified β-actin to a greater extent than ONOOCO$_2^-$. In contrast β-tubulin was modified to a greater extent by ONOOCO$_2^-$ than ONOOH. Most probably β-actin and β-tubulin proteins were also nitrated by ONOOH and ONOOCO$_2^-$ in the lysate sample since nitrated proteins were detected around the 42 and 50 kDa (Figure 4.15A & B).

**Figure 4.15** 3NT formation on various proteins of HCAEC lysates treated with ONOOH either in the absence (A) or presence (B) of 25 mM NaHCO$_3$.

HCAEC lysates were incubated with 0-500 µM ONOOH and decomposed 500 µM ONOOH for 5 min at 21 °C and pH 7.4 either in the absence (A) or presence (B) of 25 mM NaHCO$_3$. Subsequently 3NT formation was detected using an anti-3NT antibody and Western blotting. HO represents lysates that were not treated with ONOOH or NaOH. De represents lysates that were treated with decomposed 500 µM ONOOH. Multiple nitrated proteins have been detected when lysates were treated with increasing concentrations of ONOOH. β-Actin (42 kDa) and β-tubulin (50 kDa) were employed as a loading controls, however both antibodies significantly lost their recognition when exposed to increasing ONOOH concentrations (A & B). Similar results were obtained using 3 different HCAEC donors in 3 independent experiments.
Figure 4.16 Densitometric analysis showing 3NT formation in the HCAEC lysates exposed to ONOOH (A) along with β-actin (B) and β-tubulin (C) protein levels.

HCAEC lysates were incubated with 0-500 μM ONOOH and decomposed 500 μM ONOOH for 5 min at 37 °C and pH 7.4 either in the absence (black bars) or presence (red bars) of 25 mM NaHCO₃. Western blotting was performed to detect 3NT formation using an anti-3NT antibody. β-Actin (42 kDa) was detected using an anti-β-actin antibody and β-tubulin (50 kDa) was detected using an anti-β-tubulin antibody. Densitometric analysis was performed on the entire lane of nitrated proteins (A) using Image J software. Densitometric analysis was also performed on the individual β-actin (B) and β-tubulin (C) protein bands. HO represents lysates that were not treated with ONOOH or NaOH. De represents intact HCAEC treated with decomposed 500 μM ONOOH. * and # represent significant (p < 0.05) increase in nitrated protein’s pixel density or loss of control proteins pixel density relative to 0 μM ONOOH, as determined by one way ANOVA, with Dunnett’s post-hoc test. The effect of 25 mM NaHCO₃ was determined by repeated measures ANOVA, with Bonferroni post-hoc test. ^ represent a significant difference in the pixel density in the presence of 25 mM NaHCO₃. Values are means ± standard deviation and represent results obtained using 3 different HCAEC donors in 3 independent experiments.
4.3.4.3 Cellular fractions of intact cells

In the previous sections, it has been shown that significant 3NT formation was detected when intact HCAEC and lysates were treated with ONOOH and ONOOCO$_2^-$.

It was therefore of interest to investigate whether and which cytosolic or membrane proteins of HCAEC are being nitrated by these oxidants. The data presented in the previous Section is consistent with modification of β-actin and β-tubulin by ONOOH and ONOOCO$_2^-$.

HCAEC were seeded at a cell density of 4 x 10$^5$ cells per well in a 6-well plate at 37 °C and pH 7.4 overnight. Cells were washed with PBS and treated with 0-1000 μM ONOOH as well as decomposed 1000 μM ONOOH for 5 min at 37 °C and pH 7.4. This step was followed by the separation of cytosolic and membrane fractions using a commercial kit. The efficiency of separation of the cytosolic and membrane fractions was then confirmed using anti-ABCG1 and anti-VEGF antibodies respectively as described in Section 4.3.2.3.

ABCG1 and VEGF were detected in the membrane and cytosolic fractions respectively (Figure 4.17A). A similar result was detected in the presence of 25 mM NaHCO$_3$ (Figure 4.17B). This confirms that the separation of cytosolic and membrane proteins was efficient and the fractions were not significantly cross-contaminated. The levels of each of these proteins do not appear to affected by the increasing concentration of ONOOH (perhaps with the exception of VEGF at the highest ONOOH concentrations). This is in contrast to the situation with β-actin and β-tubulin, where recognition by these antibodies was lost as ONOOH concentrations increased.
Figure 4.17 Detection of ABCG1 and VEGF in the cytosolic and membrane fractions isolated from intact HCAEC exposed to ONOOH.

Intact HCAEC (4 x 10^5 cells) were incubated with 0-1000 µM ONOOH and decomposed 1000 µM ONOOH for 5 min at 37 °C and pH 7.4 followed by separation of cytosolic and membrane fractions. The protein concentrations were equalised in both fractions via the BCA Assay. Western blotting was performed after SDS-PAGE. ABCG1 (a membrane protein) and VEGF (a cytosolic protein) were detected using anti-ABCG1 and anti-VEGF antibodies respectively. HO represents cells not treated with ONOOH or NaOH. Decomp (1000) µM represents cells treated with decomposed 1000 µM ONOOH. Similar results were obtained using 3 different HCAEC donors in 3 independent experiments.
3NT formation was detected via Western blotting after SDS-PAGE using an anti-3NT antibody. In both fractions, no significant 3NT formation was detected in the controls (HCAEC only, treatment with 0 µM ONOOH or with decomposed 1000 µM ONOOH) (Figure 4.19 & 4.20). Similar results were obtained in the presence of 25 mM NaHCO₃.

Direct observations suggest that multiple cytosolic (Figure 4.18A) and membrane (Figure 4.19A) proteins were nitrated when intact HCAEC were treated with increasing concentrations of ONOOH. 3NT formation increased as ONOOH concentrations increased. Similar results were obtained in the presence of 25 mM NaHCO₃ (Figure 4.18B & 4.19B) indicating that ONOOH and ONOOCO$_2^-$ can nitrate multiple proteins. Interestingly some cytosolic and membrane proteins appear to be selectively nitrated by both ONOOH and ONOOCO$_2^-$. 

Densitometric analysis of the entire lane of nitrated proteins was performed. Due to high background in the control samples, this technology failed to show a significant increase in nitrated cytosolic and membrane proteins as ONOOH and ONOOCO$_2^-$ concentrations increased. Owing to this technical limitation, it was difficult to detect which concentrations of ONOOH and ONOOCO$_2^-$ induced quantitatively significant 3NT formation in intact HCAEC after a 5 min incubation period. Thus it was not possible to assess whether cytosolic or membrane proteins are nitrated to a greater extent by ONOOH and ONOOCO$_2^-$. 

However visual comparison of the cytosolic and membrane data suggests that nitration is occurring to a significant extent at lower concentrations of ONOOH in the cytosolic preparations than in the membrane (Figure 4.18A and 4.19A). Furthermore in the membrane fraction experiments, greater nitration appears to occur with lower levels of ONOOH in the presence of NaHCO₃ than in its absence (Figure 4.19A and B).

Overall these data indicate that under these experimental conditions, ONOOH and ONOOCO$_2^-$ induce 3NT formation on selected cytosolic and membrane proteins of intact HCAEC within 5 min. The exact identities of the nitrated cytosolic and membrane proteins are unknown at this stage.
Figure 4.18 3NT formation in the cytosolic fraction of intact HCAEC treated with ONOOH.

Intact HCAEC (4 x 10^5 cells) were incubated with 0-1000 µM ONOOH and decomposed 1000 µM ONOOH for 5 min at 37 °C and pH 7.4 both in the absence (A) and presence (B) of 25 mM NaHCO₃. The cytosolic and membrane fractions were then separated. The protein concentrations were equalised in both fractions via the BCA Assay. Western blotting was performed to detect 3NT formation in the cytosolic fraction using an anti-3NT antibody after SDS-PAGE. HO represents cells that were not treated with ONOOH or NaOH. Decomp (1000) µM represents cells that were treated with decomposed 1000 µM ONOOH. Multiple nitrated cytosolic proteins have been detected when cells were treated with ONOOH. Similar results were obtained using 3 different HCAEC donors in independent experiments.
Figure 4.19 3NT formation in the membrane fraction of intact HCAEC treated with ONOOH.

Intact HCAEC (4 x 10⁵ cells) were incubated with 0-1000 µM ONOOH and decomposed 1000 µM ONOOH for 5 min at 37 °C and pH 7.4 both in the absence (A) and presence (B) of 25 mM NaHCO₃. The cytosolic and membrane fractions were then separated. The protein concentrations were equalised in both fractions via the BCA Assay. Western blotting was performed to detect 3NT formation in the membrane fraction using an anti-3NT antibody after SDS-PAGE. HO represents cells that were not treated with ONOOH or NaOH. Decomp (1000) µM represents cells that were treated with decomposed 1000 µM ONOOH. Multiple nitrated membrane proteins have been detected when cells were treated with ONOOH. Similar results were obtained using 3 different HCAEC donors in independent experiments.
4.3.4.4 Cell lysate experiments

The results described in the previous Section indicated that significant 3NT formation occurs on the cytosolic and membrane proteins of intact HCAEC treated with ONOOH and ONOOO$^{-2}$. It was therefore of interest to investigate whether cytosolic and/or membrane proteins were nitrated to the same extent if these oxidants were generated in excess intracellularly in the absence of an intact cell membrane.

HCAEC were seeded at a cell density of $4 \times 10^5$ cells per well in a 6-well plate at 37 °C and pH 7.4 overnight. The cytosolic and membrane fractions were separated using a commercial kit. The amount of proteins in both fractions were equalised via the BCA assay and the separated fractions were then treated with 0-1000 µM ONOOH as well as decomposed 1000 µM ONOOH for 5 min at 21 °C.

Antibodies against ABCG1 (membrane protein) and VEGF (cytosolic protein) were used to confirm efficient fraction separation (Figure 4.20). As previously there doesn't appear to be significant loss of recognition of these proteins with increasing ONOOH concentrations, unlike the situation with β-actin and β-tubulin though there may be some loss of ABCG1 in both cases (Figure 4.20A and B) at the highest ONOOH concentrations.

3NT formation was detected via Western blotting after SDS-PAGE using an anti-3NT antibody. In both fractions no significant 3NT formation was detected in the controls (HCAEC only, treatment with 0 µM ONOOH or with decomposed 1000 µM ONOOH) (Figure 4.21 & 4.22). Multiple nitrated proteins were detected in the cytosolic (Figure 4.21) and membrane (Figure 4.22) fractions when these fractions were treated with high concentrations of ONOOH. In both fractions, nitration of multiple proteins increased as the ONOOH concentrations were increased.
Figure 4.20 Detection of ABCG1 and VEGF in separated HCAEC fractions exposed to ONOOH.

HCAEC cytosolic and membrane fractions were separated and the protein concentrations were equalised in both fractions via the BCA Assay. Both fractions were treated with 0-1000 µM ONOOH and decomposed 1000 µM ONOOH for 5 min at 21 °C both in the absence (A) and presence (B) of 25 mM NaHCO₃. Western blotting was performed after SDS-PAGE. ABCG1 (a membrane protein) and VEGF (a cytosolic protein) were detected using anti-ABCG1 and anti-VEGF antibodies. HO represents fractions not treated with ONOOH or NaOH. Decomp (1000) µM represents fractions treated with decomposed 1000 µM ONOOH. Similar results were obtained using 3 different HCAEC donors in independent experiments.
The presence of 25 mM NaHCO$_3$ appears to make a significant difference to ONOOH-mediated 3NT formation on the cytosolic and membrane proteins. In the cytosolic fraction, proteins marked A and F appear to be nitrated by ONOOH and not by ONOOCO$_2^-$ (Figure 4.21). Other cytosolic proteins marked B, C and E appear to be nitrated to a greater extent by ONOOH than ONOOCO$_2^-$ . Interestingly the protein marked D appear to be nitrated to a greater extent by ONOOCO$_2^-$ than ONOOH. Whilst in the membrane fraction, the proteins in regions X and Y appear to be nitrated to a greater extent by ONOOH than ONOOCO$_2^-$ (Figure 4.22). These data indicate that some cytosolic and membrane proteins appear to be selectively nitrated by either ONOOH or ONOOCO$_2^-$ . Multiple proteins also seem to be nitrated to a greater extent by either ONOOH or ONOOCO$_2^-$.

Densitometric analysis of the entire lane of nitrated cytosolic and membrane proteins was performed by the use of Image J software both in the presence and absence of 25 mM NaHCO$_3$. Due to the high background values obtained for the control samples, this analysis failed to show a significant increase in 3NT formation on the cytosolic and membrane proteins as the ONOOH and ONOOCO$_2^-$ concentrations increased. Due to this technical limitation, it was not possible to assess in a quantitative manner which concentrations of ONOOH and ONOOCO$_2^-$ induced significant 3NT formation on cytosolic and membrane proteins relative to the control samples. It was also difficult to determine whether cytosolic or membrane proteins (in total) are nitrated to a greater extent in the absence of an intact cell membrane; it is however clear that there are major variations in the extent of nitration of proteins within each fraction.

These data overall suggest that in the absence of an intact cell membrane, both ONOOH and ONOOCO$_2^-$ induced detectable nitration of multiple cytosolic and membrane proteins simultaneously after 5 min incubation period. The exact identities of these nitrated cytosolic and membrane proteins are unknown.
Figure 4.21 3NT formation in the cytosolic fraction treated with ONOOH.

HCAEC cytosolic and membrane fractions were separated and the protein concentrations were equalised in both fractions via the BCA Assay. Both fractions were treated with 0-1000 µM ONOOH and decomposed 1000 µM ONOOH for 5 min at 21 °C both in the absence (A) and presence (B) of 25 mM NaHCO₃. Western blotting was performed to detect 3NT formation in the cytosolic proteins using an anti-3NT antibody after SDS-PAGE. HO represents cytosolic fraction not treated with ONOOH or NaOH. De represents cytosolic fraction treated with decomposed 1000 µM ONOOH. Similar results were obtained using 3 different HCAEC donors in independent experiments.
Figure 4.22 3NT formation in the membrane fraction treated with ONOOH.

HCAEC cytosolic and membrane fractions were separated and the protein concentrations were equalised in both fractions via the BCA Assay. Both fractions were treated with 0-1000 µM ONOOH and decomposed 1000 µM ONOOH for 5 min at 21 °C both in the absence (A) and presence (B) of 25 mM NaHCO₃. Western blotting was performed to detect 3NT formation in the membrane fraction using an anti-3NT antibody after SDS-PAGE. HO represents membrane fraction not treated with ONOOH or NaOH. De represents membrane fraction treated with decomposed 1000 µM ONOOH. Similar results were obtained using 3 different HCAEC donors in independent experiments.
4.4 Discussion

Previous literature reports suggest that thiol oxidation and 3NT formation are major protein modifications induced by ONOOH (63, 117). In addition, ONOOH also appears to induce protein aggregation, crosslinking and fragmentation in some situations (63, 118, 181, 264). The results reported in the previous Chapter indicate that ONOOH and ONOOCO$_2$\(^-\) can induce significant thiol oxidation, 3NT formation and other protein modifications such as fragmentation and aggregation.

This Chapter aimed to investigate the modifications induced by ONOOH to HCAEC after a short, 5 min incubation period. In vivo ONOOH can be generated both intra- and extracellularly, so the effect of excess extracellular and intracellular generated ONOOH was investigated by treating intact HCAEC and lysates with varying concentrations of ONOOH in both the absence and presence of 25 mM NaHCO$_3$.

Excess extracellular ONOOH formation has been shown in the experiments reported here to induce cytotoxic effects as evident by MTT assay to HCAEC only at high concentrations. Thus exposure of cells to up to 500 µM ONOOH for 5 min did not induce significant cell death. Whereas when cells were exposed to 1250 µM ONOOH and ONOOCO$_2$\(^-\) for 5 min, a significant loss of cell viability (15 %) and loss of thiol residues (20 %) were detected. Fractional centrifugation experiments have indicated that the exposure of intact HCAEC to 750 µM ONOOH induced oxidation of approximately 17 % of cytosolic protein thiols and 30 % of membrane protein thiols. The presence of 25 mM NaHCO$_3$ made no significant difference to ONOOH-mediated thiol oxidation. In intact cells, the membrane protein thiols seem to be preferentially oxidised when compared to cytosolic protein thiols. This is as might be expected if ONOOH is entering the cell from the extracellular medium.

In the absence of an intact cell membrane, exposure to 5 µM ONOOH and ONOOCO$_2$\(^-\) caused oxidation of 25 and 50 % of thiol residues respectively. 35 % of cytosolic and 25 % of membrane thiols were oxidised by 10 µM ONOOH. The presence of 25 mM NaHCO$_3$ made no significant difference to ONOOH-mediated thiol oxidation. In the absence of an intact cell membrane the overall extent of thiol oxidation was much greater then with the intact cells and the cytosolic thiols appear to be oxidised to a greater extent than membrane thiols.
ONO0H-mediated protein modifications were detected via silver staining after proteins were separated via SDS-PAGE. No significant (detectable) protein modifications appeared when intact cells were exposed to up to 1250 µM concentrations of ONOOH for 5 min. This result was further confirmed by fractional centrifugation experiments where exposure of intact cells to up to 1250 µM ONOOH for 5 min induced no significant (detectable) modification to cytosolic or membrane proteins.

In contrast, when HCAEC lysates were exposed to varying concentrations of ONOOH, protein smears were detected with 50 µM or higher concentrations of ONOOH. This is indicative of either protein aggregation and/or fragmentation. Protein aggregation and/fragmentation was detected to a greater extent in the presence of 25 mM NaHCO₃. These suggest that ONOOCO₂⁻ may induce protein aggregation and/fragmentation to a greater extent than ONOOH in the absence of an intact cell membrane after 5 min incubation period and that intracellular ONOOH has much more damaging effects than extracellular ONOOH formation.

Fractional centrifugation experiments have indicated that the intensity of the silver staining of multiple cytosolic and membrane proteins appear to become less intense when separated fractions were directly exposed to high concentrations of ONOOH. Interestingly, more membrane protein bands appear to be affected compared to cytosolic proteins as ONOOH concentrations increased when the separated fractions were directly exposed to ONOOH. This may indicate that more membrane proteins were being modified by intracellularly generated ONOOH than cytosolic proteins. Densitometric analysis for the silver staining was performed on selected protein bands of cytosolic and membrane proteins. This analysis failed to show any significant decrease in band intensities when proteins were exposed to high ONOOH concentrations. This is most probably due to the high background or differences in protein expression between the 3 different HCAEC donors. These results indicate that ONOOH/ONOOCO₂⁻ induce protein modifications to HCAEC proteins, with these modified to a greater extent when the cell membrane was disrupted.

3NT has been used as a “gold standard” biomarker for ONOOH-mediated damage for over two decades (143, 148). 3NT formation was detected on multiple proteins when intact cells were treated with 100 µM or greater concentrations of ONOOH. Nitration of many proteins increased as the ONOOH concentrations were increased. Owing to the
high background, the densitometric analysis could not provide any quantitative data on
the extent of increase in 3NT formation on proteins of intact HCAEC. β-Actin is an
abundant cellular protein that was examined as a potential target of ONOOH. The
densitometric analysis also failed to show a significant decrease in β-actin protein levels
as ONOOH concentrations increased. Examination of the Western blots indicated that
nitration of multiple cytosolic and membrane proteins occurred when intact cells were
exposed high concentrations of ONOOH and ONOOCO$_2$ with the level of nitration
increased as the concentrations of these oxidants increased.

In the absence of an intact cell membrane, direct observations detected significant 3NT
formation occurring on multiple HCAEC proteins when exposed to 100 µM or higher
concentrations of ONOOH and ONOOCO$_2$$. A significant loss of β-actin and β-tubulin
were detected when lysates were exposed to 250 and 100 µM ONOOH respectively. In
the presence of 25 mM NaHCO$_3$, β-tubulin loss occurred to a greater extent. ABC-G1
and VEGF do not appear to be affected by ONOOH except at high ONOOH
concentrations. Exposure of the proteins present in the cytosolic fraction to 500 µM
ONOOH induced significant (detectable) 3NT formation. Exposure of the membrane
fraction to 250 µM ONOOH induced significant 3NT formation on proteins present in
this fraction. In the presence of 25 mM NaHCO$_3$, significant 3NT formation was detected
in the membrane fraction when lysates were exposed to 500 µM ONOOH. Thus
membrane proteins appear to be nitrated by ONOOH and ONOOCO$_2$ to a greater extent
than cytosolic proteins.

Previous studies have shown ONOOH-mediated cysteine oxidation occurring in isolated
proteins, cells or animal models. Vascular endothelial growth factor (VEGF) is an
important regulator of physiological angiogenesis that is important for embryogenesis,
skeletal growth and reproductive roles (367). It has been reported that under
physiological conditions, low concentrations of ONOOH mediates VEGF’s signal and
function in microvascular endothelial cells via thiol oxidation (368). However no major
change in VEGF was seen here at low ONOOH concentrations. Protein tyrosine
phosphatases participate in signalling, cell growth and differentiation and the cell cycle
(369). These enzymes have critical thiol residues in the active site that is important for
their activity. A previous study has reported that ONOOH oxidised important thiol
residues in protein tyrosine phosphatases, which resulted in rapid and irreversible inactivation of this enzyme (370).

Analogously other studies have reported ONOOH-mediated thiol oxidation and subsequent inactivation of thiol-containing enzymes such as alcohol dehydrogenase (371), creatine kinase (124), succinate dehydrogenase (372), glyceraldehyde-3-phosphate dehydrogenase (123), NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochrome c reductase (complex III) and ATP synthase (complex V) of the mitochondrial respiratory chains (125, 126). In contrast, ONOOH-mediated cysteine oxidation can also activate enzymes such as matrix metalloproteinases. These events may play a role in ONOOH-mediated toxicity in heart diseases (127). The results of this Chapter agree with these studies since thiol loss or oxidation was detected when HCAEC were exposed to ONOOH. It would be interesting to examine in further studies which proteins were modified (see also Chapter 6) and whether HCAEC proteins are either activated or inactivated by ONOOH-mediated thiol oxidation under our experimental conditions.

Several studies have shown previously that ONOOH-mediated tyrosine nitration is observed in endothelial cells. A study reported that exposure of bovine aortic endothelial cells to arsenic caused ONOOH formation and nitration of cyclooxygenase-2 (COX-2) (373). COX-2 is a key inflammatory regulator that is believed to be associated with inflammation, pain, parturition and some types of cancer (374). Another study showed that exposure of bovine pulmonary artery endothelial cells to ONOOH increased the intensity of nitrated proteins and reduced the intensity of tyrosine-phosphorylated proteins (338). The results detected in this Chapter agree with these previous studies since exposure of HCAEC to ONOOH increased the band intensity of nitrated proteins. The majority of the studies have indicated that tyrosine nitration is associated with loss of function, with nitration resulting in inactivation of about 140 mammalian proteins whose activity depends on tyrosine residues (338). It would be interesting to identify which HCAEC proteins are nitrated by ONOOH and to investigate whether nitration induces loss of function of these proteins.

