Role of myeloperoxidase-derived oxidants in the induction of vascular smooth muscle cell damage

Konstantina Flouda a, John Mercer b, Michael J. Davies a, Clare L. Hawkins a, *

a Department of Biomedical Sciences, University of Copenhagen, Panum, Blegdamsvej 3B, Copenhagen N, DK-2200, Denmark
b Institute of Cardiovascular & Medical Sciences, University of Glasgow, 126 University Place, Glasgow, G12 8TA, United Kingdom

A R T I C L E   I N F O
Keywords:
Myeloperoxidase
Hypochlorous acid
Hypothiocyanous acid
Inflammation
Atherosclerosis

A B S T R A C T
Myeloperoxidase (MPO) is released by activated immune cells and forms the oxidants hypochlorous acid (HOCl) and hypothiocyanous acid (HOSCN) from the competing substrates chloride and thiocyanate. MPO and the overproduction of HOCl are strongly linked with vascular cell dysfunction and inflammation in atherosclerosis. HOCl is highly reactive and causes marked cell dysfunction and death, whereas data with HOSCN are conflicting, and highly dependent on the nature of the cell type. In this study we have examined the reactivity of HOCl and HOSCN with human coronary artery smooth muscle cells (HCASMC), given the key role of this cell type in maintaining vascular function. HOCl reacts rapidly with the cells, resulting in extensive cell death by both necrosis and apoptosis, and increased levels of intracellular calcium. In contrast, HOSCN reacts more slowly, with cell death occurring only after prolonged incubation, and in the absence of the accumulation of intracellular calcium. Exposure of HCASMC to HOCl also influences mitochondrial respiration, decreases glycolysis, lactate release, the production of ATP, cellular thiols and glutathione levels. These changes occurred to varying extents on exposure of the cells to HOSCN, where evidence was also obtained for the reversible modification of cellular thiols. HOCl also induced alterations in the mRNA expression of multiple inflammatory and phenotypic genes. Interestingly, the extent and nature of these changes was highly dependent on the specific cell donor used, with more marked effects observed in cells isolated from diseased compared to healthy vessels. Overall, these data provide new insight into pathways promoting vascular dysfunction during chronic inflammation, support the use of thiocyanate as a means to modulate MPO-induced cellular damage in atherosclerosis.

1. Introduction
Neutrophils are the most abundant leukocytes in the circulation, and are generally considered to be the first line of defense in the innate immune system [1]. Myeloperoxidase (MPO), a member of the superfamily of heme peroxidases, is present in the phagolysosomal compartment and released extracellularly by neutrophils, and catalyzes the reaction of H2O2 and halides (Cl−, Br−, I−) or the pseudohalide thiocyanate (SCN−) to produce the respective hypohalous acids: hypochlorous acid (HOCl), hypobromous acid (HOBr), hypoiodous acid (HOI) or hypothiocyanous acid (HOSCN) [2]. While HOCl is believed to be the most abundant hypohalous acid formed in vivo, due to the higher concentrations of Cl− in plasma (100–140 mM Cl−) compared with other halides (20–100 μM Br−, 1 μM I− and 20–150 μM SCN−), HOSCN is also formed, as SCN− is the favored substrate for MPO [3]. Thus, it has been estimated that similar concentrations of HOCl and HOSCN can be produced by MPO under normal physiological conditions [3].

The formation of MPO-derived oxidants has an important role in neutrophil antimicrobial defense, as these oxidants rapidly kill invading pathogens [4]. However, the over-production of HOCl resulting from the infiltration and activation of neutrophils at inflammatory sites induces tissue damage and is associated with the development of many diseases [2, 4]. There is clear evidence for a role of MPO and HOCl in the progression of atherosclerosis and the development of atherosclerotic plaques (reviewed [5]). MPO is elevated in human lesions [6] and its enzymatic activity contributes to lesion formation [7]. The presence of increased levels of HOCl-modified proteins, and the biomarkers 3-chloro-tyrosine and 5-chloro-uracil in human lesions, further supports a role for this MPO oxidant in the development of disease [5–13].