There is also evidence in the literature that indicates that ONOOH induces protein aggregation, fragmentation and cross-linking. Perlecan is a major heparan sulphate proteoglycan that plays an important role in vascular homeostasis by stabilising and
organising the ECM and controls the adhesion, differentiation and proliferation of vascular cells (267). It has been reported that ONOOH induced perlecan aggregation in a concentration dependent manner (264). The results obtained in this Chapter agree with these studies since protein modifications (such as aggregation and fragmentation) were detected when HCAEC were exposed to ONOOH, though it should be noted that these are very different protein types (extracellular matric versus cellular).

The data reported in this Chapter indicate that ONOOH and ONOOCO$_2^-$ can induce HCAEC cell death at high concentrations using an incubation time of 5 min. Low concentrations of ONOOH and ONOOCO$_2^-$ in the absence of an intact cell membrane can induce significant thiol loss, 3NT formation and protein modifications in HCAEC. In contrast, high concentrations of ONOOH and ONOOCO$_2^-$ generated extracellularly are required to induce a significant thiol loss, 3NT formation and protein modifications in intact HCAEC. Extracellularly generated ONOOH can nitrate multiple membrane as well as cytosolic proteins. Regardless of whether ONOOH formation occurs intra- or extracellularly, selective cytosolic and membrane proteins are nitrated by ONOOH and ONOOCO$_2^-$.

Thiols groups of HCAEC proteins appear to be major targets of ONOOH and ONOOCO$_2^-$. For instance in the absence of an intact cell membrane, 5 µM ONOOH and ONOOCO$_2^-$ induced significant thiol loss, whereas 100 µM or higher concentration of oxidants were required to induce significant 3NT formation under identical conditions. 250 and 500 µM ONOOH and ONOOCO$_2^-$ induced significant 3NT formation on membrane and cytosolic proteins, and 50–500 µM ONOOH and ONOOCO$_2^-$ caused protein smearing. When the cytosolic and membrane proteins were separated, multiple protein bands appear to become less intense when silver stained. These data indicate that significant ONOOH-mediated thiol oxidation occurs prior to 3NT formation and other protein modifications (aggregation and/or fragmentation) in HCAEC. All of these ONOOH- and ONOOCO$_2^-$-mediated modifications in HCAEC may be associated with subsequent cell death though this has not been examined in detail.

The results of the previous Chapter showed that thiols (cysteine) are a major target of ONOOH followed by methionine, tryptophan and tyrosine. The results of this Chapter showed that ONOOH and ONOOCO$_2^-$ modified thiols (cysteine) prior to modification of
tyrosine (3NT formation) and other protein modifications (aggregation and/or fragmentation) in HCAEC within 5 min.

In the previous Chapter, ONOOH also induced protein fragmentation in BSA. In this Chapter some HCAEC proteins appeared to be modified by ONOOH. The data obtained using the cell system in this Chapter agree with the data obtained using isolated BSA in the previous Chapter. However these ONOOH- and ONOOCO$_2^-$-mediated modifications to HCAEC were investigated using only a short incubation time of 5 min and used bolus doses of oxidants. It was therefore of interest to investigate the effects of prolonged exposure of HCAEC to these oxidants. Consequently in the next Chapter, studies were carried out to investigate the effect of slow continued generation of ONOOH and ONOOCO$_2^-$ on HCAEC over a 4 hour period.
Chapter 5: Damage Induced by SIN-1 to Human Coronary Artery Endothelial Cells
5.1 Introduction

In the previous Chapter, the damaging effects of intra- and extra-cellular ONOOH and ONOOCO$_2^-$ formation on intact HCAEC, lysates or subcellular fractions (cytosolic or membrane) were investigated. The accumulating results suggest that ONOOH induces thiol oxidation followed by 3NT formation and other protein modifications (such as aggregation and/or fragmentation) to HCAEC proteins. However these data were obtained after bolus addition of reagent ONOOH and ONOOCO$_2^-$ with a 5 min incubation period, rather than as a result of sustained exposure to lower levels of this oxidant that may better reflect conditions *in vivo*. It was therefore of interest to investigate the damaging effects of prolonged low levels of ONOOH and ONOOCO$_2^-$ formation *in vivo*.

Because of the short half-life and instability of authentic ONOOH, 3-morpholinosydnonimine-N-ethylcarbamide (SIN-1) is widely used to generate ONOOH slowly in aqueous solutions to investigate oxidative and nitrosative stress *in vitro* and *in vivo* (74, 180, 181, 186, 375-377). SIN-1 is an active metabolite of the vasodilatory drug molsidomine, which decomposes slowly in solution. SIN-1 is commercially available and is commonly used in cell culture experiments because SIN-1 can easily penetrate cells. Numerous cell culture studies have used SIN-1 as a ONOOH source (186, 378-380).

In aqueous solution, SIN-1 undergoes base-catalysed hydrolysis of the sydnonimine ring to form SIN-1A (Scheme 5.1) (381). SIN-1A is oxidised by O$_2$ to give the SIN-1 cation-radical and O$_2$$^-$. The former species liberates NO$^*$ and eventually forms 3-morpholinoiminoacetonitrile (SIN-1C), a stable end product. In solution SIN-1 has a half-life of about 30 min and liberates NO$^*$ and O$_2$$^-$ with a 1:1 stoichiometry. NO$^*$ and O$_2$$^-$ react at near diffusion-controlled rates (rate constant of ca. $10^{10}$ M$^{-1}$s$^{-1}$) to form peroxynitrous anion (ONOO$^-$) and hence ONOOH (58, 59). The biological effects of SIN-1 are generally attributed to the actions of ONOOH (376).
Scheme 5.1 SIN-1 decomposition and peroxynitrite anion formation [taken from (381)].

5.2 Aims

This Chapter investigated whether cytosolic and/or membrane proteins are targeted by ONOOH and ONOOCO$_2^-$ if these oxidants are generated in excess extra- or intracellularly over a longer period of time using SIN-1. Extracellular ONOOH-mediated damage was investigated by treating intact cells with increasing concentrations of SIN-1, whereas intracellular ONOOH-mediated damage was investigated by treating lysates with varying concentrations of SIN-1. We aimed to mimic prolonged ONOOH and ONOOCO$_2^-$ formation on HCAEC via SIN-1 decomposition either in the absence or presence of 25 mM NaHCO$_3$ respectively. The damage induced by SIN-1 was assessed by detecting thiol oxidation (using the ThioGlo assay), 3NT formation (by Western blotting) and other protein modifications (e.g. protein fragmentation and/or aggregation) by silver staining.
5.3 Results

5.3.1 Incubation time

SIN-1 has an estimated half-life of about 30 min (378). After 4 h approximately 0.4 % of SIN-1 would remain while 99.6 % of SIN-1 would have decomposed. Consequently 4 h (or 24 h in the initial thiol experiments) was an ideal time to study long-term effects of ONOOH and ONOOCO$_2$$^-$ formation (generated via SIN-1 decomposition) on HCAEC.

5.3.2 Cell viability

Initial experiments investigated SIN-1 mediated HCAEC cytotoxicity via the MTT assay, as described in the previous Chapter (Section 4.3.1). HCAEC were seeded at a cell density of $4 \times 10^5$ cells per well in a 6-well plate at 37 °C overnight. Cells were treated with varying concentrations of SIN-1 (0-1000 µM) for 4 h at 37 °C and pH 7.4 both in the presence and absence of 25 mM NaHCO$_3$. Excess oxidant was then removed and then the MTT assay was performed. No significant loss of HCAEC viability was detected when cells were exposed to up to 1000 µM SIN-1 (Figure 5.1). In order to investigate the effect of CO$_2$ on ONOOH-mediated cytotoxicity, this experiment was also performed in the presence of 25 mM NaHCO$_3$. In the presence of NaHCO$_3$, exposure of HCAEC to 0-500 µM SIN-1 did not induce loss of cell viability. However, about 75 % loss of cell viability was detected when HCAEC were exposed to 1000 µM SIN-1 in the presence of 25 mM NaHCO$_3$. Overall 25 mM NaHCO$_3$ made a significant difference to SIN-1 mediated cytotoxicity, with ONOOCO$_2$$^-$ found to be more cytotoxic to HCAEC than ONOOH over this long-term incubation period.

5.3.3 Separation of sub-cellular fractions after SIN-1 exposure

Prior to examining SIN-1 mediated damage to HCAEC cytosolic and membrane proteins, it was crucial to investigate whether the cytosolic and membrane proteins were separated efficiently after cells were exposed to increasing concentrations of SIN-1. HCAEC were therefore seeded at a cell density of $4 \times 10^5$ cells per well in a 6-well plate at 37 °C and pH 7.4 overnight, and then treated with 0-1000 µM SIN-1 for 4 h at 37 °C and pH 7.4 both in the presence and absence of 25 mM NaHCO$_3$. 

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Figure 5.1 Loss of cell viability in HCAEC treated with SIN-1.

HCAEC (4 x 10^5 cells) were treated with SIN-1 (0-1000 µM) for 4 h at 37 °C and pH 7.4 followed by determination of cell viability using the MTT Assay. The red and black bars represent the cell viability either in the presence or absence of 25 mM NaHCO₃ respectively. Results are expressed as percentage of viable cells relative to the untreated control (0 µM SIN-1). * and # represent significant loss (p < 0.05) of cell viability relative to the untreated control, as determined by one way ANOVA, with Dunnett’s post-hoc test. The effect of 25 mM NaHCO₃ was assessed using repeated measures ANOVA, with Bonferroni post-hoc test. Values are means ± standard deviation and represent results obtained using 3 different HCAEC donors from 3 independent experiments.

The cytosolic and membrane fractions were separated via a commercial kit and the protein concentrations were equalised in both fractions via the BCA assay. The cytosolic and membrane proteins were separated via SDS PAGE. Western blotting was then performed using the anti-ABCG1 and anti-VEGF antibodies to detect ABCG1 (a membrane protein) and VEGF (a cytosolic protein) respectively.

The data obtained indicate that when intact cells were exposed to 0–1000 µM SIN-1 for 4 h at 37 °C and pH 7.4, VEGF was detected in the cytosolic fraction while ABCG1 was detected in the membrane fractions (Figure 5.2A). A similar result was detected in the presence of 25 mM NaHCO₃ (Figure 5.2B). This indicates that the separation method used in the earlier studies using ONOOH (see Chapter 4) also works well for cells exposed to SIN-1 over a longer incubation time. Interestingly, the staining density of
ABC-G1 and VEGF decreased when intact cells were exposed to 1000 µM SIN-1 in the presence of 25 mM NaHCO₃ (Figure 5.2B). This may be related to loss of antibody recognition as these proteins may be modified by ONOOCO₂⁻ after prolonged exposure. This may also reflect loss of cell viability, which is observed particularly at high doses of SIN-1 in the presence of 25 mM NaHCO₃. During short term (5 min) exposure only VEGF appeared to be modified by high concentrations of bolus ONOOH and ONOOCO₂⁻ (Chapter 4).

Figure 5.2 Detection of ABCG1 and VEGF in intact HCAEC exposed to SIN-1.

Intact HCAEC (4 x 10⁵ cells) were incubated with 0-1000 µM SIN-1 for 4 h at 37 °C and pH 7.4 both in the absence (A) and presence (B) of 25 mM NaHCO₃. The cytosolic and membrane fractions were then separated and the protein concentrations were equalised in both fractions via the BCA Assay. Western blotting was performed after SDS-PAGE. ABCG1 (a membrane protein) and VEGF (a cytosolic protein) were detected using anti-ABCG1 and anti-VEGF antibodies. Similar results were achieved using 3 different HCAEC donors in 3 independent experiments.
5.3.4 Thiol oxidation

5.3.4.1 HCAEC lysates

The results obtained in the previous Chapter clearly indicated that thiol residues of HCAEC are being modified by bolus ONOOH after an incubation time of 5 min. In the light of these data, the damaging effects of prolonged ONOOH formation (from SIN-1 decomposition) on thiol levels in HCAEC was examined. HCAEC were seeded at a cell density of 4 x 10^5 cells per well in a 6-well plate at 37 °C overnight. The cells were then lysed in cold (4 °C) npH2O and the lysates were exposed to 0-250 µM SIN-1 for up to 24 h at 37 °C. Under these experimental conditions the cellular components in the lysates were directly exposed to SIN-1.

The initial thiol concentrations in the untreated lysates were approximately 66 µmoles/g of protein (Figure 5.3). There was no significant thiol loss detected in the lysates over a 24 h period in the absence of added SIN-1. In contrast, whilst exposure of lysates to 50 µM SIN-1 induced no significant thiol loss up to 1 h, after 2 h exposure, about 40 µmole/g (60 %) of thiols were oxidised. More thiols were lost as time increased.

When lysates were treated with 100 and 250 µM SIN-1, about 15 and 26 µmoles of thiols/g thiols were lost respectively at time = 0 h. 0 h corresponds to thiol concentrations quantified immediately after addition of SIN-1 solutions. 15 and 26 µmoles/g of thiols corresponds to about 23 and 39 % of the initial thiol concentration in the absence of SIN-1. This thiol loss is most probably due to ONOOH formed in the SIN-1 solutions during preparation even though each solution was prepared immediately before use. Increasing amounts of thiols were lost over the 24 h period in a dose- and time-dependent manner, indicating that ONOOH generated from SIN-1 decomposition can oxidise thiols in lysates over long time periods (up to 24 h). However the majority of thiol loss occurred over the first 4 h incubation period. Consequently in the subsequent experiments, SIN-1 mediated thiol loss in the cytosolic or membrane fractions were investigated only over a 4 h period.
Figure 5.3 Thiol loss in HCAEC lysates exposed to increasing concentrations of SIN-1.

HCAEC lysates were incubated with 0-250 µM SIN-1 for up to 24 h at 37 °C followed by determination of residual thiols using the ThioGlo assay. The white, blue, red and black bars represent treatment with 0, 50, 100 and 250 µM SIN-1 respectively. Results are expressed as concentration of thiols over the respective protein concentrations. * p < 0.05, ** p < 0.01, ***/*** p < 0.001 represents the first time-point at which significant loss of thiols relative to the control was detected, as determined by one way ANOVA, with Dunnett’s post-hoc test. Data for subsequent time points was omitted for clarity. Values are means ± standard deviation and represent results obtained using 3 different HCAEC donors from 3 independent experiments.

5.3.4.2 Cellular fractions of intact cells

Initial experiments examined thiol oxidation in intact cells in the absence of SIN-1 over 4 h to determine if thiols were stable or being oxidised by other species generated within HCAEC over this time period. HCAEC were seeded at a cell density of 4 x 10⁵ cells per well in a 6 well plate overnight. Cells were washed twice with 750 µL of warm PBS and incubated in 725 µL of HBSS (± 25 mM NaHCO₃). 25 µL of npH₂O was then added and the cells were incubated for 4 h at 37 °C and pH 7.4. The cytosolic and membrane fractions were then separated using a commercial kit. A significant loss (7 µmoles/g) of HCAEC cytosolic protein thiols was detected over this time period (Figure 5.4). 6 µmoles/g of cytosolic thiols were oxidised in the presence of 25 mM NaHCO₃ over the same time period. This could be due to oxidation by other oxidants (e.g. H₂O₂) generated within these cells. In contrast, no significant loss of membrane protein thiols was detected over the 4 h. A similar result was also detected in the presence of 25 mM NaHCO₃.
Figure 5.4 Thiol loss in the cytosolic and membrane fractions of intact HCAEC controls. Intact HCAEC (4 x 10^5 cells) were incubated in HBSS and treated with npH2O for 0 and 4 h at 37 °C and pH 7.4. The subcellular fractions were then separated using a commercial kit, followed by determination of residual thiols using the ThioGlo assay. The blank and shaded bars represent thiol concentrations after 0 and 4 h respectively. The effect of time was determined by repeated measures 2 way ANOVA, with Bonferroni post-hoc test. Values are means ± standard deviation and represent results obtained using 3 different HCAEC donors from 3 independent experiments.

After examining thiol loss in the absence of SIN-1, subsequent experiments investigated the effect of SIN-1 on cytosolic- and membrane-protein thiols of intact HCAEC exposed to SIN-1. HCAEC were seeded at a cell density of 4 x 10^5 cells per well in a 6 well plate overnight. Cells were washed twice with 750 µL of warm PBS. Intact HCAEC were incubated in 725 µL HBSS (± 25 mM NaHCO3) and treated with 25 µL of 0-1000 µM of SIN-1 for 4 h at 37 °C and pH 7.4 both in the presence and absence of 25 mM NaHCO3. The cytosolic and membrane fractions were then separated and thiol loss was detected via ThioGlo assay using the ThioGlo 1™ reagent.

Exposure of intact cells to up to 500 µM SIN-1 did not induce significant cytosolic protein thiol loss. In contrast a significant loss (35 % reduction) of cytosolic thiols was detected when intact cells were treated with 1000 µM SIN-1 compared to the respective control (Figure 5.5A). These data suggest that SIN-1 or ONOOH (generated via SIN-1 decomposition) passed through the cell membrane and oxidised the cytosolic thiols of HCAEC. The presence of 25 mM NaHCO3 made no significant difference to the loss of
thiols in the cytosolic fractions of intact cells. This indicates that ONOOH (or radical species generated by hemolysis) and ONOOCO₂⁻ (or species derived from this) may have similar thiol oxidising capabilities under these conditions.

Exposure of intact cells to 1000 µM SIN-1 did not induce a significant loss of membrane protein thiols compared to the respective control (Figure 5.5B). However in the presence of 25 mM NaHCO₃, about 30 % of the membrane protein thiols were oxidised when cells were exposed to 1000 µM SIN-1 for up to 4 h. Statistical analysis indicated that the presence of 25 mM NaHCO₃ did not make any significant difference to the thiol loss induced by SIN-1 for up to 4 h.

These data overall suggest that in intact HCAEC, the cytosolic protein thiols were slightly more susceptible towards ONOOH (generated via SIN-1 decomposition) mediated oxidation than those present on membrane proteins. Therefore if ONOOH formation occurs extracellularly over a prolonged period of time, the cytosolic protein thiols may be preferentially targeted over membrane-bound thiols.
Figure 5.5 Loss of thiols in the cytosolic (A) and membrane (B) fractions of intact HCAEC treated with SIN-1.

Intact HCAEC (4 x 10^5 cells) were incubated with 0-1000 µM SIN-1 for 4 h at 37 °C and pH 7.4 followed by separation of cytosolic and membrane fractions. Immediately afterwards the residual thiols were quantified using the ThioGlo assay and ThioGlo™ 1 reagent. The red and black bars represent loss of thiols in the presence or absence of 25 mM NaHCO₃ respectively. Results are expressed as concentration of thiols relative to 0 µM SIN-1. * and # represent significant loss (p < 0.05) of thiols relative to 0 µM SIN-1, as determined by one way ANOVA, with Dunnett’s post-hoc test. The effect of 25 mM NaHCO₃ was determined by repeated measures ANOVA, with Bonferroni post-hoc test. Values are means ± standard deviation and represent results obtained using 3 different HCAEC donors from 3 independent experiments.
5.3.4.3 Cell lysate experiments

The results obtained in the previous Section indicated that intact cells thiol residues were being modified by high concentrations of SIN-1. It was therefore of interest to investigate SIN-1 mediated thiol loss in the absence of an intact cell membrane. Before proceeding however, it was important to investigate thiol loss in separated cytosolic and membrane fractions over the 4 h period in the absence of SIN-1.

HCAEC were seeded at a cell density of 4 x 10^5 cells per well in a 6 well plate at 37 °C overnight. The cytosolic and membrane fractions were then separated as described earlier. The thiol concentrations of both fractions were equalised by use of the ThioGlo assay and NaHCO₃ was added to a portion of both fractions to a final concentration of 25 mM. The fractions were then incubated at 37 °C for 4 h. After 1, 2 and 4 h, the thiol concentrations were quantified in both fractions via the ThioGlo assay. No significant thiol loss was detected in both fractions over the 4 h period though there was a trend towards a decrease with increasing time (Figure 5.6). A similar result was detected in the presence of 25 mM NaHCO₃.

After detecting minimal thiol loss in the separated fractions over the 4 h period, SIN-1 mediated thiol oxidation was then investigated in the cytosolic and membrane proteins. In these experiments, the aim was to identify whether the cytosolic and/or membrane thiols are mostly targeted by exposure to ONOOH (generated by SIN-1) over a longer incubation period in the absence of a cell membrane. The effect of ONOOCO₂⁻ on long term exposure to separated fractions was also investigated.

HCAEC were seeded at a cell density of 4 x 10^5 cells per well in a 6 well plate overnight. The cells were then washed with PBS. The cytosolic and membrane fractions were then separated. The thiol concentrations in both fractions were equalised by use of the ThioGlo assay and the fractions were then incubated with 0-1000 μM SIN-1 for 4 h at 37 °C both in the presence and absence of 25 mM NaHCO₃. The thiol concentrations in both fractions were quantified after 0, 1, 2 and 4 h incubation with SIN-1.

Under these experimental conditions at the initial time point (time = 0 h), no significant thiols loss was detected when the cytosolic fractions were exposed to 0-1000 μM SIN-1 (Figure 5.7). Exposure of the cytosolic fraction to 250 μM SIN-1 for 1 h, oxidised about
50% of thiols. Interestingly in the presence of 25 mM NaHCO₃, exposure of the cytosolic fractions to 50 μM SIN-1 oxidised approximately 95% of the thiols. More thiols were lost as the SIN-1 concentration and reaction time were increased.

No significant thiol loss was detected when the membrane fractions were exposed to 0-1000 μM SIN-1 at 0 h (Figure 5.8). However exposure of membrane fractions to 50 μM SIN-1 for 1 h induced 25% thiol oxidation. In the presence of 25 mM NaHCO₃, 50 μM SIN-1 induced oxidation of about 70% of thiols. More thiols were oxidised as the SIN-1 concentration and reaction time were increased, both in the presence and absence of 25 mM NaHCO₃. These results suggest that ONOOCO₂⁻ induces thiol oxidation of cytosolic- and membrane-bound proteins to a greater extent than ONOOH.

A comparison was made to determine whether cytosolic or membrane thiols were oxidised to a greater extent. The results indicate that up to 2 h of exposure with 0-1000 μM SIN-1, the cytosolic and membrane thiols were lost to the same extent (Figure 5.9). In contrast after 4 h of incubation with 50 μM or higher concentrations of SIN-1, cytosolic thiols were oxidised to a greater extent than membrane thiols, with 78 and 65% of the cytosolic and membrane thiols oxidised respectively.

When both fractions were exposed to 50 μM SIN-1 in the presence of 25 mM NaHCO₃, about 95 and 70% of cytosolic and membrane thiols were oxidised respectively (Figure 5.10). The thiol loss occurred in a SIN-1 concentration and time-dependent manner. ONOOH and ONOOCO₂⁻ (generated from SIN-1 decomposition) seem to target the cytosolic thiols to a greater extent than membrane thiols. In this case, ONOOCO₂⁻ appears to be a more potent thiol oxidising species than ONOOH.
Figure 5.6 Loss of thiols in the cytosolic (A) and membrane (B) fractions incubated in the absence of SIN-1.