Whether HOSCN also plays a role in the development of atherosclerosis is not well understood, and the data are conflicting. In particular, there are no data regarding the reactivity of this oxidant with vascular smooth muscle cells (VSMC). This is significant, given the...
differences in cellular reactivity of this oxidant with specific mammalian cell types [11–13]. In contrast to HOCI, there are no specific biomarkers for HOSCN to assess its formation in vivo [13]. Cyanate (OCN⁻), a HOSCN decomposition product, modifies proteins by carbamylation, resulting in higher levels of homocitrulline, which can be used as an indirect indicator of HOSCN formation [14]. Elevated levels of homocitrulline are present in lesions and have been implicated in the development of atherosclerosis, though this product can also be formed independently of MPO and HOSCN [14]. The formation of HOSCN in vivo is dependent on the availability of SCN⁻, which can be influenced by smoking, drugs and diet [15,16]. Interestingly, smokers with higher plasma SCN⁻ levels have greater macropage foam cell populations [17], and more extensive deposition of oxidized low-density lipoprotein (oxLDL) compared to non-smokers [18], consistent with a role for HOSCN in lesion development. However, it is possible that this association reflects the formation of other toxic metabolites in smokers, as supplementation with SCN⁻ in murine models of atherosclerosis (in the absence of smoking) is protective and decreases lesion formation [19, 20]. In addition, in humans, there is a correlation between elevated plasma SCN⁻ and a decrease in long-term, all-cause mortality in patients following an initial myocardial infarction [21].

Smooth muscle cell dysfunction plays a key role in the pathogenesis and progression of atherosclerosis. The switching of VSMCs from a contractile phenotype to a synthetic and proliferative phenotype promotes cell migration to the growing lesion to promote formation of a fibrous cap, which is essential in the formation of stable plaques [22]. The synthetic VSMCs produce ECM proteins such as fibronectin, laminins and collagen, which increases the thickness and stability of the fibrous cap [23]. However, the infiltration of macrophages and other immune cells, including neutrophils, into the developing lesion is associated with an acceleration of VSMC apoptosis, which is a major contributor to plaque instability [24]. HOCI is a strong oxidant and is known to induce significant damage to cells, including VSMC (reviewed in [2,12]). HOCI also damages components of the extracellular matrix (ECM), including fibronectin, which results in decreased adhesion and increased proliferation of VSMC [25,26], and can regulate the VSMC phenotypic switch from the contractile to the synthetic phenotype [27].

However, less is known about the mechanisms involved in HOCI-induced VSMC death, or the ability of this oxidant to modulate the expression of pro-inflammatory and/or phenotypic genes, which could represent an important pathway to vascular dysfunction under chronic inflammatory conditions. In addition, there are a lack of data regarding the reactivity of HOSCN with this cell type, which is significant in light of the growing interest in the therapeutic use of SCN⁻ as a means to modulate inflammatory damage in the context of atherosclerosis [19, 20]. HOSCN is typically regarded as a milder oxidant, which reacts selectively with thiol and selenol groups [13,28,29]. Exposure of cells to HOSCN results in rapid depletion of cellular thiols and GSH and inactivation of thiol-dependent enzymes, which is linked to modulation of function [30–33]. HOSCN can react with thiols in a reversible manner, forming sulfenic acids and related products, which have been suggested to be repaired by cellular antioxidant systems [32–34]. Using a murine macrophage-like cell line model, it was recently shown that the re-incubation of cells previously exposed to HOSCN can result in regeneration of the reduced thiol pool when compared to cells analyzed immediately following oxidant treatment [35], and a lower extent of cell death [30,35]. However, whether these reactions occur in primary human vascular cell models has yet to be established.

Therefore, in this study, we compared the effects of HOCI and HOSCN on the function of human coronary artery smooth muscle cells (HCASMC), as a primary vascular cell model, using multiple cell donors together with a range of physiological and pathological oxidant concentrations. We have examined the consumption of each oxidant by the cells, and the resulting impact on cellular viability, together with determining the extent and nature of thiol oxidation, and alterations in Ca²⁺ homeostasis, mitochondrial respiration and markers of cell phenotype. These studies provide new insight into understanding how the over-production of MPO-derived oxidants contributes to lesion formation, and provide support for a potential modulatory role of SCN⁻ under chronic inflammatory conditions. The data also highlight the importance of comparing the reactivity of primary human vascular cells sourced from multiple donors in redox-related research, particularly when quantifying alterations in the expression of inflammatory and phenotypic genes, as divergent, donor-dependent results can be observed.

2. Materials and methods

2.1. Preparation and quantification of hypohalous acids

All buffers and aqueous solutions were prepared with nano-pure water (npH₂O) from a MilliQ system (Millipore). All chemicals were purchased from Sigma – Aldrich/Merck (St. Louis, MI, USA) unless stated otherwise. Hypochlorous acid (HOCI) was used directly after dilution of a concentrated stock solution of sodium hypochlorite (10–15% NaOCl, Acros Organics, Waltham, MA, USA). HOCI concentrations were determined by measuring the absorbance at 292 nm with a molar extinction coefficient of 350 M⁻¹ cm⁻¹ at pH 11 [36]. HOSCN was prepared enzymatically, by using lactoperoxidase (LPO, from bovine milk), as previously [37]. HOSCN concentrations and cellular oxidant consumption were measured by quantification of the loss of 5-thio-2-nitrobenzoic acid (TNB), at 412 nm, as described previously [38] using an
extinction coefficient of 14,150 M⁻¹ cm⁻¹ [39].

2.2. Cell culture

All experiments were carried out with HCASMC, purchased from Cell Applications (San Diego, CA, USA) from 3 individual donors, and were used between passages 2 and 5. The characteristics of each cell donor are given in Table S1. The cells were cultured in HCASMC growth medium (Cell Applications) in 75-cm² or 175-cm² tissue culture flasks in a humidified environment of 5% CO₂ at 37 °C. Prior to experimentation, the cells were harvested with trypsin/EDTA solution (0.025% trypsin, 0.01% EDTA, in PBS), centrifuged at 220 g for 5 min and plated down to adhere overnight, at a density of 1 x 10⁵ cells mL⁻¹ in 6-, 12- or 96-well plates using volumes of 2 mL, 1 mL or 100 μL, respectively. Before the treatments, the cell media was removed and the cells washed with warm HBSS. HCASMC were exposed to increasing concentrations of HOCl (0–100 μM) for 15 min, and to HOSCN (0–200 μM) for 15 min and/or 1 h, with all dilutions of the oxidant in HBSS.

2.3. Metabolic activity

The metabolic activity was assessed using a commercial MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt] reagent (CellTiter 96® AQueous One Solution Assay, Promega, WI, USA). HCASMC (1 × 10⁵ cells) were plated in 96-well tissue culture plates overnight and exposed to HOCl and HOSCN for 15 min and/or 1 h. After the incubation times, the treatment solution (100 μL) was removed and the cells were washed with warm HBSS. To measure the metabolic activity directly after the treatments, cell growth medium (100 μL) containing MTS reagent (10 μL) was added into the wells and incubated for 4 h at 37 °C, before measuring the change in absorbance at 490 nm, using a microplate reader (Spectra Max i3x, Molecular Devices, San Jose, CA, USA).