HCAEC cytosolic and membrane fractions were separated followed by equalisation of the thiol concentrations in both fractions using the ThioGlo assay. The separated fractions were treated with 0 µM SIN-1 (npH$_2$O) for 4 h at 37 °C and immediately the residual thiols were quantified after 1, 2 and 4 h. The black and red bars represent thiol concentrations in the absence and presence of 25 mM NaHCO$_3$. Results were expressed as thiols concentrations at different time points. Investigation into thiol loss over the 4 h period was performed by one-way ANOVA, with Dunnett’s post-hoc test. The effect of 25 mM NaHCO$_3$ was determined using repeated measures ANOVA, with Bonferroni post-hoc test. Values are means ± standard deviation and represent results obtained using 3 different HCAEC donors from 3 independent experiments.
Figure 5.7 Loss of thiols in the HCAEC cytosolic fractions treated with SIN-1.

HCAEC cytosolic and membrane fractions were separated followed by equalisation of the thiol concentrations in both fractions using the ThioGlo assay. The separated cytosolic fractions were treated with 0-1000 µM SIN-1 for up to 4 h at 37 °C. The residual thiols were quantified after 0, 1, 2 and 4 h. The black and red bars represent thiol concentrations in the absence and presence of 25 mM NaHCO₃ respectively. Results are expressed as thiol concentrations relative to 0 µM SIN-1. * and # represent significant loss (p < 0.05) of thiols relative to 0 µM SIN-1, as determined by one way ANOVA, with Dunnett’s post-hoc test. The effect of 25 mM NaHCO₃ was determined by repeated measures ANOVA, with Bonferroni post-hoc test. Values are means ± standard deviation and represent results obtained using 3 different HCAEC donors from 3 independent experiments.
Figure 5.8 Loss of thiols in the HCAEC membrane fractions treated with SIN-1

HCAEC cytosolic and membrane fractions were separated followed by equalisation of the thiol concentrations in both fractions using the ThioGlo assay. The separated membrane fractions were treated with 0-1000 µM SIN-1 for up to 4 h at 37 °C. The residual thiols were quantified after 0, 1, 2 and 4 h. The black and red bars represent thiol concentrations in the absence and presence of 25 mM NaHCO₃ respectively. Results are expressed as thiols concentrations relative to 0 µM SIN-1. * and # represent significant loss (p < 0.05) of thiols relative to 0 µM SIN-1, as determined by one way ANOVA, with Dunnett’s post-hoc test. The effect of 25 mM NaHCO₃ was determined by repeated measures ANOVA, with Bonferroni post-hoc test. Values are means ± standard deviation and represent results obtained using 3 different HCAEC donors from 3 independent experiments.
Figure 5.9 Loss of thiols in the HCAEC cytosolic vs membrane fractions treated with SIN-1.

HCAEC cytosolic and membrane fractions were separated followed by equalisation of the thiol concentrations in both fractions using the ThioGlo assay. The separated cytosolic and membrane fractions were treated with 0-1000 µM SIN-1 for up to 4 h at 37 °C. The residual thiols were quantified after 0, 1, 2 and 4 h of incubation with SIN-1. The white and shaded bars represent thiol concentrations in the cytosolic and membrane fractions respectively. Results are expressed as thiols concentrations relative to 0 µM SIN-1. Comparisons between the fractions were made by repeated measures ANOVA, with Bonferroni post-hoc test. ^ represents a significant difference between the thiol loss detected in both fractions. Values are means ± standard deviation and represent results obtained using 3 different HCAEC donors from 3 independent experiments.
Figure 5.10 Loss of thiols in the HCAEC cytosolic vs membrane fractions treated with SIN-1 in the presence of 25 mM NaHCO₃.

HCAEC cytosolic and membrane fractions were separated followed by equalisation of the thiol concentrations in both fractions using the ThioGlo assay. The separated cytosolic and membrane fractions were treated with 0-1000 µM SIN-1 in the presence of 25 mM NaHCO₃ for up to 4 h at 37 °C. The residual thiols were quantified after 0, 1, 2 and 4 h of incubation with SIN-1. The white and shaded bars represent thiol concentrations in the cytosolic and membrane fractions respectively. Results are expressed as thiols concentrations relative to 0 µM SIN-1. Comparisons between the fractions were made by repeated measures ANOVA, with Bonferroni post-hoc test. ^ represents a significant difference between the thiol loss detected in both fractions. Values are means ± standard deviation and represent results obtained using 3 different HCAEC donors from 3 independent experiments.
5.3.5 Other protein modifications

5.3.5.1 Cellular fractions of intact cells

The results of the previous Chapter have indicated that ONOOH substantially modifies HCAEC proteins in the absence of an intact cell membrane over a short exposure time. Thus protein smears, which are indicative of aggregation and/or fragmentation, were detected when HCAEC were exposed to bolus ONOOH after a 5 min incubation period. Therefore we were interested in examining the damaging effects of prolonged ONOOH formation (from SIN-1 decomposition) on HCAEC proteins.

HCAEC were seeded at a cell density of $4 \times 10^5$ cells per well in a 6-well plate at 37 °C and pH 7.4 overnight. Cells were treated with 0-1000 µM SIN-1 for 4 h at 37 °C and pH 7.4 both in the presence and absence of 25 mM NaHCO$_3$. The cytosolic and membrane fractions were separated as described earlier and the protein concentrations were equalised in both fractions by use of the BCA assay. Cytosolic and membrane proteins were separated via SDS PAGE and then the gels were subjected to silver staining.

Silver staining detected multiple cytosolic (Figure 5.11) and membrane proteins (Figure 5.12). The overall protein band patterns detected in the cytosolic fraction were quite different to those seen with the membrane fraction, which is indicative (as expected) of the presence of different proteins in these fractions. In the incubation controls (treatment with 0 µM SIN-1 or npH$_2$O), no significant protein modifications were detected (Figure 5.11A & 5.12A). A similar result was detected in the presence of 25 mM NaHCO$_3$ (Figure 5.11B & 5.12B). This indicated that no significant cytosolic and membrane protein modifications were detected when cells were incubated in the absence of SIN-1. A similar result was also detected in the presence of 25 mM NaHCO$_3$. Thus in the absence of ONOOH and ONOOCO$_2^-$, no other reactive species appeared to be present which modify HCAEC proteins.

Visually no significant protein modifications in the cytosolic (Figure 5.11) and membrane (Figure 5.12) fractions were detected when intact cells were exposed to up to 1000 µM SIN-1 for 4 h at 37 °C. A similar result was detected in the presence of 25 mM NaHCO$_3$. To confirm this result, densitometric analysis was performed on 2 abundant but unknown cytosolic (marked A & B in Figure 5.11) and membrane (marked C & D in
Figure 5.12) proteins that gave distinct bands on the gels. The results indicated that SIN-1 did not induce a significant reduction in the pixel densities of these unknown cytosolic (A & B) and membrane (C & D) proteins (Figure 5.13). The presence of 25 mM NaHCO₃ also made no significant difference to ONOOH-mediated protein modifications. These data suggest that over the 4 h time period, no significant protein modifications were induced by ONOOH and ONOOCO₂⁻ (generated from SIN-1 decomposition) on intact HCAEC proteins that could be detectable by this methodology.

![Figure 5.11 SIN-1 mediated modification of intact HCAEC cytosolic proteins.](image)

Intact HCAEC (4 x 10⁵ cells) were incubated with 0-1000 µM SIN-1 for 4 h at 37 °C and pH 7.4 both in the absence (A) and presence (B) of 25 mM NaHCO₃. Subsequently the cytosolic and membrane fractions were separated via a commercial kit. The protein concentrations were equalised in both fractions via the BCA assay. Silver staining of the cytosolic fraction was performed after SDS-PAGE. Densitometric analysis was performed on the individual lanes of the unknown proteins A and B using Image J software. Similar results were achieved using 3 different HCAEC donors in 3 independent experiments.
Figure 5.12 SIN-1 mediated modification of intact HCAEC membrane proteins.

Intact HCAEC (4 x 10^5 cells) were incubated with 0-1000 µM SIN-1 for 4 h at 37 °C and pH 7.4 both in the absence (A) and presence (B) of 25 mM NaHCO₃. Subsequently the cytosolic and membrane fractions were separated via a commercial kit. The protein concentrations were equalised in both fractions via the BCA assay. Silver staining of the membrane fraction was performed after SDS-PAGE. Densitometric analysis was performed on the individual lanes of the unknown proteins C and D using Image J software. Similar results were achieved using 3 different HCAEC donors in 3 independent experiments.
Figure 5.13 Densitometric analysis of unknown cytosolic and membrane proteins of intact HCAEC treated with SIN-1.

Intact HCAEC (4 x 10⁵ cells) were incubated with 0-1000 µM SIN-1 for 4 h at 37 °C and pH 7.4 both in the absence (black bars) and presence (red bars) of 25 mM NaHCO₃. Subsequently the cytosolic and membrane fractions were separated via a commercial kit. The protein concentrations were equalised in both fractions via the BCA assay. Silver staining of the membrane fraction was performed after SDS-PAGE. Densitometric analysis was performed on individual bands of the unknown cytosolic (A & B) and membrane proteins (C & D) using Image J software to determine its pixel density when exposed to increasing concentrations of SIN-1. No significant decrease in the pixel density of protein bands was detected as the SIN-1 concentrations increased relative to the control (0 µM SIN-1), as determined by one-way ANOVA with Dunnett’s post-hoc test. A similar result was detected in the presence of 25 mM NaHCO₃. Overall the presence of 25 mM NaHCO₃ made no significant difference to the pixel density of these unknown proteins, as determined by repeated measures ANOVA with Bonferroni post-hoc test. Similar results were achieved using 3 different HCAEC donors in 3 independent experiments.
5.3.5.2 Cell lysate experiments

The results reported in the previous section indicate that during a 4 h incubation period, the cell membrane of HCAEC may afford protection against ONOOH-mediated damage to the cellular proteins. It was therefore of interest to investigate the effect of ONOOH and ONOOCO$_2^-$ on HCAEC proteins over 4 h in the absence of an intact cell membrane. HCAEC were seeded at a cell density of $4 \times 10^5$ cells per well in a 6-well plate at 37 °C and pH 7.4 overnight. The cytosolic and membrane fractions were separated as described above. The amount of proteins in both fractions were then equalised via the BCA assay and the separated fractions were treated with 0-1000 µM SIN-1 for 4 h at 37 °C both in the presence and absence of 25 mM NaHCO$_3$. The proteins were separated via SDS-PAGE and then silver staining was performed.

The presence of different band patterns in the cytosolic (Figure 5.14) and membrane (Figure 5.15) fractions confirmed that different proteins were present in these fractions, and indicated that efficient separation of the 2 fractions had been achieved, consistent with the data in Chapter 4. No significant protein modifications were detected in the incubation controls (treatment with 0 µM SIN-1) (Figure 5.14 & 5.15). This suggests that in the absence of ONOOH and ONOOCO$_2^-$, no other reactive species was present in the separated fractions that could induce protein modifications.

No significant decrease in the pixel density of the cytosolic proteins was detected when these were exposed to up to 1000 µM SIN-1 for 4 h (Figure 5.14). Using 2 abundant and unknown cytosolic proteins (marked as A & B), densitometric analysis was performed on the individual lanes of these proteins, to detect any loss in pixel density. No significant decrease in the pixel density of these proteins was detected as the oxidant concentration was increased (Figure 5.16 A & B). A similar result was detected in the presence of 25 mM NaHCO$_3$. These results suggest that over the 4 h incubation period, neither of these proteins were markedly affected by these concentrations of ONOOH and ONOOCO$_2^-$. 

In the case of the membrane proteins, a number of bands (e.g. those marked C & D in Figure 5.15) along with other proteins (marked with red arrows in Figure 5.15) might be modified by ONOOH over the 4 h incubation since their protein bands seem to become lighter as the SIN-1 concentrations increased. Densitometric analysis of the individual
lanes of these unknown membrane proteins (C & D) was performed using Image J software. No significant loss of pixel density was detected as the SIN-1 concentrations increased though the variability in the data (as indicated by the error bars) was large (Figure 5.16 C & D). This suggests that there is no obvious effect of SIN-1 both in the presence and absence of NaHCO$_3$ on protein degradation/aggregation as measured using the silver stain technique.

![Image: Figure 5.14 SIN-1 mediated modification of HCAEC cytosolic proteins.](image)

HCAEC cytosolic and membrane fractions were separated and the protein concentrations were equalised in both fractions via the BCA Assay. Both fractions were then treated with 0-1000 µM SIN-1 for 4 h at 37 °C and pH 7.4 both in the absence (A) and presence (B) of 25 mM NaHCO$_3$. Silver staining of the cytosolic fraction was performed after SDS-PAGE. Densitometric analysis was performed on the individual lanes of the unknown proteins A and B using Image J software. Similar results were achieved using 3 different HCAEC donors in 3 independent experiments.

Figure 5.14 SIN-1 mediated modification of HCAEC cytosolic proteins.
Figure 5.15 SIN-1 mediated modification of HCAEC membrane proteins.

HCAEC cytosolic and membrane fractions were separated and the protein concentrations were equalised in both fractions via the BCA Assay. Both fractions were then treated with 0-1000 µM SIN-1 for 4 h at 37 °C and pH 7.4 both in the absence (A) and presence (B) of 25 mM NaHCO₃. Silver staining of the membrane fraction was performed after SDS-PAGE. Densitometric analysis was performed on the individual lanes of the unknown proteins C and D using Image J software. Similar results were achieved using 3 different HCAEC donors in 3 independent experiments.
Figure 5.16 Densitometric analysis of unknown cytosolic and membrane proteins of HCAEC fractions treated with SIN-1.

HCAEC cytosolic and membrane fractions were separated and the protein concentrations were equalised in both fractions via the BCA Assay. Both fractions were then treated with 0-1000 µM SIN-1 for 4 h at 37 °C and pH 7.4 both in the absence (black bars) and presence (red bars) of 25 mM NaHCO₃. Silver staining of the cytosolic and membrane fraction was performed after SDS-PAGE. Densitometric analysis was performed on individual bands of the unknown cytosolic (A & B) and membrane proteins (C & D) to determine its pixel density when exposed to increasing concentrations of SIN-1. No significant loss in the pixel density of protein bands were detected as the SIN-1 concentrations increased, as determined by one way ANOVA with Dunnett’s post-hoc test. A similar result was detected in the presence of 25 mM NaHCO₃. The presence of 25 mM NaHCO₃ made no significant difference to the pixel density of these unknown proteins, as determined by repeated measures ANOVA with Bonferroni post-hoc test. Similar results were achieved using 3 different HCAEC donors in 3 independent experiments.
5.3.6 3-Nitrotyrosine formation

5.3.6.1 Cellular fractions of intact cells

The results presented in the previous Chapter are consistent with ONOOH and ONOOCO$_2^-$ inducing 3NT formation on cytosolic and membrane proteins in intact HCAEC after 5 min exposure. Consequently, it was of interest to determine whether similar behaviour was seen with SIN-1.

HCAEC were seeded at a cell density of 4 x 10$^5$ cells per well in a 6-well plate at 37 °C and pH 7.4 overnight. Cells were treated with 0-1000 µM SIN-1 for 4 h at 37 °C and pH 7.4 both in the presence and absence of 25 mM NaHCO$_3$. The cytosolic and membrane fractions were then separated and the protein concentrations equalised by the use of the BCA assay. The proteins were separated via SDS PAGE and Western blotting was then performed using an anti-3NT antibody to detect 3NT on cytosolic and membrane proteins.

No significant 3NT formation was detected on the cytosolic proteins in the incubation control samples (cells treated with 0 µM SIN-1) (Figure 5.17A). A similar result was detected in the presence of 25 mM NaHCO$_3$ (Figure 5.17B). These data indicate that during the 4 h incubation, in the absence of SIN-1 no other nitrating species were generated which could induce 3NT formation on the cytosolic proteins of the intact cells.

As the intact cells were exposed to increasing concentrations of SIN-1, multiple cytosolic proteins were nitrated (Figure 5.17A). The pixel density of the bands from these nitrated proteins increased as the SIN-1 concentration increased. A similar result was detected in the presence of 25 mM NaHCO$_3$ (Figure 5.17B). These data suggest that the nitration of these proteins increases as the ONOOH and ONOOCO$_2^-$ concentrations increased. Interestingly, only a limited number of the proteins seem to be nitrated by both ONOOH and ONOOCO$_2^-$ during the 4 h period.

A basal level of nitration was detected in few membrane proteins in the control samples (0 µM SIN-1) (Figure 5.18A). As the SIN-1 concentration was increased, the band intensity of the nitrated proteins increased. In the presence of 25 mM NaHCO$_3$ it appears that nitration of different membrane proteins occur when compared to the absence of 25
mM NaHCO₃ (Figure 5.18B). Particularly a membrane protein with a MW just above 50 kDa MW band appears to be nitrated more by the combination of SIN-1 and NaHCO₃ than SIN-1 alone. Nitration of membrane proteins again seems to be occurring in a selective manner with a limited number of proteins nitrated by ONOOH and ONOOCO₂⁻.

Densitometric analysis of the entire lane of nitrated cytosolic and membrane proteins was performed using Image J software. Owing to the high background in the control lanes, this technology failed to show a significant increase in nitration of the cytosolic and membrane proteins when the intact cells were exposed to increasing SIN-1 concentrations. Due to this technical limitation, it was difficult to determine which concentrations of ONOOH and ONOOCO₂⁻ induced significant 3NT formation on cytosolic and membrane proteins when intact cells were exposed to these oxidants over 4 h. It was also difficult to determine whether the cytosolic or membrane proteins were nitrated to a greater extent by SIN-1 after 4 h incubation period under these experimental conditions. In this situation visual observations were better and easier.

Direct observations of the membranes suggest that few membrane proteins are nitrated to a significant extent in non-treated HCAEC. In the presence of ONOOH and ONOOCO₂⁻ nitration is increased. ONOOCO₂⁻ appears to be nitrating both cytosolic and membrane proteins to a greater extent than ONOOH. Membrane proteins appear to be more susceptible to 3NT formation than cytosolic proteins. The exact identities of these nitrated cytosolic and membrane proteins are unknown.
Figure 5.17 3NT formation in the cytosolic fraction of intact HCAEC treated with SIN-1.

Intact HCAEC (4 x 10^5 cells) were incubated with 0-1000 µM SIN-1 for 4 h at 37 °C and pH 7.4 both in the absence (A) and presence (B) of 25 mM NaHCO₃. The cytosolic and membrane fractions were then separated. The protein concentrations were equalised in both fractions via the BCA Assay. Western blotting was performed to detect 3NT formation in the cytosolic fraction using an anti-3NT antibody after SDS-PAGE. Multiple nitrated cytosolic proteins were detected when cells were treated with SIN-1. Similar results were achieved using 3 different HCAEC donors in 3 independent experiments.
Figure 5.18 3NT formation in the membrane fraction of intact HCAEC treated with SIN-1.

Intact HCAEC (4 x 10^5 cells) were incubated with 0-1000 µM SIN-1 for 4 h at 37 °C and pH 7.4 both in the absence (A) and presence (B) of 25 mM NaHCO₃. The cytosolic and membrane fractions were then separated. The protein concentrations were equalised in both fractions via the BCA Assay. Western blotting was performed to detect 3NT formation in the membrane fraction using an anti-3NT antibody after SDS-PAGE. Multiple nitrated membrane proteins were detected when cells were treated with SIN-1. Similar results were achieved using 3 different HCAEC donors in 3 independent experiments.
5.3.6.2 Cell lysate experiments

In the previous Chapter, data has been presented consistent with 3NT formation on multiple cytosolic and membrane proteins of HCAEC in the absence of an intact cell membrane (Section 4.3.4.4). In these earlier experiments, fractions were exposed to increasing concentrations of bolus ONOOH and ONOOCO$_2^-$ for 5 min. The experiments reported here extend this work to investigate the effect of long-term exposure of these fractions to ONOOH and ONOOCO$_2^-$ generated by SIN-1. The cytosolic and membrane proteins were separated and then both fractions were exposed to varying concentrations of ONOOH and ONOOCO$_2^-$.

HCAEC were seeded at a cell density of $4 \times 10^5$ cells per well in a 6-well plate at 37 °C and pH 7.4 overnight. The cytosolic and membrane fractions were separated and the amount of proteins in both fractions were equalised by the use of BCA assay. The separated fractions were then treated with 0-1000 µM SIN-1 for 4 h at 37 °C both in the presence and absence of 25 mM NaHCO$_3$. The proteins were separated via SDS-PAGE and then Western blotting was performed using an anti-3NT antibody to detect 3NT formation on cytosolic and membrane proteins.

No significant 3NT formation was detected on the cytosolic proteins in the incubation control samples (cells treated with 0 µM SIN-1) (Figure 5.19A), in either the absence or presence of 25 mM NaHCO$_3$ (Figure 5.19B). These data indicate that during the 4 h incubation and in the absence of SIN-1, no other species caused 3NT formation on cytosolic proteins. In the presence of SIN-1, 3NT-positive bands were detected, and as the SIN-1 concentration increased, the pixel density of the 3NT-positive bands from selected cytosolic proteins increased.

A low level of basal 3NT formation was detected on membrane proteins in the incubated control samples (Figure 5.20A) but this was not detected in the presence of 25 mM NaHCO$_3$ (Figure 5.20B). As the SIN-1 concentrations increased, the pixel density of these 3NT-positive bands from selected membrane proteins increased.

It appears that the combination of SIN-1 and NaHCO$_3$ had a greater effect on nitrating the cytosolic proteins (Figure 5.19B). This is apparent when the fraction was at exposure to 500 µM SIN-1 around the 50 kDa MW band. A similar result was also seen with
membrane proteins, particularly when the fraction was exposed to 250 µM SIN-1 around the 50 kDa MW band (Figure 5.20B).

Again, densitometric analysis failed to show a significant increase in 3NT formation on cytosolic and membrane proteins in these experiments due to the high background. As a result, it was difficult to determine which concentrations of ONOOH and ONOOCO$_2^-$ induced significant 3NT formation when cytosolic and membrane proteins are directly exposed to these oxidants for 4 h in the absence of an intact cell membrane. It was also difficult to detect whether cytosolic or membrane proteins are nitrated to a greater extent when the cell membrane was disrupted using Image J analysis. It might have been advantageous to have a housekeeping protein blot or of staining the membrane after stripping antibodies, but this was not carried out. Therefore this is a limitation.

Direct observation of the gels suggests that a basal level of nitration occurs on the membrane proteins and this was not detected on the cytosolic proteins. ONOOCO$_2^-$ nitrated both cytosolic and membrane proteins to a greater extent than ONOOOH particularly at high concentrations. Under these experimental conditions, membrane proteins appear to be nitrated to a greater extent than cytosolic proteins. The exact identities of these nitrated cytosolic and membrane proteins are unknown.
Figure 5.19 3NT formation in the cytosolic fraction treated with SIN-1.