2.4. Lactate dehydrogenase (LDH) release

Assessment of cell death was evaluated by the LDH release assay, after the initial oxidant treatments, followed by 3 and 24 h re-culture of the cells in growth medium. After the incubation times, the media was collected, the cells were washed with HBSS and lysed with nH₂O₂. Both media and cell lysates were centrifuged at 2000 g for 5 min (4 °C) to remove cell debris. Cell lysates, media and blanks containing growth medium or nH₂O₂ (10 μL), were incubated with 200 μL of reaction buffer containing 225 μM NADH and 2.5 mM sodium pyruvate. The NADH absorbance was measured at 340 nm for 30 min at 5 min intervals, using a microplate reader. Cell viability was calculated according to the following equation:

\[
\% \text{ viability} = \frac{\Delta \text{ intracellular activity}}{\Delta \text{ intracellular activity} + \Delta \text{ extracellular activity}} \times 100
\]

2.5. Flow cytometry

Apoptosis and necrosis was assessed using the APC Annexin V Apoptosis Detection Kit with propidium iodide (PI) (BioLegend, San Diego, CA, USA). Analysis of cell death, was performed immediately after the treatments, and after 24 h re-culture in growth medium. Briefly, cells were harvested with trypsin/EDTA solution (0.025% trypsin, 0.01% EDTA, in phosphate buffered saline), resuspended in binding buffer containing APC Annexin V and PI, incubated in the dark at 22 °C for 30 min and analyzed with a FACSVerse™ flow cytometer (BD, Franklin Lakes, NJ, USA). Cytosolic Ca²⁺ levels were measured using the cell permeant probe, Fluo-4AM (Thermo Fisher Scientific, Waltham, MA, USA). After the treatment, cells were incubated in the dark at 37 °C with 5 μM Fluo-4AM in HBSS (Ca²⁺ free) for 45 min. Cells were then detached from the tissue culture plates by gently scraping and were subsequently analyzed on the flow cytometer.

2.6. Quantification of cellular thiols

Cellular thiols were measured using the ThioGlo 1 reagent (Berry & Associates Inc, Dexter, MI, USA) in HCASMC exposed to HOCl (15 min) and to HOSCN (15 min and/or 1 h), immediately after the treatment, and after oxidant treatment with re-culture in growth medium for 3 h and 24 h. Cells were washed with warm HBSS and lysed on ice with nH₂O₂, before incubation with the ThioGlo 1 reagent (13 μM) for 5 min in the dark (22 °C) and measuring the fluorescence at λₜₐ₅ 384 nm and λₑₘ₅ 513 nm with a microplate reader [38]. Thiols were quantified by using a GSH standard curve and were normalized to total protein concentration, measured by the Pierce BCA Protein Assay (ThermoFisher Scientific, Waltham, MA, USA).

2.7. Determination of GSH and GSH/GSSG

Intracellular GSH was measured in cell lysates with mono- bromobimane derivatization and HPLC separation, after the initial treatment, and after oxidant treatment with re-culture of the cells in growth medium for 24 h. The samples were prepared as described by Kariya et al. [40] and analyzed on an HPLC system (Shimadzu, Kyoto, Japan) with a fluorescence detector (RF10A-XL) with λₜₐ₅ 390 nm and λₑₘ₅ 480 nm. Samples were eluted from a Syngery Hydro-RP C-18 column (4 μm, 150 × 4.6 mm; Phenomenex, Torrance, CA, USA) at 30 °C, with a flow rate of 1 mL min⁻¹. Mobile phase A contained 1% (v/v) acetic acid and 5% (v/v) acetonitrile and mobile phase B contained 1% (v/v) acetic acid and 20% (v/v) acetonitrile as previously [30]. GSH levels were quantified by a GSH standard curve and normalized to the total cell protein concentration. Loss of GSH and generation of GSSG was also quantified using the GSH/GSSG-Glo™ Assay Kit (Promega, WI, USA). In this case, HCASMC were plated in white, opaque 96-well tissue culture plates overnight and exposed to HOCl and HOSCN as described above. Cells were lysed according to the manufacturer’s instructions, directly after the oxidant treatment and after 24 h re-culture in growth media. The change in luminescence was then detected using a microplate reader.