HCAEC cytosolic and membrane fractions were separated and the protein concentrations were equalised in both fractions via the BCA Assay. Both fractions were treated with 0-1000 µM SIN-1 for 4 h at 37 °C both in the absence (A) and presence (B) of 25 mM NaHCO₃. Western blotting was performed to detect 3NT formation on the cytosolic proteins using an anti-3NT antibody after SDS-PAGE. Similar results were achieved using 3 different HCAEC donors in 3 independent experiments.
HCAEC cytosolic and membrane fractions were separated and the protein concentrations were equalised in both fractions via the BCA Assay. Both fractions were treated with 0-1000 µM SIN-1 for 4 h at 37 °C both in the absence (A) and presence (B) of 25 mM NaHCO₃. Western blotting was performed to detect 3NT formation on the membrane proteins using an anti-3NT antibody after SDS-PAGE. Similar results were achieved using 3 different HCAEC donors in 3 independent experiments.
5.4 Discussion

It is well established that thiol oxidation and 3NT formation are major protein modifications induced by ONOOH (63, 117). ONOOH also appears to induce protein aggregation, crosslinking and fragmentation under certain circumstances (63, 118, 181, 264). The results outlined in the previous Chapters indicate that ONOOH and ONOOCO$_2^-$ can induce cell death, thiol oxidation, protein modifications and 3NT formation after an exposure time of 5 min. In light of these data, it was of interest to investigate whether similar changes occurred on long-term exposure to ONOOH and ONOOCO$_2^-\$ and therefore SIN-1 was used to generate ONOOH over a prolonged period (4 h). Experiments were performed using both intact cells and cell lysates to mimic extra- or intracellular ONOOH formation respectively with the latter experiments carried out to investigate whether the cell membrane had any protective effect.

In the experiments using a 5 min incubation period, ONOOH and ONOOCO$_2^-$ induced $\sim$10 % cell death. In contrast after 4 h, no significant cell death was detected when cells were exposed to up to 1000 $\mu$M SIN-1 in the absence of 25 mM NaHCO$_3$. Interestingly the species derived from 1000 $\mu$M SIN-1 induced 70 % cell death in the presence of 25 mM NaHCO$_3$. These data suggest that ONOOCO$_2^-$ is mainly responsible for this cytotoxic effect towards HCAEC and this oxidant was more cytotoxic than ONOOH. These data are consistent with previous studies that have shown that high concentrations of SIN-1 are cytotoxic to PC12 cells (378), bovine pulmonary artery endothelial and smooth muscle cells (375), human microvascular endothelial cells (377) and a mixed cortical cell culture (composed of both neurons and astrocytes) (382).

Interestingly, a previous study has reported that cell culture medium was cytotoxic to PC12 cells even after almost complete SIN-1 decomposition (378). A later study indicated that bicarbonate ions play critical roles in the generation of cytotoxic substances that persist in the cell culture medium after almost complete SIN-1 decomposition (376). The results obtained in the current study agree with this latter study because HCAEC cell death was detected to a greater extent in the presence of bicarbonate ions than in its absence. It is however unclear whether the SIN-1 mediated cytotoxicity detected in the current study was mainly due to ONOOCO$_2^-$ or the radicals generated by this oxidant.
The results reported in Chapter 4 indicated that when intact cells are exposed to 1250 µM ONOOH for 5 min, about 20 % of total thiol residues were oxidised and that exposure of cells to 750 µM ONOOH, oxidised about 17 and 30 % of the separated cytosolic and membrane protein thiols respectively. In the current study, exposure of intact cells to 1000 µM SIN-1 for 4 h induced 35 and 30 % thiol reduction of the cytosolic and membrane thiol concentrations, respectively. The presence of 25 mM NaHCO₃ did not make any significant difference to the extent of thiol oxidation. Overall therefore ONOOH and ONOOCO₂⁻ induced thiol oxidation to a similar extent during prolonged exposure. In this situation, these oxidants targeted cytosolic protein thiols to a greater extent than membrane thiols. This behaviour is in clear contrast to the cell viability data above where ONOOCO₂⁻ had more marked effects. Since SIN-1 can cross the plasma membrane, it is difficult to determine whether majority of the oxidants are generated intra- or extra-cellularly when cells were exposed to SIN-1 for prolong periods of time.

When lysates were exposed to 5 µM ONOOH and ONOOCO₂⁻ for 5 min about 25 and 50 % of total thiol residues were oxidised respectively (see Chapter 4), and 81 % of cytosolic and 66 % of membrane thiols were oxidised by 50 µM ONOOH. The presence of 25 mM NaHCO₃ made no significant difference to ONOOH-mediated thiol oxidation under these conditions. In contrast, after 1 h incubation of lysates with 50 µM SIN-1, about 14 and 95 % of cytosolic thiols were oxidised both in the absence and presence of 25 mM NaHCO₃ respectively. Exposure of the separated membrane fractions to 50 µM SIN-1 for 1 h oxidised 26 and 68 % of thiol residues in the absence and presence of 25 mM NaHCO₃ respectively. As SIN-1 concentrations increased, a dose- and time-dependent thiol loss was detected in both fractions. The thiol residues of both fractions were oxidised to a greater extent in the presence of 25 mM NaHCO₃. After 4 h incubation period, SIN-1 oxidised cytosolic thiols to a greater extent than membrane thiols and this difference was more pronounced in the presence of 25 mM NaHCO₃.

This thiol oxidation by ONOOH and ONOOCO₂⁻ may be of both physiological and pathological importance. Thus in mammalian cells, one of the major signalling pathways involves the activation of sphingomyelinases, which induces the hydrolysis of sphingomyelin to form ceramide (383). This signalling pathway is involved in several cellular responses such as the T-cell receptor-signalling pathway (384). The neutral sphingomyelinase 1 (NSM1) is involved in apoptosis induced by T-cell receptor activation. A previous study has indicated that SIN-1 (which generated ONOOH)
induced thiol oxidation in NSM1 resulting in the irreversible inactivation of this enzyme (385).

Similarly exposure of human teratocarcinoma cells derived from neuronal cells to SIN-1 induced cell death and thiol loss (386) and another study showed that SIN-1 induced thiol oxidation in isolated peptides such as glutathione, cysteine, cysteamine, homocysteine, cysteine methyl ester, cysteine ethyl ester, N-acetylcysteine, penicillamine, ammonium thioglycolate, captopril, N-acetylpenicillamine, mesna (sodium 2-mercaptoethanesulfonate) and tiopronin (N-(2-mercapto-propionyl)glycine) (387). Interestingly this study indicated that SIN-1 is 30 times more potent in inducing thiol oxidation than 2,2′-azo-bis (2-amidinopropane) hydrochloride. These studies agree with the data reported in this Chapter because SIN-1 induced thiol oxidation in HCAEC in a dose-dependent manner.

In the previous Chapter (Chapter 4) it was also shown that exposure of intact HCAEC to 1250 µM ONOOH for 5 min did not induce detectable protein modifications such as aggregation, crosslinking and fragmentation in a manner detectable by SDS PAGE with silver staining. A similar result was detected in the current studies when these cells were exposed to 1000 µM SIN-1 for 4 h. A prolonged exposure of HCAEC to ONOOH and ONOOCO$_2^-$ does not therefore appear to induce significantly greater or different modifications to the bolus oxidant, as assessed by SDS PAGE.

In contrast to the above intact cell data, exposure of HCAEC lysates to high ONOOH concentrations for 5 min, resulted in the formation of protein smears, which is consistent with protein aggregation and/or fragmentation. In the absence of an intact cell membrane, multiple cytosolic and membrane proteins appear to be affected with increasing ONOOH concentration. When separated fractions from the HCAEC were exposed to increasing concentrations of SIN-1, a limited number of membrane proteins but few, if any, cytosolic proteins appeared to be modified by SIN-1 after the 4 h incubation period. SIN-1 mediated protein fragmentation of tissue inhibitor of metalloproteinase 1 has been reported previously (388). These data are consistent with that observed in this Chapter, since SIN-1 was observed to induce protein modifications such as fragmentation over a prolonged incubation period.
The previous Chapter reported that detectable increases in 3NT formation were seen on multiple HCAEC cytosolic and membrane proteins when intact cells were exposed to high concentrations of ONOOH and ONOOCO$_2^-$ over a 5 min incubation period. Multiple cytosolic and membrane proteins were also nitrated by ONOOH and ONOOCO$_2^-$ in the absence of an intact cell membrane. Under these experimental conditions, the membrane proteins appear to be nitrated by ONOOH and ONOOCO$_2^-$ to a greater extent than cytosolic proteins.

The experiments reported in this Chapter demonstrated that SIN-1 also induced 3NT formation on selected cytosolic and membrane proteins, particularly at high concentrations after 4 h. This was detected with both intact cells and lysates exposed to SIN-1. Examination of the Western blots suggests that a basal level of membrane protein nitration occurs in HCAEC. In the presence of ONOOH or ONOOCO$_2^-$ the level of nitration increased over the 4 h incubation period with ONOOCO$_2^-$ appearing to nitrate both cytosolic and membrane proteins to a greater extent than ONOOH. The membrane proteins were nitrated to a greater extent than cytosolic proteins. Interestingly, it appears that the exposure of cells to the combination of SIN-1 and NaHCO$_3$ results in nitration of different membrane proteins than SIN-1 alone. This could be due to selective nitration of different membrane proteins by ONOOH and ONOOCO$_2^-$. Overall, both short- and long-term incubation with ONOOH and ONOOCO$_2^-$ seems to result in nitration of membrane proteins to a greater extent than cytosolic proteins. This data are in accordance with previous studies that have reported SIN-1 mediated 3NT formation occurring in human umbilical vein endothelial cells (389), a mixed cortical cell culture (composed of both neurons and astrocytes) (382), neuronal nitric oxide synthase (390), thioredoxin 1 (391) and extracellular matrix derived from vascular smooth muscle cells (181).

Overall the results of this Chapter collectively suggest that during prolonged exposure (4 h) of HCAEC to ONOOH and ONOOCO$_2^-$, the latter species seems to be more cytotoxic, and induces thiol oxidation and 3NT formation (particularly on membrane proteins) to a greater extent than ONOOH. Compared to ONOOH, SIN-1 induced alterations in protein over a longer period of time and this was also seen in a previous study (180). ONOOH-mediated modifications were detected on multiple proteins but the identities of these proteins are unknown. Therefore the next Chapter investigates which proteins are being modified in HCAEC via mass spectrometry (LC/MS/MS) using both direct and enrichment approaches.
Chapter 6: Mass Spectroscopic Identification and
Characterisation of Cellular Targets of Peroxynitrous Acid
6.1 Introduction

In the previous Chapters thiol oxidation and 3NT formation was detected on various cytosolic and membrane proteins by the use of thiol assays and Western blotting. However the exact identities of these modified proteins were unknown. Using mass spectroscopy (MS) we aimed to identify which proteins were modified by ONOOH as well as identify other possible protein modifications caused by ONOOH after a 5 min incubation period with HCAEC.

The results of the previous 2 Chapters have indicated that membrane proteins appear to be nitrated to a greater extent than cytosolic proteins during short- and long-term exposure to ONOOH. The membrane proteins are likely to be directly exposed to oxidants generated extracellularly. However it has also been shown that these proteins are present at much lower levels than cytosolic proteins and are known to be difficult to analyse (392). For these reasons, initial studies were carried out with cytosolic proteins, which are easy to isolate and are present in high abundance.

In this work, prior to MS analysis, proteins were extracted from samples and separated via SDS-PAGE (Figure 6.1). The proteins were then subjected to in-gel trypsin digestion. Trypsin was used because this is a well-characterized and efficient enzyme that generates peptides (an average of 5-25 amino acids in length) well suited to fragmentation by collision-induced dissociation (CID) in tandem mass spectrometry (393). This enzyme cleaves peptide chains on the carboxyl side of lysine and arginine, except when either lysine or arginine is followed by proline. The tryptic peptides generated are then subjected to LC/MS/MS analysis.

A mass spectrometer consists of an ion source, a mass analyser that quantifies the mass-to-charge ratio (m/z) of the ionised peptides and detector that detects the number of ions at each m/z value. All measurements are conducted on ionised peptides in the gas phase. Peptides are ionised using either electrospray ionisation (ESI) (394) or matrix-assisted laser desorption/ionisation (MALDI) (395). ESI ionises peptides out of a volatile solution and is generally combined with a liquid-based separation tool such as chromatography (394). MALDI uses laser pulses to ionise peptides out of a dry crystalline matrix (395). MALDI-MS is generally employed to analyse simple peptide mixtures while ESI-MS (such as LC/MS/MS) is used to analyse complex samples (393).
Figure 6.1 General outline for MS based proteomics [taken from (393)].

The mass analyser is critical and its important parameters include high sensitivity, high mass resolution, high mass accuracy and the ability to produce information-rich ion mass spectra from peptide fragments. Currently there are 4 types of mass analyser employed that include ion trap, time-of-flight (TOF), quadrupole and Fourier transform ion cyclotron analysers. Each of these analysers has a different design, performance, advantages and disadvantages. These analysers can either be used alone or in combination with other analysers (393).

Liquid chromatography-tandem mass spectrometry (LC/MS/MS) has high specificity, sensitivity and speed. Hunt and colleagues invented LC/MS/MS for the analysis of drugs, metabolites and complex peptide mixtures such as cell samples, plasma, blood, serum, urine and tissue. Currently this method is the core of MS-based proteomics (396, 397). In this project, LC/MS/MS was used to examine ONOOH-mediated modifications in HCAEC after 5 min exposure.
In this LC/MS/MS analysis, a quadrupole TOF instrument was used due to its high sensitivity, resolution and mass accuracy. The TOF analysers employ an electric field to accelerate ions through the same potential and subsequently quantify the time taken by ions to reach the detector. If the ions have the same charge, their masses determine their velocity. Lower mass ions tend to reach the detector first. Different ions with different mass-to-charge ratios reach the detector in rapid sequence at slightly different times and a complete mass spectrum is generated in a short time (398).

6.2 Aims

The aim of experiments presented in this Chapter was to investigate which proteins and which amino acids are modified by ONOOH when intact HCAEC cells were exposed to 500 µM ONOOH for 5 min at 37 °C and pH 7.4 using LC/MS/MS. ONOOH-mediated modifications include tyrosine nitration, cysteine oxidation and methionine oxidation. We also attempted to enrich the sample for nitrated proteins via immunoprecipitation and then identify which proteins are nitratated by ONOOH.

All the LC/MS/MS analysis was carried out in collaboration with Dr Matthew Padula at the Proteomics Core Facility at the University of Technology Sydney. Dr Padula ran all the samples through the LC/MS/MS and provided guidance with the data extraction and analysis.

6.3 Results

Ideally modifications should only be detected in all treated and not in any control samples. In this project, when a particular modification was detected in all treated and not in any of the control samples, it was classified as a “confirmed modification”. However multiple non-reproducible ONOOH-mediated modifications were also observed including modifications not occurring in one of the HCAEC treated samples or modifications being detected in all treated and one control sample. These modifications are most likely mediated by ONOOH under these experimental conditions, however further analysis is needed to confirm these results.
6.3.1 MS Analysis

Each LC/MS/MS run generates large and rich data files that require specialised software to efficiently process them, extract the important data and give the best proteome coverage. The data obtained was then searched in the Mascot database, a powerful search engine, which identifies proteins from their primary sequence databases. The data was analysed using the Peaks post translation modification algorithm. Peptide scores of 20 or above were considered good scores, and peptides with scores below 20 were not considered further. Thousands of proteins were detected in all HCAEC samples. The modifications induced by ONOOH to HCAEC included cysteine oxidation, methionine oxidation and tyrosine nitration.

6.3.2 Direct approach

Initial experiments analysed HCAEC that were treated with 0 and 500 µM ONOOH for 5 min. HCAEC were seeded at a cell density of 4 x 10^5 cells per well in a 6 well plate overnight. Cells were washed twice with 750 µL of warm PBS and incubated in 725 µL of HBSS. Cells were then treated with 500 µM ONOOH for 5 min at 37 °C and pH 7.4. Cells were lysed in lysis buffer and the proteins were precipitated using 50 % (w/v) trichloroacetic acid. The proteins were separated via SDS-PAGE, and then subjected to either Western blotting or Coomassie staining.

After SDS-PAGE, Western blotting was performed to detect 3NT using an anti-3NT antibody. Multiple nitrated proteins were detected via Western blotting using the anti-3NT antibody (Figure 6.2B), which confirms 3NT formation on multiple HCAEC proteins. Simultaneously, separate gels were subjected to Coomassie staining to detect proteins. The Coomassie staining detected multiple proteins both in the treated and control samples (Figure 6.2A). The individual entire lanes of the Coomassie stained gel containing treated and control samples were excised, sliced, destained and reduced and alkylated. The gel pieces were then subjected to trypsin in-gel digestion and subsequently LC/MS/MS analysis was performed. These experiments were performed using 3 different HCAEC donors (2315, 2286 and 2366).
Figure 6.2 Coomassie staining (A) and Western blotting (B) of intact HCAEC cells exposed to ONOOH.

Intact HCAEC cells were treated with 0 and 500 µM ONOOH for 5 minutes at 37 °C and pH 7.4. Multiple proteins were detected using Coomassie staining (A) and subsequently the Coomassie stained gels were subjected to in gel tryptic digest and LC/MS/MS. 3NT formation was detected using anti 3NT antibody and Western blotting after SDS-PAGE (B). Similar results were achieved using 3 different HCAEC donors in 3 separate experiments performed on 3 different days.

6.3.2.1 Confirmed modifications

- Cysteine oxidation at C328 in vimentin

The results obtained via LC/MS/MS detected peptides spanning about 69 – 85 % of the sequence coverage of vimentin (53 kDa, accession number P08670) in all HCAEC treated and control samples (Figure 6.3). 43 - 56 peptides described this protein, within these 41 - 46 peptides were unique to this protein. There were different numbers of specific peptides in different samples, and the total number of peptides (including unique peptides) differed between samples. Such high percentage recovery, number of peptides and number of unique peptides confirmed that this protein was present in all samples and this protein is expressed by HCAEC.

In the samples treated with 500 µM ONOOH, 1 spectrum containing an oxidised cysteine residue at position C328 was present in all HCAEC donors. The cysteine residue was oxidised to cysteic acid. The scores for these spectra ranged from 22.55 – 31.26, which indicates that this modification is a significant change. A spectrum showing an oxidised
cysteine residue is shown in Figure 6.4A. Spectra containing non-oxidised cysteine residues were also detected multiple times on the same peptide in all treated samples. This is not surprising as it is unlikely that the oxidation yield will be 100 %. These samples were reduced and alkylated; hence there should be no unmodified cysteine residues unless the alkylation was incomplete.

In the control samples, no spectrum containing an oxidised cysteine at C328 with a score above 20 was detected. Many peptides without an oxidised cysteine (due to incomplete alkylation) were present. These data suggest that cysteine oxidation is not likely to be occurring in the controls. Overall the presence of spectra containing cysteine oxidation with high scores in the treated samples suggests that 500 µM ONOOH induces cysteine oxidation at C328 in vimentin of HCAEC.

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<td>1 (43.81), 1 (29.83)</td>
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<td>No. of peptides</td>
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<td>46</td>
<td></td>
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<tr>
<td>No. of unique peptides</td>
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<td>43</td>
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<td>2 (41.40), 1 (28.45)</td>
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Figure 6.3 LC/MS/MS analysis showing ONOOH-mediated cysteine oxidation at C328 in vimentin.

- Methionine oxidation at M240 in alpha actinin 4

In all treated or control samples, 50 - 68 % sequence coverage of alpha actinin 4 (105 kDa, accession number O43707) was detected (Figure 6.5). In all the samples, 33 - 50 peptides described this protein of which 21 – 32 peptides were unique to this protein. Different numbers of specific peptides were detected in different samples. These high percentages of sequence coverage, number of peptides and number of unique peptides indicate that alpha actinin 4 was present in all HCAEC treated and control samples.
A: Treated sample

B: Control sample

Figure 6.4 LC/MS/MS spectrum of treated (A) and control (B) samples showing ONOOH-mediated cysteine oxidation at position C328 in vimentin.
All HCAEC treated samples contained multiple spectra with an oxidised methionine residue at position M240 (Figure 6.5). These spectra scores ranged from 34.74 – 49.58, which suggests that this modification is highly likely to be mediated by ONOOH under these experimental conditions. A spectrum of treated samples containing an oxidised methionine is shown in Figure 6.6A. The treated samples also contained peptides that did not have this modification. This is not surprising as it would be unexpected if oxidation occurred with 100 % yield at particular sites.

In the control samples, no peptides containing an oxidised methionine at position M240 were detected. A spectrum from a control sample is shown in Figure 6.6B. Peptides without this particular modification were detected in all control samples and these peptides had high scores (34.26 – 42.26). These results indicate that methionine oxidation at position M240 did not occur in the control samples in the absence of ONOOH, further supporting the conclusion that this modification is not an artifact of sample preparation. Overall the presence of multiple peptides containing modifications with high scores in the treated samples suggest that 500 µM ONOOH induces methionine oxidation at position M240 in alpha actinin 4 of HCAEC under these experimental conditions.

<table>
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<td>No. of peptides</td>
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<td>No. of spectra without this modification (score)</td>
<td>1 (42.26)</td>
<td>1 (34.26)</td>
<td>1 (36.76)</td>
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</table>

**Figure 6.5** LC/MS/MS analysis showing ONOOH-mediated methionine oxidation at M240 in alpha actinin 4.
Figure 6.6 LC/MS/MS spectrum of treated (A) and control (B) samples showing ONOOH-mediated methionine oxidation at position M240 in alpha actinin 4.
6.3.2.2 Non-reproducible modifications

- **Actin alpha cardiac muscle 1**

The LC/MS/MS analysis detected peaks covering 38 – 48 % of the protein sequence of actin alpha cardiac muscle 1 (42 kDa, accession number P68032) in all the control and treated samples (Figure 6.7A-C). About 19 – 30 peptides described this protein with between 1 and 6 peptides unique to this protein. All samples had different numbers of peptides and unique peptides. These data suggest that actin alpha cardiac muscle 1 protein is expressed by HCAEC.

In all treated samples, multiple spectra containing an oxidised methionine at position M192 were detected (Figure 6.7A). These spectra had scores between 25.46 and 30.54, which indicate that this modification was induced by ONOOH. A spectrum containing this modification is shown in Figure 6.8A. The treated samples also contained a few spectra of the same peptide that did not have an oxidised methionine. The control samples did not contain any spectra with an oxidised methionine that had a score above 20 (Figure 6.7A). The control sample from donor 2366 had a spectrum without an oxidised methionine and the score of this spectrum was 27.76. Likewise the 2315 control sample had a spectrum without an oxidised methionine and the score of this spectrum was 31.83. These data suggest that significant methionine oxidation did not occur in the control samples where ONOOH was absent.

Likewise in all treated samples, multiple spectra indicating the presence of a nitrated tyrosine at position Y220 from this protein were detected (Figure 6.7B). These spectra had scores that ranged from 25.09 – 32.32. A spectrum containing a nitrated tyrosine is shown in Figure 6.8B. Apart from this, multiple spectra without a nitrated tyrosine were also detected in the treated samples. The control samples did not yield any spectra with a nitrated tyrosine residue at position Y220 with a score above 20 (Figure 6.7B). These data suggest that in the absence of ONOOH, this tyrosine residue was not nitrated to a significant extent. No nitrated tyrosine residues from this peptide were detected in the control spectra from donor 2315.

Multiple spectra containing an oxidised methionine at position M229 were detected in the treated samples (Figure 6.7C). Most of the spectra scores were between 22.73 and 28.83. A spectrum is shown in Figure 6.8C. The presence of such high scores and good
spectra in the treated samples suggests that this modification is highly likely to be mediated by ONOOH. Several spectra without this particular modification were also detected in the treated samples. On the other hand, no spectra that had an oxidised methionine at position M229 and a score above 20 were detected in the control samples. Spectra without this modification were also detected in the control samples.

In contrast to the above data, this protein was not detected in the control sample from donor 2286 so the data set from this donor is incomplete. However, overall these data suggest that 500 µM ONOOH induces methionine oxidation at position M192 and M229 as well as tyrosine nitration at position Y220 in actin alpha cardiac muscle 1 of HCAEC since this modification was detected in all treated samples and not detected in the majority of control samples.

- **Isoform 2 of clathrin heavy chain**

The LC/MS/MS analysis detected 27-45 % of sequence coverage of isoform 2 of clathrin heavy-chain (187 kDa, accession number Q00610-2) in all HCAEC treated and control samples (Figure 6.9A). 35 – 54 peptides described this protein of which about 34 – 43 peptides were unique to this protein. Different numbers of peptides (including unique peptides) were detected in different samples. Such high sequence coverage, number of peptides and number of unique peptides suggest that this protein is expressed by HCAEC and this protein was present in all samples.

In the treated samples, all HCAEC donors contained spectra that had an oxidised methionine at position M181 (Figure 6.9A). These spectra had high scores that ranged from 47.95 – 51.03. A spectrum containing an oxidised methionine residue is shown in Figure 6.9B. These results imply that this particular modification is most likely induced by ONOOH. Interestingly, all treated samples also contained spectra that did not have this modification. The scores of these latter spectra ranged from 39.46 – 49.63. This is because the modification yield is not 100 %.
### A: Methionine oxidation @ M192

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### B: Tyrosine nitration @ Y220

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### C: Methionine oxidation @ M229

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<td>3 (19.05)</td>
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Figure 6.7 LC/MS/MS analysis showing ONOOH-mediated modifications in actin alpha cardiac muscle 1.
Figure 6.8 LC/MS/MS spectrum of ONOOH-mediated modifications in actin alpha cardiac muscle 1.
Control samples from two donors (2286 and 2366) did not contain any spectrum that had an oxidised methionine at position M181. However the control sample from donor 2315 had 1 spectrum that contained an oxidised methionine and the score of this spectrum was 52.23. This is a significant score, which implies that this particular methionine residue was oxidised in this control sample in the absence of ONOOH. The possible routes of this methionine oxidation include oxidation during the experiment or oxidation during sample handling and processing. Methionine residues are known to be easily oxidised during sample preparation (399, 400). This control sample also had a spectrum without an oxidised methionine at position M181 and the score of this spectrum was 52.71. This implies that this particular control sample from donor 2315 has a mixed population of oxidised and non-oxidised methionine residues at position M181.

Although these results are incomplete, overall these results suggest that 500 µM ONOOH induces methionine oxidation at position M181 in isoform 2 of clathrin heavy-chain of HCAEC since this modification was detected in all treated samples and not in majority of the control samples.

- **Endoplasmin**

Approximately 34 – 56 % sequence coverage of endoplasmin (92 kDa, accession number P14625) was detected in the control and treated samples (Figure 6.10A). 27 – 45 peptides from this protein were detected in all control and treated samples of which 26 – 43 peptides were unique to this protein. There were different numbers of specific peptides in different samples. Such high sequence coverage, the number of peptides and number of unique peptides suggest that this protein was present in the sample.

In the treated samples, all HCAEC donors had spectra with an oxidised methionine at position M154. These spectra scores ranged from 38.10 – 50.95. A spectrum containing an oxidised methionine residue is shown in Figure 6.10B. The high scores and good spectrum suggest that 500 µM ONOOH induced methionine oxidation at this particular residue. These treated samples also had spectra that did not have this modification.
A

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B

Figure 6.9 LC/MS/MS analysis (A) and spectrum (B) of ONOOH-mediated methionine oxidation at position M181 in isoform 2 of clathrin heavy chain.

In the control samples from donors 2315 and 2286, no spectra with an oxidised methionine at position M154 were detected (Figure 6.10A). Both of these donors had spectra without a modified methionine and their scores (44.87 and 43.92) were very high. These data indicate that this modification did not occur in these donors in the absence of 500 µM ONOOH. In contrast, the control sample from donor 2366 had a spectrum with an oxidised methionine and the score of this particular spectrum was 42.69. This high score indicates that this particular methionine was oxidised in this sample in the absence of ONOOH. This is most probably due to methionine oxidation occurring during sample
preparation or the experiment. Interestingly, this latter sample also had a spectrum without an oxidised methionine and this spectrum had a high score of 49.84. This indicates that in this control sample from donor 2366, there are unmodified methionine residues at position M154 present.

These results are incomplete as methionine oxidation was detected in all treated samples and the control samples from donor 2366. However overall these data suggest that 500 µM ONOOH induces methionine oxidation at position M154 in endoplasmin of HCAEC.

Eukaryotic initiation factor 4A-1

About 23 – 37 % of protein sequence coverage of eukaryotic initiation factor 4A-1 (46 kDa, accession number P60842) was detected in all treated and control samples (Figure 6.11A). 6 – 13 peptides were detected, of which 5 – 9 were unique peptides. Different numbers of specific peptides were present in different samples. These suggest that this protein was present in the HCAEC samples.

The treated samples from donors 2286 and 2366, had spectra with an oxidised methionine at position M398 (Figure 6.11A). The scores of these spectra were 24.75 and 50.63. Such high scores suggest that methionine oxidation most likely occurred on this particular residue. These samples also had spectra without an oxidised methionine. In the treated samples from donor 2315, no spectra were detected which had an oxidised methionine. None of the control samples from all 3 donors had spectra with an oxidised methionine at position M398. In contrast, all of these control samples had spectra without this particular modification. These latter spectra had scores of 24.16 and 28.07. These data suggest that in the absence of ONOOH, methionine oxidation did not occur at this particular methionine residue.

These results are not reproducible and incomplete because methionine oxidation at position M398 was detected in the treated samples from donors 2286 and 2366, and not detected in the treated sample from the donor 2315. Overall these data may be indicating that 500 µM ONOOH induces methionine oxidation at position M398 in the eukaryotic initiation factor 4A-1 of HCAEC.
Figure 6.10 LC/MS/MS analysis (A) and spectrum (B) of ONOOH-mediated methionine oxidation at position M154 in endoplasmin.
Figure 6.11 LC/MS/MS analysis (A) and spectrum (B) of ONOOH-mediated methionine oxidation at position M398 in eukaryotic initiation factor 4A-1

Elongation factor 1 – alpha 1

The LC/MS/MS analysis detected approximately 39 – 62 % of protein sequence coverage of elongation factor 1-alpha 1 (50 kDa, accession number P68104) in all treated and control samples (Figure 6.12A). 15 – 26 peptides were detected with 6 – 26 of these being unique. There were different numbers of specific peptides in different samples. These data suggest that this protein was present in all treated and control samples.
The treated samples from donors 2286 and 2366, had 1 spectrum with an oxidised cysteine residue at position C111 and the scores of these spectra were 22.98 and 25.77 respectively. The cysteine residue was oxidised to cysteic acid. A spectrum containing an oxidised cysteine is shown in Figure 6.12B. These samples also had spectra that did not have an oxidised cysteine. In the treated sample from donor 2315, no spectra containing an oxidised cysteine at position C111 were detected. In this sample, spectra without an oxidised cysteine were detected.

In the control samples from all donors, no spectra containing an oxidised cysteine at position C111 were detected. Multiple spectra without cysteine oxidation were detected in the control samples, which indicates that in the absence of 500 µM ONOOH, this particular cysteine was not oxidised. These results are incomplete, but nevertheless these data suggest that under these experimental conditions, 500 µM ONOOH induces cysteine oxidation at position C111 in elongation factor 1 – alpha 1 of HCAEC.

Translational endoplasmic reticulum ATPase
Approximately 23 – 45 % sequence coverage of translational endoplasmic reticulum ATPase (89 kDa, accession number P55072) was detected in all HCAEC treated and control samples. 13 – 29 peptides described this protein, with all of them being unique. In all control and treated samples, different numbers of specific peptides were present. These data suggest that this protein was present in the samples.

The treated samples from HCAEC donors 2286 and 2366 had a spectrum each with an oxidised methionine at position M332 in translational endoplasmic reticulum ATPase (Figure 6.13A). These spectra had high scores (36.53 and 38.25) and a spectrum containing this modification is shown in Figure 6.13B. No spectra containing an oxidised methionine were detected in the treated sample from donor 2315. All the treated samples had multiple spectra without an oxidised methionine.

None of the control samples had a spectrum with an oxidised methionine. These control samples had multiple spectra without this modification. These data suggest that in the absence of ONOOH, methionine oxidation of this particular residue did not occur. These results are not reproducible, however these data suggest that 500 µM ONOOH induces
methionine oxidation at position M332 in translational endoplasmic reticulum ATPase of HCAEC.

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**Figure 6.12** LC/MS/MS analysis (A) and spectrum (B) of ONOOH-mediated cysteine oxidation at position C111 in elongation factor 1 – alpha 1
A

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B

Figure 6.13 LC/MS/MS analysis (A) and spectrum (B) of ONOOH-mediated methionine oxidation at position M332 in translational endoplasmic reticulum ATPase.
6.3.2.3 HCAEC donor variations

There is evidence in the literature that suggests that different donors of a particular cell type have different responses to the same stimuli. This is due to genetic differences, age, racial background and health conditions between the individual donors. Donors variations have been reported in human induced pluripotent stem cells (401), hepatocytes derived from human induced pluripotent stem cells (402), human skin-derived mesenchymal stem cells (403), lymphoblastoid cells lines (404), human corneal endothelial cells (405), human umbilical vein endothelial cells (406) and human aortic endothelial cells (407).

In this project 3 commercially available HCAEC donors were used (Table 6.1). A previous study identified two distinguished populations of HCAEC, which had different gene expression and different responses to TNF-alpha (408). A number of variations have been detected in this project whereby cells from one donor were more susceptible to a particular modification compared to the cells from the other donors. The following subsections discuss how some ONOOH-mediated modifications may occur specifically in one HCAEC donor and not others.

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<tr>
<td>2366</td>
<td>60 year old caucasian male</td>
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Table 6.1 Description of HCAEC donors used in this project.
Modifications detected in cells from donor 2366

1. Cysteine oxidation in myosin 9
LC/MS/MS detected cysteine oxidation at position C988 in myosin 9 (226 kDa, accession number P35579) only in treated samples from donor 2366 and not in the other treated samples (donors 2315 and 2286) or controls (donors 2315, 2286 and 2366). 38 - 55 % sequence coverage was detected. 62 – 108 peptides described this protein with 25 – 85 of these being unique to this protein (Figure 6.14A).

In the treated sample from donor 2366, one spectrum (score was 23.52) was detected that had an oxidised cysteine residue at position C988 (Figure 6.14B). The cysteine residue was oxidised to cysteic acid. The high percentage of sequence coverage along with fragmentation spectra of the peptide containing C988 suggests that this particular modification occurred at this residue. This treated sample also had a spectrum (score 39.88) without an oxidised cysteine residue at position C988. None of the other treated or control samples had any spectrum with an oxidised cysteine residue at position C988. However all of these latter samples had spectra without an oxidised cysteine residue. The scores of these latter spectra ranged from 22.50 – 49.48 (Figure 6.14A). Such high scores indicate that in these samples cysteine oxidation did not occur at C988. Overall these data suggest that ONOOH induces cysteine oxidation at C988 in myosin-9, specifically in HCAEC donor 2366.
Figure 6.14 LC/MS/MS analysis (A) and spectrum (B) of ONOOH-mediated cysteine oxidation at position C988 in myosin-9 detected in the treated sample from donor 2366.
2. Methionine oxidation in alpha actinin 4

The LC/MS/MS detected methionine oxidation at position M379 in alpha actinin 4 (105 kDa, accession number O43707) in the treated sample from donor 2366. In contrast, this particular modification was absent in the other treated samples (donors 2315 and 2286) and all control samples. 52 – 68 % sequence coverage was detected (Figure 6.15A). 33 – 50 peptides were detected which described this protein and 21 – 32 of these were unique. The number of specific peptides differed between samples. These high percentages of sequence coverage and number of peptides suggest that this protein was present in the sample.

The 2366 treated sample had a spectrum with an oxidised methionine residue at position M379 (Figure 6.15B) and the score of this spectrum was 48.87. This high score and good fragmentation spectrum suggest that ONOOH most likely induced methionine oxidation at this particular residue in the treated sample of this donor. No spectra without this modification were detected in treated sample from this donor.

The other treated samples (donors 2315 and 2286) and all controls either had no spectra with an oxidised methionine at position M379 or had spectra with very low scores (below 20). This suggests that methionine oxidation most probably did not occur in these samples. These latter samples except 2366 control samples, had spectra with no modified methionine residue at position M379. The control samples from donor 2366 had no spectra with an oxidised methionine at this particular residue. Although these data are incomplete, they indicate that ONOOH induces methionine oxidation at M379 in alpha actinin 4, particularly in HCAEC donor 2366.

3. Methionine oxidation in eukaryotic initiation factor 4A-1

The LC/MS/MS analysis indicate that ONOOH induced methionine oxidation at position M149 in eukaryotic initiation factor 4A-1 (46 kDa, accession number P60842) only in the treated sample from donor 2366 and not in other donors (2315 and 2366) and controls (donors 2315, 2286 and 2366). 23 – 37 % of sequence coverage of this protein was detected in all treated and control samples (Figure 6.16A). 6 - 13 peptides described this protein and 5 – 9 unique peptides were detected. Different samples had different numbers of specific peptides.
Figure 6.15 LC/MS/MS analysis (A) and spectrum (B) of ONOOH-mediated methionine oxidation at position M379 in alpha actinin 4 detected in the treated sample from donor 2366.
2 spectra with an oxidised methionine at position M149 were detected in the donor 2366 treated samples (Figure 6.16B). These spectra had scores of 54.48 and 43.37. This sample also had a spectrum without an oxidised methionine residue at M149. None of the other treated samples (donors 2315 and 2366) or controls (donors 2315, 2286 and 2366) had an oxidised methionine at M149. All of these latter samples had spectra (with high scores that ranged from 39.18 – 52.40) without an oxidised methionine at M149. These results indicate that ONOOH induces methionine oxidation at M149 in eukaryotic initiation factor 4A-1, specifically in donor 2366.

4. Methionine oxidation in elongation factor 1 alpha 1
ONOOH-mediated oxidation methionine 102 in elongation factor 1 alpha 1 (50 kDa, accession number P68104) was detected via LC/MS/MS only in the treated sample from 2366 and not in the other samples. 39 – 62 % of sequence coverage of this protein was detected in all treated and control samples. 15 – 26 peptides described this protein of which 6 – 26 were unique. The total number of peptides (including unique peptides) differed between samples.

2 spectra had an oxidised methionine residue at position M102 in the treated sample from donor 2366 and not in the other samples (Figure 6.17). This sample also had multiple other spectra that did not have an oxidised methionine at position M102. All other treated or control samples either had no spectra or had spectra with low scores (less than 20). In these latter samples, multiple spectra without an oxidised methionine residue were detected. These data overall indicate that ONOOH induces tyrosine oxidation at position Y85 and methionine oxidation at position M102 in elongation factor 1 alpha 1, particularly in HCAEC donor 2366.
Figure 6.16 LC/MS/MS analysis (A) and spectrum (B) of ONOOH-mediated methionine oxidation at position M149 in eukaryotic initiation factor 4A-1 detected in the treated sample from donor 2366.
A

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<td>2 (37.90), 1 (21.76)</td>
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</table>

B

Figure 6.17 LC/MS/MS analysis (A) and spectrum (B) of ONOOH-mediated methionine oxidation at position M102 in elongation factor 1 – alpha 1 detected in the treated sample from donor 2366.
5. Sulfone formation in actin cytoplasmic 1 and 2

Two different actin isoforms were detected in all HCAEC donors, which includes actin cytoplasmic 1 and 2. Methionine oxidation by ONOOH results in methionine sulfoxide formation. This product then gets further oxidised by ONOOH to form methionine sulphone (167).

The LC/MS/MS detected sulphone formation at M305 in actin cytoplasmic 1 (41 kDa, accession number P60709) only in the treated sample from donor 2366 and not the other samples. 66 – 79 % of sequence coverage of this protein was detected in all treated and control samples (Figure 6.18A). 43 – 53 peptides described this protein and 1 unique peptide was also detected. There were different numbers of specific peptides in different samples. One spectrum with sulphone formation at M305 was also detected in the treated sample from donor 2366. This spectrum had a high score of 44.41 and a fully labelled spectrum is shown in Figure 6.18B. Apart from this, this sample also had several spectra without sulphone formation at this particular methionine residue. All the other treated samples (donors 2315 and 2286) and controls (2315, 2286 and 2366) did not have any spectra with a sulphone at this residue.

The LC/MS/MS detected sulphone formation at M305 in actin cytoplasmic 2 (41 kDa, accession number P63261) only in the treated sample from donor 2366 and not the other samples. 66 – 79 % of sequence coverage was detected (Figure 6.19A). 43 – 53 peptides described this protein and 1 – 2 unique peptides were also detected. One spectrum had sulphone formation at M305 only in the treated sample from donor 2366. The score of this spectrum was 44.41 and the fully labelled spectrum is shown in Figure 6.19B. This sample also had other spectra that did not have this modification. None of the other treated samples (donors 2315 and 2286) or controls had sulphone formation. However these samples had multiple spectra without sulphone formation. These data suggest that ONOOH induces methionine oxidation at M305 in both actin cytoplasmic 1 and 2, specifically in donor 2366.
Figure 6.18 LC/MS/MS analysis (A) and spectrum (B) of ONOOH-mediated sulphone formation at M305 in actin cytoplasmic 1 detected in the treated sample from donor 2366.
Figure 6.19 LC/MS/MS analysis (A) and spectrum (B) of ONOOH-mediated sulphone formation at M305 in actin cytoplasmic 2 detected in the treated sample from donor 2366.

Modification detected in cells from donor 2315

LC/MS/MS detected ONOOH-mediated methionine oxidation at M295 in alpha actinin 4 (105 kDa, accession number O43707) only in the treated sample from donor 2315 and not in the other samples. 50 – 68 % of sequence coverage was detected in all treated and control samples (Figure 6.20A). 32 – 50 peptides were detected which described this protein with 21 – 32 being unique. The total number of specific peptides differed between samples.
One spectrum in the 2315 treated sample had an oxidised methionine residue at M295. The score of this spectrum was 34.52 and the spectrum is shown in Figure 6.20B. This high score suggests that this residue is most likely modified by ONOOH. This sample also had another spectrum that did not have an oxidised methionine at this particular position. This is due to modifications not being 100% resulting in a mixed population of modified and non-modified peptides.

All other treated samples (donors 2286 and 2366) and controls (2315, 2286 and 2366) either did not have any spectrum with an oxidised methionine residue or had a spectrum with a low score (below 20). These data suggest that under these experimental conditions, ONOOH induces methionine oxidation at M195 in alpha actinin 4, particularly in HCAEC donor 2315.

The results outlined so far in this Chapter have indicated that 500 µM ONOOH induced multiple modifications such as cysteine and methionine oxidation, sulphone formation and tyrosine oxidation and nitration to various HCAEC proteins. Some ONOOH-mediated modifications appear to be donor specific.

6.3.3 Enrichment approach

The results outlined in Chapter 3 and 4 indicate that ONOOH induced 3NT formation on multiple HCAEC proteins during short (5 min) and prolonged (4 h) exposure. In the results presented so far in this Chapter, the samples used to perform LC/MS/MS analysis were not enriched for nitrated proteins and only a limited number of positive identifications for this modification could therefore be made. As a consequence, an enrichment approach for nitrated proteins using immunoprecipitation was employed.

HCAEC were seeded at a cell density of 4 x 10^5 cells per well in a 6 well plate overnight. Cells were washed twice with 750 µL of warm PBS and incubated in 725 µL of HBSS. Cells were then treated with 500 µM ONOOH for 5 min at 37 °C and pH 7.4. Immunoprecipitation was performed using a commercial kit, the Dynabeads Protein G Immunoprecipitation Kit (Life Technologies). Nitrated proteins were captured using an anti-3NT antibody. The captured nitrated proteins were then separated via SDS-PAGE and gels were subjected to either Western blotting to detect the presence of 3NT on
proteins or Coomassie staining to visualise all protein bands. The Coomassie stained gel was then subjected to trypsin in-gel digestion and LC/MS/MS analysis.

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### Figure 6.20

- **A**
  - LC/MS/MS analysis (A) and spectrum (B) of ONOOH-mediated methionine oxidation at position M295 in alpha actinin 4 detected in the treated sample from donor 2315.
The Western blotting results showed few nitrated proteins in the control lane (Figure 6.21A). This suggests that a basal or low level of nitration occurs on HCAEC proteins. Nitration of proteins increased when cells were treated with 500 μM ONOOH. This was confirmed by an increase in the pixel density of nitrated proteins in the treated lane. When this membrane was further exposed, the nitrated bands became more evident. The light chains of primary antibodies are about 25 kDa. The dense protein band around the 25 kDa mark was suspected to be the light chain of the 3NT antibody which may have coeluted with the sample.

In order to test whether the dense band was the light chain of the 3NT antibody, Western blotting was performed without the primary antibody (3NT antibody). It was expected that if the light chain of the 3NT antibody was present in the coeluted sample, the secondary antibody (whole antibody of anti-mouse IgG) would bind to it. In these experiments a nitrated protein band around the 25 kDa protein was detected (Figure 6.21B). This result confirmed that the light chain of the 3NT antibody unexpectedly coeluted with the sample during immunoprecipitation and this was responsible for the dense nitrated band around 25 kDa. The nitrated higher molecular mass proteins (about 70 kDa) are most likely the heavy chain of the 3NT antibody.

Multiple HCAEC proteins were detected in the Coomassie stained gel after immunoprecipitation both in the treated and control lanes (Figure 6.21C). In the previous Chapters, no 3NT formation was detected on proteins exposed to 0 μM ONOOH or 20 mM NaOH (the vehicle of ONOOH). Consequently during immunoprecipitation while enriching for nitrated proteins, we did not expect 0 μM ONOOH to induce nitration of HCAEC proteins. It was also not expected that non-nitrated proteins would be recognised by the anti-3NT antibody. Surprisingly in the control lane, many proteins were detected (Figure 6.21C), which indicates that the anti-3NT antibody non-specifically bound to other non-nitrated proteins when this antibody should ideally only bind to nitrated proteins. In the treated lane, the presence of multiple nitrated proteins was expected and the Western blotting results confirmed that the proteins present in the treated samples were nitrated by ONOOH.
Figure 6.21 Nitrated HCAEC proteins were detected after immunoprecipitation (A). 3NT antibody was detected coeluting with the nitrated proteins; Western blotting was performed without anti-3NT antibody (B). The gel shown on the right side is the magnified versions of the upper part (above 25 kDa) of the gel shown on the left side. Coomassie staining detected multiple HCAEC proteins (C).
6.3.3.1 Non-reproducible modifications

After enriching for nitrated proteins, multiple non-reproducible modifications were detected and these modifications are discussed in the following subsections.

- **Mitochondrial ATP synthase beta subunit**

The LC/MS/MS analysis detected 25 – 53 % sequence coverage of mitochondrial ATP synthase beta subunit (56 kDa, accession number P06576) in all treated and control immunoprecipitated samples (Figure 6.22A). 9 – 20 peptides described this protein and all of these peptides were unique to this protein. The number of specific peptides varied between samples. These indicate that this protein is expressed by HCAEC.