2.8. High resolution respirometry, ATP and glycolysis

Measurements of mitochondrial O₂ respiration were conducted using the Oxygraph-2k (O2k, Oroboros Instruments, Innsbruck, Austria), at 37 °C. After the treatment with HOCl (15 min) or HOSCN (1 h), cells were harvested with trypsin/EDTA solution (0.025% trypsin/0.01% EDTA) and re-suspended in respiration buffer (MiR05 (110 mM KPO₄, 20 mM HEPES, 1 mg mL⁻¹ BSA, pH 7.1)). Respiration of intact cells was measured using a coupling control protocol [41]. Cell suspensions were added to the oxygen chambers containing respiration buffer (total volume 2 mL) and the chambers were sealed. After stabilization of the signal (ROUTINE respiration, R), oligomycin (2.5 μM), an inhibitor of mitochondrial ATP synthase, was added to obtain a measure of the proton leak respiration (LEAK respiration, L). The maximum electron transfer – pathway capacity (ETS capacity, E) was measured by titration of the protonophoric uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP, 0.5 μM steps). Finally, rotenone (0.5 μM) and antimycin A (2.5 μM), inhibitors of Complex I and III, respectively, were added to obtain the residual oxygen consumption (ROX) or non-mitochondrial respiration. ATP levels were quantified using the ATP lite Luminescence kit (Perkin-Elmer, Waltham, MA, USA) according to the manufacturer’s instructions. The effect of HOCl and HOSCN on glycolysis was examined using the glycolysis cell-based assay kit (Cayman Chemicals, Ann Arbor, MI, USA),
with the secretion of l-lactate into the extracellular environment recorded at 490 nm.

2.9. Quantitative real-time polymerase chain reaction (qPCR)

RNA from HCASMC was extracted using a RNAeasy® Mini Kit (Qiagen, Hilden, Germany) as per the manufacturer’s instructions, including a DNase digestion step (RNase-Free DNase Set, Qiagen). The RNA concentration was quantified with a 24-Microplate, SpectraDrop Micro-Volume Starter Kit (Molecular Devices) and reverse transcription was performed using a SensiFAST cDNA Synthesis Kit (Bioline, London, UK). For qPCR, the SensiFAST SYBR Hi-ROX Kit (Bioline) was used with the following conditions: 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s for 39 cycles, followed by dissociation curve analysis. Data were normalized to two housekeeping genes, 18S ribosomal RNA and beta-2-microglobulin (B2M), and expressed as a fold change compared to the control cells, using the $2^{-\Delta\Delta Ct}$ method. Housekeeping and target gene primer sequences, are listed in Table S2.

2.10. Enzyme-linked immunosorbent assay (ELISA)

HCASMC were treated with HOCl (0 and 50 μM) for 15 min and with HOSCN (0 and 100 μM) for 1 h, followed by re-incubation in cell growth media for 24 h, as described above. The supernatant (cell media) was collected after centrifugation at 1000 g for 20 min (4 °C). Cells were lysed in RIPA buffer (Sigma-Aldrich) containing 1% protease inhibitors (Sigma-Aldrich) and were centrifuged at 1000 g for 15 min (4 °C) to remove cell debris. Levels of interleukin 6 (IL-6) and osteopontin (OPN) were quantified using a commercial Human IL-6 ELISA kit (BioLegend, San Diego, CA, USA) and Human OPN ELISA kit (Nordic BioSite, Copenhagen, DK) according to the manufacturer’s instructions.

2.11. Statistical analysis

All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, USA) using either 1-way or 2-way ANOVA with Dunnett’s comparison post hoc test, with p < 0.05 taken as significant. Data represent mean of triplicate determinations ± SEM from 3 individual donors, unless otherwise stated in the figure legends.

Fig. 1. Metabolic activity, oxidant consumption and viability observed on exposure of HCASMC to HOCl and HOSCN.