All treated samples (from donors 2315, 2286 and 2366) had spectra that contained an oxidised methionine at position M272. These spectra scores ranged from 22.48 – 46.19. One of the spectra is shown in Figure 6.22B. Such high scores and the presence of numerous good spectra suggest that ONOOH most likely induced methionine oxidation in these treated samples.

Interestingly the control sample from donor 2286 also had a spectrum with an oxidised methionine at position M272 and this spectrum’s score was 22.12 (Figure 6.22A). There is insufficient information to determine whether this methionine oxidation occurred during sample preparation. This sample also had another spectrum with even higher score of 50.29, which did not show methionine oxidation occurring at this particular residue. The other control samples (from donors 2315 and 2366) did not have any spectra showing an oxidised methionine at M272, however these samples had spectra without an oxidised methionine. These samples spectra also had very high scores (46.12 and 53.17).

The presence of methionine oxidation at M272 in all treated as well as one control sample (from donor 2286) does not make these results reproducible. These data are incomplete, but imply that ONOOH induces methionine oxidation at M272 in the mitochondrial ATP synthase beta subunit of HCAEC.
Figure 6.22 LC/MS/MS analysis (A) and spectrum (B) of ONOOH-mediated methionine oxidation at position M272 in mitochondrial ATP synthase beta subunit detected in the enriched samples.
Heat shock cognate 71 kDa protein

LC/MS/MS analysis detected 14 – 30 % sequence coverage of heat shock cognate 71 kDa protein (accession number P11142) in all treated and control samples after immunoprecipitation (Figure 6.23A). 7 – 16 peptides described this protein, of which 3 – 12 peptides were unique. Different numbers of specific peptides were detected in different samples.

All the treated samples had multiple spectra with an oxidised methionine at position M61 (Figure 6.23B). These spectra had scores ranging from 32.87 – 45.94. These data suggest that ONOOH induces methionine oxidation at this particular methionine residue. These samples also had spectra without an oxidised methionine.

The control sample from donor 2366 also had a spectrum with an oxidised methionine at position M61 and the score of this spectrum was 49.50. This particular modification may have occurred during sample preparation. This sample also had a spectrum without an oxidised methionine and the score of this spectrum was 44.03. All the other control samples did not have any spectra with an oxidised methionine but did have spectra without any oxidised methionine. This modification is not reproducible because methionine oxidation was detected in one control sample as well as all treated samples, but the data suggest that ONOOH induces methionine oxidation at M61 in heat shock cognate 71 kDa protein of HCAEC.

Nestin

The LC/MS/MS analysis detected 8 – 16 % sequence coverage of nestin (177 kDa, accession number P48681) in all treated and control samples (Figure 6.24A). 11 – 21 peptides described this protein and all of these were unique to this protein. This suggests that this protein was present in the sample. In all treated samples from all donors, multiple spectra were present with an oxidised methionine at position 200. The scores of these spectra ranged from 37.35 – 43.97. A spectrum is shown in Figure 6.24B. These samples also had few spectra without an oxidised methionine at this particular residue.

The control sample from donor 2366 also gave spectra with an oxidised methionine at M200. None of the other control samples showed this, but these samples did give spectra without an oxidised methionine at M200. Overall this modification is not reproducible as
methionine oxidation was detected in one control sample, but these data suggest that ONOOH induces methionine oxidation at M200 in nestin.

<table>
<thead>
<tr>
<th></th>
<th>Treated samples</th>
<th>Control samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCAEC donor</td>
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<td>2315</td>
</tr>
<tr>
<td>Sequence coverage (%)</td>
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<td>2286</td>
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<tr>
<td>No. of peptides</td>
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<tr>
<td>No. of unique peptides</td>
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<tr>
<td></td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>No. of spectra containing modification (score)</td>
<td>2 (45.94)</td>
<td>2 (32.87)</td>
</tr>
<tr>
<td>No. of spectra without this modification (score)</td>
<td>1 (34.93)</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 6.23 LC/MS/MS analysis (A) and spectrum (B) of ONOOH-mediated methionine oxidation at position M61 in heat shock cognate 71 kDa protein detected in the enriched samples.
Figure 6.24 LC/MS/MS analysis (A) and spectrum (B) of ONOOH-mediated methionine oxidation at position M200 in nestin detected in the enriched samples.
6.3.3.2 HCAEC donor variations

In the immunoprecipitated samples, HCAEC donor variations were also detected. The following sections discuss HCAEC donor variations detected after enriching the samples for nitrated proteins.

 Modifications detected in the enriched samples from donor 2315

1. Tyrosine nitration in actin cytoplasmic 1

In the treated and enriched sample from donor 2315, ONOOH-mediated tyrosine nitration was detected using LC/MS/MS at Y198 in actin cytoplasmic 1 (41 kDa, accession number P60709) (Figure 6.25A). 51 – 66 % sequence coverage of this protein was detected in all treated and control samples. 19 – 30 peptides were detected which described this protein of which 1 – 13 peptides were unique to this protein. The spectra from the treated sample had a score of 41.28 and a spectrum is shown in Figure 6.25B. This sample also yielded spectra without a nitrated tyrosine at this particular residue. All the other treated and control samples did not yield spectra with a nitrated tyrosine at Y198. However all of these samples had multiple spectra without a nitrated tyrosine residue.

2. Methionine oxidation in talin-1

LC/MS/MS analysis detected 11 – 16 % sequence coverage of talin-1 (269 kDa, accession number Q9Y490) in all treated and control samples. 19 – 30 peptides described this protein and 19 – 27 peptides unique to this protein were detected. The total number of specific peptides differed between samples.

In the treated and enriched sample from donor 2315, one spectrum with an oxidised methionine residue at position M2290 was detected (Figure 6.26A). This spectrum’s score was 36.35 and the spectrum is shown in Figure 6.26B. This sample also had another spectrum showing the methionine residue at M2290 that was not modified. All the other treated samples and controls did not have any spectra with an oxidised methionine at position M2290. However all of these samples had multiple spectra with an unmodified methionine residue at position M2290.
Modifications detected in the enriched samples from donor 2366

1. Tyrosine nitration in vimentin

In the treated and enriched samples from donor 2366, tyrosine nitration was detected at position Y85 in vimentin (53 kDa, accession number P08670) (Figure 6.27A). 78 – 87% sequence coverage was detected via LC/MS/MS analysis. 44 – 69 peptides described this protein with 36 – 60 peptides being unique. There were different numbers of specific peptides in different samples. In the treated and enriched sample form donor 2366, one spectrum containing a nitrated tyrosine residue at position Y85 was detected (Figure 6.27B). The score of this spectrum was 21.26. This sample also yielded other spectra without a nitrated tyrosine residue at this position. All the other treated samples and controls did not give spectra with a nitrated tyrosine residue at position Y85. In contrast, all of these latter samples had multiple spectra without a nitrated tyrosine at Y85.

Immunoprecipitation was performed to enrich the samples for nitrated proteins using an anti-3NT antibody. Most of the modifications mentioned so far in this Section are methionine oxidation and not 3NT formation. Methionine oxidation is occurring at other sites but interestingly proteins containing this particular modification were pulled out during immunoprecipitation by an anti-3NT antibody. This suggests that other non-nitrated proteins are non-specifically binding to the 3NT antibody. This further confirms the results shown in Figure 6.21C where non-nitrated proteins in the control samples were captured by the anti-3NT antibody.

Overall these data suggest that ONOOH-mediated tyrosine nitration of actin cytoplasmic 1 and vimentin was only detected after enriching the samples for nitrated proteins suggesting that the modified version of this protein occurs in low abundance. ONOOH induces tyrosine nitration at Y198 in actin cytoplasmic 1 particularly in cells from donor 2315. In addition to this, ONOOH also induces tyrosine nitration at Y85 in vimentin specifically in the cells from donor 2366. The above data also suggest that ONOOH induces methionine oxidation at M2290 in talin-1 particularly in cells from donor 2315. Like ONOOH-mediated tyrosine nitration, methionine oxidation may also be occurring at low abundance in HCAEC.
Figure 6.25 LC/MS/MS analysis (A) and spectrum (B) of ONOOH-mediated tyrosine nitration at position Y198 in actin cytoplasmic 1 detected in the enriched treated sample from donor 2315.
Figure 6.26 LC/MS/MS analysis (A) and spectrum (B) of ONOOH-mediated methionine oxidation at position M2290 in talin-1 detected in the enriched treated sample from donor 2315.
Figure 6.27 LC/MS/MS analysis (A) and spectrum (B) of ONOOH-mediated tyrosine nitration at position Y53 in vimentin detected in the enriched treated sample from donor 2366.
6.4 Discussion

The data outlined in the previous two Chapters (4 and 5) indicate that ONOOH induces thiol oxidation, 3NT formation and other protein modifications such as aggregation and/fragmentation after both short (bolus) and prolonged (SIN-1) exposure. The identities of these modified proteins were unknown. Consequently the experiments outlined in this Chapter aimed to identify which HCAEC cellular proteins were being modified by 500 µM ONOOH after a 5 min incubation period at pH 7.4 and 37 °C via LC/MS/MS. We were also interested in enriching the samples for nitrated proteins via immunoprecipitation to increase the possibility of detecting nitrated proteins. The following sections discuss the types and identities of proteins that were modified in HCAEC by 500 µM ONOOH after a 5 min incubation period (Table 6.2).

A) Cytoskeletal proteins

<table>
<thead>
<tr>
<th>Proteins modified</th>
<th>Type of modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin alpha cardiac muscle 1</td>
<td>Methionine oxidation, Tyrosine nitration</td>
</tr>
<tr>
<td>Actin cytoplasmic 1</td>
<td>Sulphone formation, Tyrosine nitration</td>
</tr>
<tr>
<td>Actin cytoplasmic 2</td>
<td>Sulphone formation</td>
</tr>
<tr>
<td>Alpha actinin 4</td>
<td>Methionine oxidation</td>
</tr>
<tr>
<td>Myosin</td>
<td>Cysteine oxidation</td>
</tr>
<tr>
<td>Nestin</td>
<td>Methionine oxidation</td>
</tr>
<tr>
<td>Talin-1</td>
<td>Methionine oxidation</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Cysteine oxidation, Tyrosine nitration</td>
</tr>
</tbody>
</table>

B) Chaperone/stress proteins

<table>
<thead>
<tr>
<th>Proteins modified</th>
<th>Type of modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endoplasmin</td>
<td>Methionine oxidation</td>
</tr>
<tr>
<td>Heat shock cognate 71 kDa protein</td>
<td>Methionine oxidation</td>
</tr>
<tr>
<td>Translational endoplasmic reticulum ATPase</td>
<td>Methionine oxidation</td>
</tr>
</tbody>
</table>

C) Mitochondrial proteins

<table>
<thead>
<tr>
<th>Proteins modified</th>
<th>Type of modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria ATP synthase β-subunit</td>
<td>Methionine oxidation</td>
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</tbody>
</table>
D) Proteins involved in protein synthesis

<table>
<thead>
<tr>
<th>Protiens modified</th>
<th>Type of modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elongation factor 1 - α1</td>
<td>Cysteine oxidation, Methionine oxidation, Tyrosine nitration</td>
</tr>
<tr>
<td>Eukaryotic initiation factor 4A-1</td>
<td>Methionine oxidation</td>
</tr>
<tr>
<td>Isoform 2 of clathrin heavy chain</td>
<td>Methionine oxidation</td>
</tr>
</tbody>
</table>

Table 6.2 Different types and identities of HCAEC proteins are modified by 500 µM ONOOH after a 5 min incubation period at 37 °C and pH 7.4.

6.4.1 Cytoskeletal proteins

The cellular cytoskeleton is a network of fibres that are composed of proteins. The cytoskeleton has 3 different components including the microfilaments (actin), microtubules (tubulin) and the intermediate filaments (vimentin, keratin and neurofilament) (409).

6.4.1.1 Alpha actinin 4

The proteins of the alpha actinin family are multifunctional cytoskeletal proteins that are required for cell shape and motility. These proteins are part of the filamentous actin (F-actin) crosslinking proteins. It is believed that alpha actinin connects the actin cytoskeleton to the cell membrane and particularly to transmembrane proteins, and this regulates the activity of multiple receptors and links the cytoskeleton with signalling pathways and adhesion molecules (410). 4 isoforms of this protein have been previously identified (alpha actinin-1, -2, -3 and -4). Alpha actinin 4 is an actin-bundling protein that is associated with cell motility. This protein interacts with other F-actin proteins and various cytoskeletal and membrane proteins, which organise the cell cytoskeleton and are found adjacent to adherent junctions (411).

It has been reported previously that ONOOH induces alpha actinin nitration in human myocardial cells (412). In this previous study, it was postulated that this modification led to contractile dysfunction. However the tyrosine residues or the isoform of alpha actinin that was nitrated were not identified. Another study has reported that ONOOH-mediated tyrosine nitration via SIN-1 decomposition of human alpha actinin 1, and that this
process resulted in inhibition of platelet adhesion (413). Previous studies have shown that chloramine-T- and hydrogen peroxide-mediated oxidation of methionine residues of actin can disrupt actin polymerisation and inhibit specific noncovalent interactions that stabilise the actin filament structure (414, 415), with this affecting actin filament stability and resulting in rapid depolymerisation.

The LC/MS/MS analysis carried out in this project has indicates that 500 µM ONOOH induces methionine oxidation at M240 in alpha actinin 4 contained in HCAEC. This is a confirmed modification because this modification was detected in all treated and none of the control samples. Incomplete data were also obtained, which suggests that 500 µM ONOOH induces methionine oxidation at M379 (in donor 2366) and at M295 (in donor 2315) of this protein in HCAEC.

It has been proposed that methionine oxidation may modulate multiple biological processes. In cells, methionine residues participate in the antioxidant defense system (165), modulate cellular functions via reversible oxidation and reduction, participate in regulation of enzymatic activities, are involved in cell signalling and direct proteins towards proteolytic degradation (416). The thioether groups of methionine side chains are weak nucleophiles that are readily oxidised to methionine sulphone, which in turn can be further oxidised to methionine sulphone (166) by oxidising agents such as ONOOH (167). In proteins, methionine oxidation appears in many cases to increase the surface hydrophobicity, and disturb the native folding pattern of the protein (168) resulting in changes in protein conformation.

Like chloramine-T and hydrogen peroxide, ONOOH may also oxidise critical methionine residues of HCAEC actin. This process could result in the inhibition of actin polymerisation and disruption of critical specific noncovalent interactions that stabilise this highly abundant cytoskeletal protein. All of these events may lead to functional impairment of actin in HCAEC.

6.4.1.2 Vimentin

Intermediate filaments are a major component of the cytoskeleton. The most widely distributed of all intermediate filaments are composed of vimentin, which are the class III intermediate filaments (417). These proteins are present in mesenchymal cells and some
non-epithelial cell types such as fibroblasts, leukocytes and endothelial cells of blood vessels (418). Within cells, vimentin is connected to the nucleus, endoplasmic reticulum and mitochondria. This protein supports the cell membrane, maintains organelles at a fixed position and transmits membrane receptor signals to the nucleus (419). This protein is also involved in cell attachment, migration and signalling (419). Vimentin binds to naturally occurring DNA sequences with high affinity (420). Consequently it has been postulated that vimentin also participates in gene expression and replication. 3 different isoforms of vimentin have been reported (421). Vimentin contains a single cysteine residue (C328) that participates in covalent crosslinking outside of the coiled-coil dimer (between the staggered dimer molecules) of intermediate filaments (422).

The LC/MS/MS data obtained in this project indicate that 500 μM ONOOH induces oxidation of C328 of vimentin in HCAEC. This was observed in all treated samples and none of the controls. Incomplete data also attained which indicates that 500 μM ONOOH also causes tyrosine nitration at Y85 (in donor 2366) of this protein. There is little literature data on ONOOH-mediated modification of vimentin, but it has been reported that hydrogen peroxide mediated thiol oxidation in vimentin induces substantial alterations to the vimentin cytoskeleton in multiple cells (423), and this occurs in preference to damage to other cytoskeletal proteins. It has also been shown that the N-terminal of vimentin protects the mitochondria against oxidative stress (424). Modification of reactive cysteine residues can change protein structure and function as well as enzymatic activity (121). Therefore the ONOOH-mediated oxidation of C328 and tyrosine nitration of Y85 in vimentin detected here may affect the covalent crosslinking, which may disrupt the coiled-coil dimer structure and the overall structure and functions of vimentin in HCAEC.

6.4.1.3 Actin

Actin is the most abundant protein in most eukaryotic cells (425). This family of highly conserved proteins are involved in multiple cellular functions including cell motility, maintenance of cellular shape and regulation of transcription (425). 6 different isoforms of actin have been reported (426). Rohn and his colleagues presented evidence for ONOOH-mediated nitration of actin in human neutrophil proteins (427), with this causing inhibition of actin polymerisation (428) and modification of migration, phagocytosis and respiratory burst activity. Another study has reported that ONOOH
induces nitration of actin and myosin (148, 429). Nitration of actin has also been shown to affect actin assembly (430).

The vascular endothelium creates a barrier that inhibits uncontrolled passage of fluids and macromolecules across the blood vessel wall. Exposure of porcine pulmonary artery endothelial cells to ONOOH and SIN-1 has been reported to induce nitration of beta actin and beta catenin as well as oxidation of multiple proteins (431). Overall this study indicated that ONOOH directly modified endothelial cytoskeletal proteins, which resulted in barrier dysfunction and increased endothelium permeability. Another study revealed that tumor necrosis factor alpha induces ONOOH-dependent increase in permeability of pulmonary microvessel endothelial monolayers (432), with this involving nitration of beta actin. Overall these data suggest that actin is a major target of ONOOH. This is most probably due at least in part to the high abundance of actin in cells.

- **Actin alpha Cardiac Muscle 1**
  Alpha cardiac muscle 1 has been reported previously to be present in cardiac muscle (433, 434), skeletal muscle (435, 436) and microvascular endothelial cells (437). Not much is known about ONOOH modification of this protein. The LC/MS/MS data obtained in this project suggest that 500 µM ONOOH induces methionine oxidation at M192 and M229 as well as tyrosine nitration at Y220 on this protein in HCAEC, although these results are incomplete. These modifications may affect cell motility and shape in HCAEC and also inhibit actin assembly and polymerization in HCAEC.

- **Actin cytoplasmic proteins 1 and 2**
  The LC/MS/MS analysis indicate that exposure of HCAEC to 500 µM ONOOH induces sulphone formation at M305 (particularly in the donor 2366) and tyrosine nitration at Y198 (particularly in donor 2315) on actin cytoplasmic 1 in HCAEC. Not much is known about ONOOH-mediated modification of actin cytoplasmic 1 or 2. 500 µM ONOOH most likely oxidised the methionine residue at position M305 to methionine sulfoxide and this product was further oxidised to sulphone in one particular donor (2366). The other modification, tyrosine nitration, was only detected after enriching the samples for nitrated proteins in one particular donor (2315). Actin cytoplasmic 2 is an isoform of actin cytoplasmic 1. The LC/MS/MS analysis indicate that ONOOH also
induces sulphone formation at M305 in actin cytoplasmic 2 in the donor 2366. These results are incomplete, but they indicate that these modifications may be induced in HCAEC under these experimental conditions, but are low abundance modifications.

6.4.1.4 Myosin

Myosin are abundant contractile proteins in muscles. These multimeric complexes are composed of two heavy and four light chains. These proteins have structural and enzymatic roles in both muscle contraction and intracellular motility. Myosin interacts with actin to induce cell movements, cell division, axoplasmic organelle movements and intracellular material transportation. Myosin exists in multiple isoforms (438-440). Myosin 9 is a non-muscle myosin which is involved in cytokinesis, cell shape and specialised functions including secretion and capping. This isoform is known to be present in many tissues including platelets (441).

Subfragment-1 (S1) is the globular head of myosin, which contains ATP and actin binding sites. During contractile activity, S1 converts ATP chemical energy into mechanical work (442). It has been shown that sub-micromolar fluxes of ONOOH generated via SIN-1 decomposition caused extensive cysteine oxidation, partial S1 unfolding and inactivation of the actin stimulated myosin ATPase activity in skeletal muscle purified myosin subfragment -1 (443). A later study indicated that when myosin was exposed to ONOOH, this oxidant also caused tyrosine nitration and protein carbonyl formation (444). Our experimental data indicate that 500 µM ONOOH mediates oxidation of C988 in myosin-9 in the cells from donor 2366. Although this is an incomplete result, it indicates that this protein can be a target of ONOOH in HCAEC.

6.4.1.5 Nestin

Nestin is an intermediate filament protein, which is expressed mostly in actively dividing progenitor cells of developing and regenerating tissues. Nestin plays crucial roles in the regulation of cellular structure, co-localises with vimentin (445), connects the microfilaments, microtubules and intermediate filaments and coordinates changes in cell dynamics (446). Nestin expression has been linked with increased cytoplasmic trafficking in progenitor cells. Expression of nestin has been reported in vascular endothelial cells (447), but nothing is known about how ONOOH modifies nestin. The
LC/MS/MS analysis of this project has indicated that 500 μM ONOOH most likely induces methionine oxidation at M200 in nestin in HCAEC. These data are incomplete, but such modification may affect this protein's structure and its ability to connect the microfilaments, microtubules and intermediate filaments and coordinate changes in cell dynamics.

6.4.1.6 Talin-1

Talin is a cytoskeletal protein, which localises at cell-extracellular matrix associations known as focal contacts. Talin connects the integrin receptors to the actin cytoskeleton and also participates in the assembly of actin filaments as well as spreading and migration of multiple cell types (448). Talin-1 is one of the isoforms of this protein and has been reported to be expressed by human umbilical vein endothelial cells (449). There are no previous reports on ONOOH-mediated modification of talin. Our experimental data indicate that 500 μM ONOOH induces methionine oxidation at M2290 in talin-1 in cells from donor 2315. This is an incomplete data set however it indicates that ONOOH-mediated modification may change the protein conformation of this protein, which may have detrimental effects on its function.

6.4.2 Mitochondrial proteins

6.4.2.1 Mitochondrial ATP synthase beta subunit

Mitochondria regulate intracellular calcium homeostasis and generate ATP to fuel the ATPases that secrete calcium out of cells or into other organelles such as the endoplasmic reticulum (450). Mitochondrial ATP synthase generates ATP from ADP and inorganic phosphate in the presence of a proton gradient across the membrane (451). The mitochondrial ATP synthase beta subunit is the catalytic substrate-binding site, which undergoes conformational changes that results in ATP generation from ADP (452). 3 different isoforms of mitochondrial ATP synthase are known (453) and it has been reported that vascular endothelial cells express mitochondrial ATP synthase (454, 455).

Using mitochondrial membranes from bovine heart, it has been shown previously that ONOOH-induces tyrosine nitration in this protein (456). Our experimental data indicate that ONOOH probably induces methionine oxidation at M272 on this protein in HCAEC.
This modification was detected in immunoprecipitated samples suggesting that this is a low abundant modification. This is an incomplete result, but if this result is confirmed, this methionine oxidation may indicate that ONOOH could modify ATP generation in HCAEC.

### 6.4.3 Chaperone or stress proteins

#### 6.4.3.1 Endoplasmin

The endoplasmic reticulum stores calcium for intracellular signalling during multiple physiological processes. Endoplasmin (also known as GRP94) is an abundant calcium-binding glycoprotein and a molecular chaperone present in the endoplasmic reticulum (457, 458). Multiple isoforms of GRP94 have been previously detected (459). This protein assists in the folding of proteins under normal physiological conditions (460) and is a component of the unfolded protein response and participates in apoptosis. Endoplasmin has been reported to be expressed in bovine pulmonary endothelial cells, human pulmonary endothelial cells and human umbilical vein endothelial cells (461).

There are no previous reports on ONOOH-mediated modification of endoplasmin. The incomplete LC/MS/MS analysis in this project indicates that 500 µM ONOOH induces methionine oxidation at M154 in endoplasmin in HCAEC. This may change the protein confirmation and inhibit endoplasmin’s ability to act as a molecular chaperone and fold proteins under physiological and stressful conditions in HCAEC.

#### 6.4.3.2 Heat shock cognate 71 kDa protein

In prokaryotic and eukaryotic cells, heat shock cognate proteins are expressed during normal growth and development. These proteins are formed in response to increases in temperature above physiological levels and exposure to multiple toxic agents (462). The heat shock cognate 71 kDa protein is also known as heat shock 70 kDa protein 8 (HSA8). Expression of this protein has been reported in human umbilical vein endothelial cells (463), but there are no previous data on ONOOH-mediated modification of this protein. The incomplete LC/MS/MS data suggest that ONOOH induces methionine oxidation at M61 in this protein in HCAEC. This modification was detected after
immunoprecipitation consistent with this being a low abundance modification that may affect this protein's structure and function in cells exposed to ONOOH.

6.4.3.3 Translational endoplasmic reticulum ATPase

Translational endoplasmic reticulum ATPase (also known as valosin containing protein or VCP) is a ubiquitously expressed protein that belongs to the AAA+ (ATPases linked with multiple activities) protein family (464). VCP has been implicated in multiple cellular functions such as autophagy, cell cycle regulation, nuclear envelope generation, Golgi biogenesis and the ubiquitin-proteasome system (465). VCP acts as a molecular chaperone (466) and induces protein aggregate trafficking in cells. Disruption in VCP activity causes accumulation of ubiquitinated proteins and impairment of endoplasmic reticulum-associated degradation (467).

It has been shown previously that ONOOH generated via SIN-1 reduced VCP ATPase activity by modification of cysteine residue (468). In contrast, our incomplete experimental data suggest that 500 µM ONOOH induces oxidation at M332 in this protein in HCAEC. This modification may disrupt this protein's capability to act as a molecular chaperone and alter protein aggregate trafficking to inclusion body, but this modification needs to be confirmed and any changes in function examined.

6.4.4 Proteins involved in protein synthesis

6.4.4.1 Elongation factor 1 – alpha 1

During protein synthesis, elongation factor 1 – alpha 1 promotes the binding of aminoacyl-tRNA to the A-site of 80S ribosomes via a GTP-dependent pathway. During this process GTP is converted to GDP (469). Naïve T cells differentiate into T helper 1 (Th1) and Th2 cells. The Th1 cells are involved in cell-mediated immunity and are important for controlling intracellular pathogens such as viruses and bacteria. Elongation factor 1 – alpha 1 also forms a complex with poly (ADP-ribose) polymerase 1 and tyrosine protein kinases. This complex acts as a Th1 cell-specific transcription factor and attaches to the promoter of IFN-gamma to control its transcription, and hence cytokine formation. Th1 cells provide cytokine-mediated “help” to T lymphocytes (470). Elongation factor 1 – alpha 1 is expressed by endothelial cells (471).
The data outlined in the current study indicate that 500 µM ONOOH most probably induces cysteine oxidation at C111 in elongation factor 1 - alpha 1 in HCAEC. The cysteine residues were oxidised to cysteic acid. The results also indicate ONOOH-mediated methionine oxidation at M102 and tyrosine oxidation at Y85 in the cells from donor 2366. These results are incomplete, and further analysis is needed, but the data obtained suggest that this modification is induced by ONOOH under these experimental conditions.

Not much is known about ONOOH-mediated damage to elongation factor 1 - alpha 1. However it has been reported that cysteine oxidation by hydrogen peroxide in elongation factor G, which is similar to elongation factor 1, caused inactivation of this protein in *Escherichia coli* (472). Therefore ONOOH-mediated cysteine oxidation in elongation factor 1 - alpha 1 in HCAEC, may result in an inhibition of protein synthesis and disruption of cell-mediated immunity.

### 6.4.4.2 Eukaryotic initiation factor 4A-1

Initiation of protein synthesis is a complex process, which requires mRNA, 40S and 60S ribosomal subunits, tRNA, ATP, GTP and multiple initiation factors (473). Eukaryotic initiation factor 4A-1 is one of the important factors required for mRNA translation. This protein is an ATP-dependent mRNA helicase that unwinds mRNA secondary structure. Subsequently mRNA cap recognition and scanning occurs prior to the binding of the mRNA to the 40S ribosomal subunit (473). This protein has been reported to be expressed by endothelial cells (474). It has been reported that oxidative stress induces phosphorylation in eukaryotic initiation factor in vascular cells (475), with this increasing protein activity and an increase in mRNA translation.

There are no previous data on ONOOH-mediated modification of this protein. Our incomplete experimental data suggest that 500 µM most likely induces methionine oxidation at M398 and also possibly at M149 (seen with donor 2366) in this protein in HCAEC. This oxidation may change the confirmation of this protein and disrupt phosphorylation of this protein, which may lead to inhibition of mRNA translation and protein synthesis in HCAEC.
6.4.4.3 Isoform 2 of clathrin heavy chain

Transfer of membrane-bound and secreted proteins between the plasma membrane and intracellular membrane necessitates budding and fusion of membrane vesicles (476). Clathrin was one of the first proteins identified that participates in this process (477). This protein is the main constituent of the cytoplasmic face of intracellular organelles, known as the coated vesicles and coated pits that initiates uptake of cell surface receptors. These specialised organelles participate in the intracellular trafficking of receptors and endocytosis of multiple macromolecules. Clathrin molecules are composed of three non-covalently bonded heavy- and light chains (478, 479). In humans, two isoforms of each of heavy- and light chain have been reported (480). Vascular endothelial cells have been reported to express clathrin (481-483), but there are no previous data on ONOOH-mediated modification of this protein.

The data obtained indicate that ONOOH probably induces methionine oxidation at M81 in isoform 2 of clathrin heavy chain of HCAEC. This may induce conformational changes in clathrin and affect its ability to bind to its substrates, and therefore disrupt trafficking of macromolecules between the plasma membrane and intracellular membranes of HCAEC.

6.4.5 Summary

Under the conditions employed in these studies, evidence has been obtained that treatment of HCAEC with 500 µM ONOOH induces cysteine oxidation, methionine oxidation, sulphone formation and tyrosine nitration on multiple HCAEC proteins. Methionine oxidation was the most prominent ONOOH-mediated modification observed on these HCAEC proteins. Only 2 confirmed and reproducible results were obtained, which includes cysteine oxidation at C328 in vimentin and methionine oxidation at M240 in alpha actinin 4. Both of these are cytoskeletal proteins. Multiple non-confirmed ONOOH-mediated modifications were also detected that need further investigation.

The results outlined in this Chapter indicate that ONOOH-mediated damage to HCAEC cellular proteins was not random but a selective process, where certain types of proteins appear to be affected more than the others. Thus this oxidant appears to selectively target cytoskeletal proteins, mitochondrial proteins, chaperone proteins and proteins involved in
protein synthesis. Cytoskeletal proteins (such as actin, alpha actinin 4, myosin, nestin, talin-1 and vimentin) appear to be major targets of this oxidant possibly because these proteins are high in abundance and are long-lived. Therefore these are highly likely to accumulate ONOOH-mediated modifications. The mitochondrial protein, mitochondrial ATP synthase beta subunit is involved in ATP generation (452). 500 μM ONOOH-mediated modification of this protein may disrupt ATP generation in HCAEC.

In addition, the chaperone or stress proteins (such as endoplasmin, heat shock cognate 71 kDa protein and translational endoplasmic reticulum ATPase) are upregulated to high concentrations when cells are stressed. These chaperone proteins are designed to restore or repair damaged proteins. Exposure of HCAEC to 500 μM ONOOH induces nitrosative stress, which may have induced the upregulation of these chaperone proteins. The proteins involved in protein synthesis also appear to be a target of ONOOH. These proteins include elongation factor 1 – alpha 1, eukaryotic initiation factor 4A-1 and isoform 2 of clathrin heavy chain. This may indicate a response to ONOOH-mediated damage that aims to generate new proteins.

In this project, 3 different HCAEC donors were used (Table 6.1) and significant variations were detected between these donors, with cells from donor 2366 appearing to be more susceptible towards ONOOH-mediated damage than the others. It is possible that this arises from the fact that this donor was the oldest of the three donors used (and hence may have lower or impaired antioxidant defences) or this may reflect other factors (possibly genetic). In particular with this donor, ONOOH appeared to modify actin cytoplasmic 1 and 2, alpha actinin 4, elongation factor-1 alpha 1, eukaryotic initiation factor 4A-1, myosin-9 and vimetin. With donor 2315, additional evidence was obtained for ONOOH-mediated modification of actin cytoplasmic 1, alpha actinin 4 and talin-1. Most studies employ isolated cells that originate from a single cell source (e.g. one cell line with no genetic diversity). Therefore the current study is possibly stronger than these previous studies as it has also examined cell-cell variation and has shown that this is a significant variation. This might be expected, as all people are different.
Chapter 7: Discussion
7.1 Overview

ONOOH is a potent oxidant capable of modifying multiple biological targets. Studies have reported that low concentrations of ONOOH are cytoprotective (72-74) whereas high concentrations are cytotoxic (69, 375). ONOOH reacts with CO$_2$ (100), low molecular mass antioxidants (103), DNA (189), proteins (63), lipids (183) and carbohydrates (180, 181). Due to their high reactivity and abundance (323), proteins are a major target of ONOOH. ONOOH oxidises cysteine, tyrosine, tryptophan, methionine, histidine and phenylalanine residues (63), but also nitrates tyrosine and tryptophan residues (140, 163). It is well established that major protein modifications induced by ONOOH include thiol oxidation and 3NT formation (117). Previous studies have also shown that in certain situations, ONOOH induces protein aggregation and fragmentation (118, 119, 264). It is unknown whether ONOOH, ONOOCO$_2^-$ or radicals derived from these oxidants are mainly responsible for the damage detected in vivo.

7.2 Effects of ONOOH on isolated peptides and proteins

Previous studies have reported that ONOOH modifies BSA by targeting multiple amino acids including cysteine, tyrosine and tryptophan (106, 118, 119, 338). However the order and efficacy of ONOOH-mediated modification was unknown. Therefore the studies reported in Chapter 3 aimed to investigate the best reagent to detect thiol loss and to determine the order as well as the efficacy of ONOOH and ONOOCO$_2^-$ mediated damage to model isolated peptides (GSH) and the protein albumin (BSA), which are potential targets in biological systems. Initial experiments investigated the best reagent to detect thiol loss using HCAEC lysates. HCAEC were treated with increasing concentrations of ONOOH for 5 min at 37 °C and pH 7.4. Under these experimental conditions, ThioGlo$^\text{TM}$ 1 reagent was found to be more sensitive and superior to ThioGlo$^\text{TM}$ 3, 5 and DTNB.

Subsequent studies examined ONOOH and ONOOCO$_2^-$ mediated modifications such as thiol oxidation, loss of other amino acids, 3NT formation and other protein modifications to GSH and BSA. Using ThioGlo$^\text{TM}$ 1 reagent, ONOOH-mediated thiol oxidation in GSH and BSA was investigated. When 7.5 µM GSH was exposed to increasing concentrations of ONOOH and ONOOCO$_2^-$, a dose-dependent oxidation of thiols occurred. Analogously, exposure of 7.5 µM BSA to increasing concentrations of
ONOOh and \( \text{ONOOCO}_2^- \) also induced a dose-dependent loss of thiol residues. Interestingly \( \text{ONOOCO}_2^- \) induced thiol oxidation in GSH and BSA to a lesser extent than ONOOH.

ONOOh-mediated modification to other amino acids (such as histidine, methionine, phenylalanine, tryptophan and tyrosine) was examined using amino acid analysis by UPLC. Under these experimental conditions, ONOOH and \( \text{ONOOCO}_2^- \) induced dose-dependent loss of tyrosine, tryptophan, histidine and methionine residues in BSA. ONOOH induced methionine sulphotide formation to a greater extent than \( \text{ONOOCO}_2^- \). Western blotting data showed that ONOOH induced significant 3NT formation on BSA. \( \text{ONOOCO}_2^- \) also induced 3NT formation but to a lesser extent than ONOOH. ONOOH induced protein fragmentation of BSA. The main conclusion from these results is that bolus ONOOH modifies cysteine residues prior to methionine, tryptophan, tyrosine, histidine and phenylalanine.

Previous studies have detected 3NT formation when BSA was exposed to increasing concentrations of SIN-1 (484, 485). The situation with slowly generated ONOOH may however be different, and hence it would be worth investigating in future studies the effect of prolonged exposure of BSA to ONOOH (via SIN-1 decomposition) to determine whether this induces amino acid modifications to a similar extent and order compared to those detected after a short term (5 min) incubation period. The formation of other products such as GSSG (GSH oxidation product) and 6-nitrotryptophan (tryptophan nitration product) could also be examined in the future.

### 7.3 Effects of ONOOH on HCAEC

The studies reported in Chapter 4 and 5 investigated ONOOH- and \( \text{ONOOCO}_2^- \)-mediated modifications (such as loss of cell viability, thiol oxidation, 3NT formation and other protein modifications e.g. aggregation and/or fragmentation) to HCAEC proteins after a short and prolonged exposure to bolus ONOOH and ONOOH generated slowly from SIN-1. In these experiments HCAEC lysates, intact cells and subcellular fractions were employed. Lysates were employed to investigate whether the cell membrane had any protective effect against ONOOH and \( \text{ONOOCO}_2^- \) mediated damage. Intact cells and lysates were used to mimic extra- and intra-cellular oxidant formation in vivo.
Subcellular fractions (cytosolic and membrane fractions) were examined to determine whether cytosolic or membrane proteins are mainly targeted by these oxidants.

7.3.1 Cell viability

ONOOh is known to be cytotoxic to cells particularly at high concentrations (375). Previous studies have reported that high concentrations of ONOOH are cytotoxic to multiple cell types such as PC12 (211), fibroblasts (228), neutrophils (234), macrophages (242), cardiomyocytes (243), vascular endothelial and smooth muscle cells (195, 240). Which concentrations of ONOOH were cytotoxic to HCAEC after short and prolonged incubation were unknown.

In this project, ONOOH-mediated loss of HCAEC cell viability was detected using the MTT assay. Treatment of HCAEC with 500 µM or lower ONOOH and ONOOCO$_2^-$ concentrations for 5 min induced no significant loss of cell viability. When these cells were exposed to 1250 µM ONOOH and ONOOCO$_2^-$ for 5 min about 14 and 9 % loss of cell viability was detected respectively (Table 7.1). Exposure of HCAEC to 1000 µM SIN-1 for 4 h caused no significant loss of cell viability. In contrast when cells were exposed to 1000 µM SIN-1 for 4 h in the presence of 25 mM NaHCO$_3$, about 75 % loss of cell viability was detected.

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<td>- NaHCO$_3$</td>
<td>+ NaHCO$_3$</td>
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<tr>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>- NaHCO$_3$</td>
<td>+ NaHCO$_3$</td>
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<tr>
<td>0</td>
<td>75</td>
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Table 7.1 Percentage loss of HCAEC cell viability induced by 1250 µM ONOOH (after 5 min incubation) and 1000 µM SIN-1 (after 4 h incubation) both in the absence and presence of 25 mM NaHCO$_3$.

The main conclusion from these data is that ONOOCO$_2^-$ is more cytotoxic to HCAEC after prolonged exposure compared to ONOOH. These data agree with a previous study, which reported that bicarbonate ions play critical roles in the generation of cytotoxic substances that persist in the cell culture medium after almost complete SIN-1
decomposition (376). It is unclear whether ONOOCO$_2^-$ or the radicals generated from this oxidant are mainly responsible for this cytotoxic effect.

7.3.2 Thiol oxidation

Thiols are known to be a major target of ONOOH (61). Thiol residues can be present at the active site of various enzymes (61). Modification of thiol residues can change protein structure and function as well as enzymatic activity (121). Previous studies have shown that ONOOH-mediated thiol oxidation caused activation of matrix metalloproteinase-2 (127) and vascular endothelial growth factor (368). In contrast, studies have also reported that ONOOH-mediated thiol oxidation can inactivate protein tyrosine phosphatases (370), alcohol dehydrogenase (371), creatine kinase (124), succinate dehydrogenase (372), glyceraldehyde-3-phosphate dehydrogenase (123), NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochrome c reductase (complex III) and ATP synthase (complex V) of the mitochondrial respiratory chains (125, 126). Similarly, it has been reported that SIN-1 can induce thiol oxidation in neutral sphingomyelinase 1 (385), human teratocarcinoma cells derived from neuronal cells (386) and in isolated peptides such as glutathione, cysteine, cysteamine, homocysteine, cysteine methyl ester, cysteine ethyl ester, N-acetylcysteine, penicillamine, ammonium thioglycolate, captopril, N-acetylpenicillamine, mesna (sodium 2-mercaptoethanesulfonate) and tiopronin (N-(2-mercaptopropionyl)glycine) (387).

To what extent ONOOH and ONOOCO$_2^-$ modify thiol residues of HCAEC in the absence and presence of an intact cell membrane was unknown. It was also unknown whether the cytosolic and/or membrane bound thiol residues were mainly targeted by these oxidants after short and prolonged exposure. In this project ONOOH-mediated thiol oxidation was detected via ThioGlo™ 1 reagent, with exposure of intact HCAEC to 1000 µM ONOOH and ONOOCO$_2^-$ for 5 min inducing significant (20 %) total thiol oxidation.

When the intact cells were treated with 1000 µM ONOOH for 5 min and the amount of thiol loss determined in the different cell fractions, 18 % of the cytosolic and 36 % of the membrane protein thiols were oxidised (Table 7.2). In the presence of 25 mM NaHCO$_3$, 1000 µM ONOOH oxidised 22 % of the cytosolic and 31 % of membrane thiols. Thus more membrane protein thiols were oxidised by bolus ONOOH than cytosolic thiols.
However when cells were exposed to bolus ONOOH in the presence of NaHCO₃, this made little difference to the percentage of thiols oxidised in the cytosolic and membrane fractions. These data suggest that ONOOH and ONOOCO₂⁻ react with thiol residues whilst entering the cells and during this process a higher percentage of membrane bound thiol residues are oxidised compared to the cytosolic thiols.

In contrast to the above data, when intact cells were exposed to 1000 µM SIN-1 for 4 h, 34 % of cytosolic and 8 % of membrane thiol residues were oxidised. In the presence of 25 mM NaHCO₃, 1000 µM SIN-1 oxidised 32 % of cytosolic and 30 % of membrane thiol in the intact cells. A small percentage of membrane thiol residues were oxidised by SIN-1 in the absence of NaHCO₃, while a significantly higher percentage of these thiol residues was oxidised by SIN-1 in the presence of NaHCO₃; thus in the presence of 25 mM NaHCO₃, SIN-1 oxidised about 30 % of membrane bound thiol residues. Therefore on prolonged exposure, ONOOCO₂⁻ appears to be more potent in oxidising membrane bound thiols than ONOOH. Compared to the membrane thiols, a greater percentage of cytosolic thiols were oxidised by SIN-1 both in the presence and absence of 25 mM NaHCO₃. These data suggest that SIN-1 most likely entered the cells and then decomposed, thereby oxidising cytosolic thiols. As a result, these oxidants oxidised a high percentage of cytosolic thiol residues. In addition, the presence of NaHCO₃ had little effect on SIN-1 mediated thiol oxidation of cytosolic thiol residues.

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<tr>
<th>Fractions</th>
<th>ONOOH</th>
<th>SIN-1</th>
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<tr>
<td></td>
<td>- NaHCO₃</td>
<td>- NaHCO₃</td>
</tr>
<tr>
<td></td>
<td>+ NaHCO₃</td>
<td>+ NaHCO₃</td>
</tr>
<tr>
<td>Cytosolic</td>
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</tr>
<tr>
<td>Membrane</td>
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<td>32</td>
</tr>
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<td>31</td>
</tr>
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<td></td>
<td>8</td>
<td>30</td>
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</tbody>
</table>

Table 7.2 Percentage of thiol loss in intact HCAEC cells induced by 1000 µM ONOOH (after 5 min incubation) and 1000 µM SIN-1 (after 4 h incubation) both in the absence and presence of 25 mM NaHCO₃.

Exposure of lysates to 5 µM ONOOH oxidised 27 % of thiol residues, and more thiols (38 %) were lost in the presence of 25 mM NaHCO₃. When the separated fractions were exposed to 50 µM ONOOH for 5 min, 81 % of cytosolic and 66 % of membrane protein

252
thiols were oxidised (Table 7.3). 50 µM ONOOCO$_2^\text{-}$ oxidised 90 % of cytosolic and 54 % of membrane fraction after 5 min. Thus HCO$_3^-$ appears to make a little difference to ONOOH-mediated thiol oxidation. After a short incubation period and in the absence of an intact cell membrane, ONOOH and ONOOCO$_2^-$ oxidise cytosolic thiol residues to a greater extent than membrane bound thiol residues. Thus ONOOH seems to mainly target membrane bound thiols whilst ONOOCO$_2^-$ appears to mainly target cytosolic thiols. This may be due to the negative charge on the latter species.

When the separated cytosolic fractions were exposed to 50 µM SIN-1 for 1 h, 14 and 95 % of cytosolic thiols were oxidised in the absence and presence of 25 mM NaHCO$_3$ respectively. In contrast, exposure of the separated membrane fractions to 50 µM SIN-1 for 1 h oxidised 26 and 68 % of thiol residues in the absence and presence of 25 mM NaHCO$_3$ respectively. Therefore when the oxidant is formed in the presence of HCO$_3^-$ and the resulting species have free access to target species, HCO$_3^-$ has a greater effect and this effect is more pronounced with SIN-1. After 1 h incubation period, 50 µM SIN-1 oxidised cytosolic and membrane bound thiols to a greater extent in the presence of HCO$_3^-$. These data indicate that prolonged exposure to ONOOCO$_2^-$ has more detrimental effects on cytosolic than membrane thiols. SIN-1 induced dose- and time-dependent thiol oxidation in both fractions over the 4 h incubation period, and more dramatic thiol oxidation occurred in the presence of HCO$_3^-$.  

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<th>Fractions</th>
<th>ONOOH</th>
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<tr>
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<td>NaHCO$_3$</td>
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<tr>
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<td>90</td>
</tr>
<tr>
<td>Membrane</td>
<td>66</td>
<td>54</td>
</tr>
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</table>

Table 7.3 Percentage of thiol loss in separated fractions of HCAEC induced by 50 µM ONOOH (after 5 min incubation) and 50 µM SIN-1 (after 1 h incubation) both in the absence and presence of 25 mM NaHCO$_3$.