The changes in metabolic activity were assessed by MTS assay after treatment of HCASMC with (A) HOCl for 15 min and (B) HOSCN for 1 h, directly after the treatment (black bars) and after treatment with re-incubation for 3 h (grey bars) or 24 h (dashed bars) in cell media. Data in (C) and (D) show the loss of HOCl (100 μM) and HOSCN (200 μM) respectively, from the cellular supernatant on exposure to HCASMC for 15–240 min (square symbol, solid lines) compared to the same concentrations of oxidants in HBSS (triangle symbol, dashed lines), by TNB assay. Graphs (E) and (F) show cell viability as assessed by the lactate dehydrogenase (LDH) release assay on exposure of HCASMC to HOCl for 15 min (E) and HOSCN for 1 h with re-incubation for 3 h (square symbol, dashed lines) and 24 h (circle symbol, solid lines) in cell media. *p < 0.01, **p < 0.001 and ***p < 0.0001, indicate a significant decrease compared to control cells, by two-way ANOVA with Dunnett’s multiple comparison post hoc test.
3. Results

3.1. HOCl and HOSCN decrease metabolic activity and HCASMC viability

For initial experiments, HCASMC were exposed to increasing concentrations of HOCl or HOSCN (10–200 μM) for 15 min and/or 1 h, before measuring the metabolic activity, over 4 h incubation with the MTS reagent. A significant dose-dependent loss in metabolic activity was observed after exposure of HCASMC to HOCl (Fig. 1A), but not to HOSCN (Supplementary Fig. S1) for 15 min. However, a significant decrease in the metabolic activity of the HCASMC was observed on exposure of the cells to the same concentrations of HOSCN for 1 h rather than 15 min (Fig. 1B). However, despite the increased reaction time, HOSCN was less damaging compared to HOCl, with ~50% compared to ~30% loss in metabolic activity seen with 100 μM HOCl and HOSCN, respectively.

To assess the effects of each oxidant on signaling events and possible repair pathways, cells were exposed to the same concentrations of HOCl and HOSCN and incubation times as above, before washing the cells with HBSS to remove any residual oxidant, and re-culturing for 3 and/or 24 h in growth medium (37 °C, 5% CO₂). After the defined re-incubation time, a significant decrease in cell viability reflected by a loss in metabolic activity, was detected for both oxidants (Fig. 1A and B). There was no significant difference between the cells examined immediately after treatment with the oxidants, and the cells analyzed following a re-incubation period, suggesting that the cells were unable to repair the damage caused by exposure to either HOCl or HOSCN. Again, the extent of loss in metabolic activity was greater with HOCl treatment following re-incubation (3 h or 24 h), compared to the same concentrations of HOSCN.

To examine whether these results reflected varying rates of reaction of each oxidant with the cells, as has been described previously for other cell types [31,42], the consumption of the oxidants was investigated by the TNB assay. HCASMCs were treated with HOCl (100 μM) or HOSCN (200 μM) and the concentration of the oxidant present in the cell supernatant at varying incubation times (0–240 min) was compared to the decomposition of the oxidant observed over identical conditions in the absence of cells. HOCl was consumed rapidly by the cells with < 50% of the initial oxidant concentration detected after 15 min incubation (Fig. 1C). In contrast, the loss of HOSCN was slower, with only ~30% of the initial oxidant consumed by the cells after 240 min (Fig. 1D). For the HOCl measurements, taurine was added (250 μM final concentration) prior to performing the TNB assay, to eliminate reactions of HOCl with the DTNB formed, by stoichiometric conversion of any residual HOCl to the taurine chloramine.

To confirm whether the changes seen in metabolic activity reflect altered viability, the extent of cell lysis after exposure of the HCASMC to HOCl (0–100 μM for 15 min) or HOSCN (0–200 μM for 1 h) followed by 3 and 24 h re-incubation in growth medium, was assessed by using the LDH release assay. Cell viability at 3 and 24 h post oxidant exposure decreased significantly with 100 μM HOCl (Figs. 1E) and 200 μM HOSCN (Fig. 1F). The loss in cell viability was less extensive with HOSCN compared to HOCl at equivalent oxidant concentrations, supporting the data from the MTS assay.