These data are in accordance with the data presented in Table 7.1, which indicated that SIN-1 induced greater loss of cell viability in the presence of 25 mM NaHCO$_3$. In this case, it is unknown whether thiol oxidation is a cause of SIN-1 mediated cytotoxicity or
merely a concurrent event. It is not possible to determine from the data obtained whether free thiols or protein bound thiols were mainly targeted by these oxidants after short or prolonged exposure. We could also not identify which proteins (cytosolic or membrane bound) thiol residues were modified. It is also unknown whether ONOOH-mediated thiol oxidations changed the structure, function or enzymatic properties (i.e. activation or inactivation) of these HCAEC proteins. These aspects could all be examined in further studies.

The main conclusion from these studies is that thiol residues in HCAEC are a major target of ONOOH and ONOOCO$_2^-$ . The cell membrane plays a crucial role in protecting HCAEC against damage induced by these oxidants. When excessive ONOOH and ONOOCO$_2^-$ formation occurs extracellularly (as in the case of intact cells), these oxidants oxidise thiol residues to a similar extent. After short and prolonged exposure to intact cells, these oxidants predominately target the membrane and cytosolic thiols respectively. In contrast, if excessive ONOOH and ONOOCO$_2^-$ formation occurs intracellularly (as mimicked to some extent by the lysate experiments), more extensive thiol oxidation occurs compared to a situation where these oxidants are generated in excess extracellularly. In the absence of an intact cell membrane, ONOOH and ONOOCO$_2^-$ induced thiol oxidation of the cytosolic and membrane fractions to a similar extent. However after prolonged exposure, ONOOCO$_2^-$ induced thiol oxidation to a greater extent than ONOOH. These oxidants oxidised the cytosolic protein thiols to a greater extent than membrane thiols during both short and prolonged exposure.

7.3.3 Other protein modifications

Previous studies have demonstrated that ONOOH can also induce other protein modifications such as aggregation, fragmentation and crosslinking (118, 119, 264). How ONOOH and ONOOCO$_2^-$ induce these types of modifications to HCAEC proteins after a short or prolonged incubation was unknown. It was also unknown whether the cell membrane of HCAEC plays a crucial role in preventing these types of ONOOH and ONOOCO$_2^-$ mediated damage.

In this project ONOOH and ONOOCO$_2^-$-mediated gross protein modifications to HCAEC was examined using silver staining. After a short incubation period (5 min), visual observation of the gels indicated that increasing concentrations of ONOOH and
ONOOCO$_2^-$ did not induce detectable protein aggregation and/or fragmentation. In this case, the cytosolic and membrane proteins did not appear to undergo significant fragmentation or aggregation by these oxidants. Similar observations were made when HCAEC were exposed to these oxidants for a prolonged period of time (4 h).

However when the HCAEC cell membrane was disrupted and all the intracellular components were directly exposed to ONOOH and ONOOCO$_2^-$ protein smears were detected on the protein gels which is indicative of aggregation and/or fragmentation. The protein smearing was more pronounced when lysates were exposed to ONOOCO$_2^-$.

When the HCAEC cytosolic and membrane fractions were separately exposed to ONOOH and ONOOCO$_2^-$ for a short period of time, a few protein bands appeared to become less intense (lighter staining) as the oxidant concentration was increased. After prolonged exposure to these oxidants, a number of membrane proteins seem to be modified, as their protein bands appear to become less intense as the oxidant concentration was increased. No detectable loss of cytosolic proteins was observed.

Differences in the susceptibility of the 3 different HCAEC cell donors to oxidation gave rise to variable results in the case of both the intact cells and lysates. Consequently, quantification of the pixel density of the gel bands using the program image J failed to show a significant loss of cytosolic and membrane proteins even though visual observations indicated that these protein bands became less intense as the oxidant concentration was increased. The main conclusion from these experiments is that both ONOOH and ONOOCO$_2^-$ can induce limited gross protein modifications such as aggregation and/or fragmentation in the absence of an intact cell membrane after both short and prolonged oxidant exposure.

### 7.3.4 3NT formation

3NT is commonly used as a “gold standard” to detect ONOOH-mediated damage (143, 148). It was however unknown whether the cytosolic and/or membrane proteins of HCAEC are mainly nitrated by ONOOH and ONOOCO$_2^-$ after short and prolonged exposure. It was also unknown whether the presence of an intact cell membrane made any difference to ONOOH- and ONOOCO$_2^-$-mediated damage. In the studies reported in Chapter 4 and 5, ONOOH-mediated 3NT formation on HCAEC proteins was detected via Western blotting using an anti-3NT antibody. The long exposure time needed to
detect 3NT in intact cells and lysates samples resulted in high levels of background staining on the gels. Due to this high background in all control lanes, the Image J analysis failed to show a significant increase in 3NT formation in all the experimental data mentioned below.

Direct examination of the Western blotting membranes clearly indicated 3NT formation on multiple HCAEC proteins when intact HCAEC were treated with 100 µM or higher concentrations of ONOOH and ONOOCO$_2^-$ after a 5 min incubation period. Multiple cytosolic and membrane proteins were selectively nitrated when intact cells were exposed to increasing concentrations of ONOOH and ONOOCO$_2^-$. Visual observations suggested that 50 µM or greater, and 250 µM or greater ONOOH concentrations were needed to induce significant 3NT formation on cytosolic and membrane proteins respectively (Table 7.4). Similar data were obtained in the presence of 25 mM NaHCO$_3$. 3NT formation increased as ONOOH and ONOOCO$_2^-$ concentrations increased. Overall lower ONOOH concentrations appear to be needed to induce significant 3NT formation on cytosolic protein than on the membrane proteins.

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<td>50</td>
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<tr>
<td>Membrane</td>
<td>250</td>
<td>250</td>
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Table 7.4 Minimum concentrations of ONOOH and SIN-1 (µM) required to induce 3NT formation on intact cell cytosolic and membrane proteins. 3NT formation was detected using visual observation of the Western blotting membranes.

When intact cells were exposed to increasing levels of ONOOH and ONOOCO$_2^-$ (via SIN-1 decomposition) for 4 h, a basal level of nitrated membrane proteins was detected in the control samples (untreated intact HCAEC). This was not detected with the cytosolic proteins. When cells were exposed to increasing concentrations of SIN-1 for 4 h, multiple cytosolic and membrane proteins were simultaneously nitrated. Direct examination of the Western blots indicate that greater than 100 µM, and greater than 500 µM SIN-1 were needed to induce 3NT formation on cytosolic and membrane proteins of
intact cells respectively (Table 7.4). Similar data were obtained in the presence of 25 mM NaHCO$_3$. As the SIN-1 concentration was increased, the band intensity of these nitrated cytosolic and membrane proteins also increased. After prolonged exposure, ONOOCO$_2^-$ induced nitration to a greater extent than ONOOH. In intact HCAEC the membrane proteins appeared to be nitrated to a greater extent than cytosolic proteins.

Analogously in the absence of an intact cell membrane, low concentrations of ONOOH and ONOOCO$_2^-$ induced significant 3NT formation on multiple HCAEC proteins after a 5 min incubation period. Visual observations indicated that 100 µM or higher concentrations of ONOOH and ONOOCO$_2^-$ induced 3NT formation on cytosolic and membrane proteins (Table 7.5). More 3NT formation was detected on these proteins as the ONOOH and ONOOCO$_2^-$ concentrations was increased. Multiple cytosolic and membrane proteins appeared to be nitrated. After a short incubation period, ONOOH appears to induce nitration to a greater extent than ONOOCO$_2^-$. After prolonged exposure of the separated fractions to high doses (500 µM) of ONOOH and ONOOCO$_2^-$, multiple cytosolic and membrane proteins were simultaneously nitrated.

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<td>Membrane</td>
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Table 7.5 Minimum concentrations of ONOOH and SIN-1 (µM) required to induce 3NT formation on separated cytosolic and membrane proteins. 3NT formation was detected using visual observations.

7.3.5 Overall

The main conclusion from these data is that if excessive ONOOH and ONOOCO$_2^-$ formation occurs extra- and intracellularly, multiple cytosolic and membrane proteins will be simultaneously nitrated by these oxidants during both short and prolonged exposure. Nitration seems to be occurring in a selective manner and only a limited number of proteins are nitrated. Nitration increased as the oxidant concentrations
increased. SIN-1 was less effective than ONOOH in inducing 3NT formation. This is most likely due to the higher flux of species (such as Tyr$^\cdot$ and NO$_2^\cdot$) generated by bolus ONOOH, which react indirectly with tyrosine residues to form 3NT (107, 140, 141). Slow generation of ONOOH via SIN-1 could be expected to limit the occurrence of these radical-radical reactions. Therefore in the latter case, it is more likely that other reactions such as thiol oxidation may be occurring.

7.3.6 Limitations

All the experiments in this project were performed using 3 different HCAEC donors. It would have been more beneficial to have more HCAEC donors. Consequently the resulting data would have generated more firm conclusions. Due to time limitation, we could not try other methods or commercial kits to enrich the protein samples using the 3NT antibody prior to running LC/MS/MS.

7.3.7 Future work

Multiple investigations can be performed in the future, which can expand the results outlined in these Chapters. Future work could include investigation into the formation of other ONOOH- and ONOOCO$_2^-$-mediated oxidation and nitration products such as methionine sulfoxide, 6-nitrotryptophan and other cysteine products such as cysteic acid and reversible products, such as sulphenic acids and disulphides. In Chapters 4 and 5, ONOOH and ONOOCO$_2^-$ were shown to induce cell death after short and prolonged exposure. It is unknown whether these oxidants induced apoptosis or necrosis under these experimental conditions. It is also unknown which signalling pathways are affected by these oxidants. Polymerase chain reactions (PCR) could be performed to investigate which genes and signalling pathways are up- or down-regulated after HCAEC are exposed to ONOOH and ONOOCO$_2^-$ for a short or prolonged period of time.

It would also be interesting to examine how HCAEC respond to ONOOH and ONOOCO$_2^-$ mediated damage. HCAEC could be treated with these oxidants for short and prolonged period of time, excess oxidants could be removed by washing the cells and subsequently the treated cells could be cultured for another 24 and/or 48 h. Then PCR experiments could be performed to investigate which genes are up- or down-regulated. The major pathways involved in the repair of damage could therefore be
identified. These experiments could also be used to determine the threshold ONOOH and ONOOCO\textsubscript{2}\textsuperscript{-} concentrations beyond which these oxidants become cytotoxic to HCAEC. In each case, it would be interesting to examine whether the treated HCAEC undergo apoptosis or necrosis, which can be done using Annexin-V and propidium iodide staining with flow cytometry for example.

The levels and activity of antioxidant and repair enzymes could be examined in the treated HCAEC. The redox status of GSH/GSSG is widely used as a marker for oxidative stress (486). ONOOH and ONOOCO\textsubscript{2}\textsuperscript{-} mediated changes in the GSH/GSSG ratio could therefore be examined. ONOOH- and ONOOCO\textsubscript{2}\textsuperscript{-}-mediated DNA and lipid oxidation could also be investigated. In addition to this, removal of damaged proteins and release of damaged materials such as free 3NT might be investigated.

It would be interesting to perform live cell imaging on HCAEC that have been exposed to ONOOH and ONOOCO\textsubscript{2}\textsuperscript{-} for a short and prolonged period of time using 3NT fluorescent probes (487-489). This technique would indicate where the proteins on which 3NT is located are in these cells. These potential future investigations could generate more information about how ONOOH and ONOOCO\textsubscript{2}\textsuperscript{-} affects HCAEC under these experimental conditions.

### 7.4 Characterisation of cellular targets of ONOOH in HCAEC

The studies described in Chapter 4 and 5 led to the investigation of cellular targets of ONOOH in HCAEC. HCAEC were exposed to 500 µM ONOOH for 5 min at 37 °C and pH 7.4, subsequently LC/MS/MS analysis was performed. These experiments identified a number of distinct classes of proteins that were modified by this treatment (Table 7.6).

Cytoskeletal proteins form a network of fibres, which maintain the cellular shape and structure (409). Both the Western blotting and LC/MS/MS analysis carried out in this project indicate that a bolus dose of 500 µM ONOOH modifies multiple cytoskeletal proteins including actin, alpha actinin 4, β-tubulin, myosin, nestin, talin-1 and vimentin (Table 7.6A). Within the actin family, actin alpha cardiac muscle 1, actin cytoplasmic 1 and 2 proteins and β-actin appear to be modified by ONOOH, suggesting that cytoskeletal proteins are a major target. The Western blotting data indicated that a dose-
dependent loss of β-actin and β-tubulin proteins was detected with increasing ONOOH concentrations, which complements the LC/MS/MS data. The cytoskeletal proteins may be a major target of ONOOH due to their high abundance, and the slow turnover of these proteins (490). Consequently the chances of detecting damage to cytoskeletal proteins may be higher than to other less abundant and rapidly turned over proteins. The role of turnover could be examined in further studies to investigate when modifications disappear by LC/MS/MS and when antibody recognition returns using Western blotting.

Chaperone proteins are a group of proteins that assist in protein folding under normal physiological and stressful conditions (491). The LC/MS/MS analysis indicates that multiple HCAEC chaperone or stress proteins are also modified by bolus 500 µM doses of ONOOH including endoplasmin, heat shock cognate 71 kDa protein and translational endoplasmic reticulum ATPase (Table 7.6B). Due to ONOOH-mediated nitrosative stress these proteins may be upregulated to high concentrations. ONOOH-mediated damage to these chaperone proteins may disrupt the structure and/or critical function of these species in HCAEC, though further studies are needed to determine whether there is a causal link between the observed modifications and any changes in function.

In cells, protein synthesis is a complex process that involves multiple proteins. The LC/MS/MS analysis indicates that 500 µM ONOOH modifies proteins that are involved in protein synthesis (e.g. elongation factor 1 – alpha 1, eukaryotic initiation factor 4A-1 and isoform 2 of clathrin heavy chain) (Table 7.6E). Due to ONOOH-mediated nitrosative stress, HCAEC may increase protein synthesis to replace damaged proteins though whether this occurs in the time frame of these experiments is unclear and could be examined in future studies.

Mitochondrial ATP synthase beta subunit is involved in ATP generation (452). The LC/MS/MS analyses indicate that mitochondrial ATP synthase beta subunit is a target of ONOOH (Table 7.6D). Consequently this oxidant may be able to disrupt ATP formation in HCAEC. A previous study has reported that ONOOH inhibited mitochondrial protein synthesis, reduced cellular ATP levels and decreased mitochondrial redox function in human umbilical vein endothelial cells (492). All of these events lead to mitochondrial dysfunction. Analogously ONOOH may be inducing mitochondrial dysfunction in HCAEC by reducing ATP levels and this event may contribute to cell death via either apoptosis or necrosis.
ATP-binding cassette sub-family G member 1 (ABCG1) is membrane protein that is involved in efflux transport of excess cholesterol (493). ABC-G1 has been shown to expressed by macrophages (494) and endothelial cells (495). Vascular endothelial cells are believed to be highly resistant to cholesterol accumulation, and cholesterol efflux pathways are critical in maintaining cholesterol homeostasis in these cells (495). It has been suggested that endothelial cells have a remarkable ability to modulate and maintain their sterol levels in the presence of high sterol levels in human plasma (496). The Western blotting data obtained in this project indicates that in HCAEC, ABC-G1 is modified by ONOOH and ONOOCO$_2^-$ (Table 7.6C). The recognition of this protein by the antibody was lost as the oxidant concentrations were increased consistent with a dose-dependent damage. Damage to this protein may inhibit transport of excess cholesterol from HCAEC to the liver for clearance, and may contribute to cholesterol accumulation in the artery wall when it is exposed to inflammatory oxidants as in the case of atherosclerosis.

Vascular endothelial growth factor (VEGF) is a key signalling protein that induces endothelial cell proliferation and differentiation, enhances cell migration and disrupts apoptosis (497, 498). It has been reported that ONOOH is critically involved in the signal transduction of VEGF via a nitration-independent and oxidation-mediated tyrosine phosphorylation in endothelial cells (368). A previous study has shown that low concentrations of ONOOH increase VEGF mRNA expression and VEGF protein levels in microvascular endothelial cells in a dose- and time-dependent manner (499). The Western blotting results obtained in this project suggest that in HCAEC, high concentrations of bolus ONOOH modify VEGF (Table 7.6F). ONOOH may disrupt the critical roles of VEGF and inhibit HCAEC proliferation, differentiation and migration, and thereby contribute to endothelial cell dysfunction in the context of the inflamed artery wall.
### A) Cytoskeletal proteins

<table>
<thead>
<tr>
<th>Proteins modified</th>
<th>Type of modification</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin alpha cardiac muscle 1</td>
<td>Methionine oxidation</td>
<td>M192, M229</td>
</tr>
<tr>
<td>Actin alpha cardiac muscle 1</td>
<td>Tyrosine nitration</td>
<td>Y220</td>
</tr>
<tr>
<td>Actin cytoplasmic 1</td>
<td>Sulphone formation</td>
<td>M305</td>
</tr>
<tr>
<td>Actin cytoplasmic 1</td>
<td>Tyrosine nitration</td>
<td>Y198</td>
</tr>
<tr>
<td>Actin cytoplasmic 2</td>
<td>Sulphone formation</td>
<td>M305</td>
</tr>
<tr>
<td>Alpha actinin 4</td>
<td>Methionine oxidation</td>
<td>M240, M295,M379</td>
</tr>
<tr>
<td>β-actin</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>β-tubulin</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>Myosin</td>
<td>Cysteine oxidation</td>
<td>C988</td>
</tr>
<tr>
<td>Nestin</td>
<td>Methionine oxidation</td>
<td>M200</td>
</tr>
<tr>
<td>Talin-1</td>
<td>Methionine oxidation</td>
<td>M2290</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Cysteine oxidation</td>
<td>C328</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Tyrosine nitration</td>
<td>Y85</td>
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</table>

### B) Chaperone/stress proteins

<table>
<thead>
<tr>
<th>Proteins modified</th>
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<th>Position</th>
</tr>
</thead>
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<tr>
<td>Endoplasmin</td>
<td>Methionine oxidation</td>
<td>M154</td>
</tr>
<tr>
<td>Heat shock cognate 71 kDa protein</td>
<td>Methionine oxidation</td>
<td>M161</td>
</tr>
<tr>
<td>Translational endoplasmic reticulum ATPase</td>
<td>Methionine oxidation</td>
<td>M332</td>
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### C) Membrane proteins

<table>
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<tr>
<th>Proteins modified</th>
<th>Type of modification</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC-G1</td>
<td>Unknown</td>
<td>Unknown</td>
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</tbody>
</table>

### D) Mitochondrial proteins

<table>
<thead>
<tr>
<th>Proteins modified</th>
<th>Type of modification</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria ATP synthase β-subunit</td>
<td>Methionine oxidation</td>
<td>M272</td>
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</table>

### E) Proteins involved in protein synthesis

<table>
<thead>
<tr>
<th>Proteins modified</th>
<th>Type of modification</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elongation factor 1 - α1</td>
<td>Cysteine oxidation</td>
<td>C111</td>
</tr>
<tr>
<td>Elongation factor 1 - α1</td>
<td>Methionine oxidation</td>
<td>M102</td>
</tr>
<tr>
<td>Elongation factor 1 - α1</td>
<td>Tyrosine nitration</td>
<td>Y85</td>
</tr>
<tr>
<td>Eukaryotic initiation factor 4A-1</td>
<td>Methionine oxidation</td>
<td>M149, M398</td>
</tr>
<tr>
<td>Isoform 2 of clathrin heavy chain</td>
<td>Methionine oxidation</td>
<td>M81</td>
</tr>
</tbody>
</table>

F) Signalling proteins

<table>
<thead>
<tr>
<th>Proteins modified</th>
<th>Type of modification</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Table 7.6 HCAEC proteins that are modified by 500 µM ONOOH after a 5 min incubation period. These results were obtained through Western blotting and LC/MS/MS analysis. Western blotting data indicated ABCG1, β-actin, β-tubulin and VEGF are modified by ONOOH however the exact type of modification and position of modification on these proteins could not be determined; these changes are therefore indicated as “unknown”.

7.4.1 Future work

There are many investigations that can be performed in the future, which can expand the data outlined in this Section. In this project, LC/MS/MS analysis was performed using HCAEC that were treated with 500 µM ONOOH for 5 min. 500 µM ONOOH is a sublethal ONOOH concentration and the damage identified in this project appears to precede overt cell necrosis. The responses of HCAEC to concentrations lower and higher than 500 µM ONOOH after a 5 min incubation could be investigated in future via LC/MS/MS. In this Thesis only qualitative data was obtained and it would clearly be beneficial to obtain quantitative data on the extent of modification.

The LC/MS/MS data outlined in this Chapter could be linked with the functional effects and this could determine whether the ONOOH-mediated changes are important or not. The repair or removal of these modified species after ONOOH exposure could also be investigated. It would be interesting to examine how rapidly damaged proteins are removed. In future if an advanced technique is developed to efficiently isolate and examine membrane proteins via LC/MS/MS, ONOOH-mediated damage to HCAEC membrane proteins could be examined.

The LC/MS/MS analysis could be extended to examine HCAEC that have been exposed to sublethal SIN-1 concentrations for 4 h in order to identify which proteins are targeted by ONOOH after prolonged and sustained exposure. In this case, dose and time...
responses to SIN-1 could be examined. The LC/MS/MS analysis with ONOOH and SIN-1 could also be performed in the presence of 25 mM NaHCO₃ to investigate the effect of HCO₃⁻ on ONOOH-mediated damage after short and prolonged exposure. This would be very sensible in light of the data obtained in this project by Western blotting suggesting that HCO₃⁻ modulates ONOOH-mediated damage. Investigations could also be performed into ONOOH-mediated cysteine modification by using a thiol-specific biotinylated or fluorescent probe (500, 501) to identify which cysteine residues are targeted by ONOOH.

LC/MS/MS analyses could also be performed using a more sensitive and advanced mass spectrometer that will detect modifications that are occurring at low abundance, or occurring on low abundant proteins. 2-dimensional gels could also be used to separate nitrated proteins based on their isoelectric point and molecular mass, before examination of the spots and LC/MS/MS analysis. This technique may yield more data on the HCAEC proteins that have been nitrated by ONOOH. In this project, one product (3NT) formation was examined. However the formation of other products such as 6-nitrotryptophan and mixed disulphides formed on HCAEC proteins could also be examined.

7.5 Summary

Overall the studies carried out and reported in this Thesis have provided new knowledge and information on the role of ONOOH (after short incubation) in damaging HCAEC. Within HCAEC, ONOOH targets the cytoskeletal proteins, chaperone proteins, membrane proteins, mitochondrial proteins, proteins that are involved in protein synthesis and signaling proteins. In these proteins, ONOOH targets cysteine residues prior to methionine, tryptophan, tyrosine, histidine and phenylalanine. In HCAEC, ONOOH induces extensive thiol oxidation and nitrates selected proteins. Multiple cytosolic and membrane proteins appear to be simultaneously affected. These data provide new insights into how the potent oxidant ONOOH, which is known to be formed during lesion development in atherosclerosis, may propagate endothelial damage. This is important, as endothelial damage is a key driver of lesion development in atherosclerosis, which is a disease of major significance in Australia and worldwide.
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