Changes in HCASMC viability were examined further by staining cells exposed to each oxidant (0–100 μM HOCl for 15 min, 0–200 μM HOSCN for 1 h) with APC Annexin V and PI, before analyzing the cell populations by flow cytometry (plots shown in Supplementary Fig. S2). Exposure of the cells to HOCl resulted in a dose-dependent decrease in

Fig. 2. HOCl and HOSCN induced necrotic and apoptotic cell death in HCASMCs. HCASMC were exposed to HOCl for 15 min (A,C) or HOSCN for 1 h (B,D) and were analyzed immediately following treatment (A,B) or after washing the cells to remove any residual oxidant and re-incubation for 24 h in cell media (C,D). The percentages of necrotic (PI positive, black bars), early apoptotic (Annexin V positive, grey bars), and late apoptotic (PI and Annexin V positive) cells in the population were assessed using the APC Annexin V apoptosis detection kit and flow cytometry analysis. **p < 0.01 and ****p < 0.0001 indicate a significant increase in apoptotic or necrotic cells as a percentage of the total cell population compared to the non-treated cells by two-way ANOVA with Dunnett’s multiple comparison post hoc test.
viability, with a significant increase in the population of PI-positive (necrotic) cells, but with no significant increase of the cell population positive only for Annexin V (early apoptotic) (Fig. 2A, Supplementary Fig. S2). However, there was a significant increase in the population of late apoptotic cells, reflected by the dual staining of the cells with Annexin-V and PI (Fig. 2A). Exposure of HCASMC to HOSCN also resulted in cell death by necrosis in a dose-dependent manner, as assessed by a significant increase in the population of PI-positive cells (Fig. 2B, Supplementary Fig. S2). Again, there was evidence for an increase in late-stage apoptosis in cells exposed to HOSCN (Fig. 2B).

Experiments were also performed to examine the extent of cell death following re-incubation of the cells in media for 24 h after the initial exposure of the HCASMC to each oxidant. In this case, the original treatment solution was removed and the cells were washed before re-incubation, which resulted in the removal of any non-adherent, dead, cells. Although the initial treatment resulted in cell death mainly by necrosis for both oxidants (Fig. 2A and B), the remaining cell populations 24 h post-oxidant exposure, showed an increase in the cellular uptake of Annexin V, consistent with apoptosis, particularly in the experiments with HOCl. Thus, with HOCl, a dose-dependent increase of the early apoptotic population, indicated by Annexin V-positive stained cells was observed, together with an increase of late apoptotic population, where cells stained positively for both Annexin V and PI (Fig. 2C, Supplementary Fig. S2). With HOSCN, an increase of the necrotic cell population and a more extensive increase of the population of late apoptotic cells was apparent (Fig. 2D, Supplementary Fig. S2). In summary, necrotic cell death was more prevalent immediately following exposure of the cells to HOCl or HOSCN, whereas the extent of apoptosis increased following prolonged incubation after the initial treatment.

3.2. HOCl and HOSCN promote both reversible and non-reversible oxidation of cellular thiols

Cellular thiols are key targets for MPO-derived oxidants with the modification of these residues recognized as a marker of oxidative stress. Unlike HOCl, HOSCN reacts in a highly selective manner with thiols, which can result in differences in the cellular behavior following exposure to each oxidant [12,28]. HCASMC were exposed to HOCl (0–100 μM) for 15 min and to HOSCN (0–200 μM) for 15 min and 1 h. The total cell thiol concentration was quantified using the ThioGlo 1 reagent and was normalized to the cell protein concentration. Reaction of the cells with the oxidants for 15 min resulted in a dose-dependent, decrease in cellular thiols, with a significant difference compared to controls seen with >50 μM HOCl (Fig. 3A) and >25 μM HOSCN (Fig. 3B). There was no significant difference in the extent of thiol loss on comparison of the two oxidants, when the thiol concentration was measured immediately following the oxidant exposure under analogous treatment conditions (Supplementary Fig. S3). This is attributed to the higher selectivity of HOSCN for cellular thiols, given the difference in consumption of each oxidant by the HCASMC over 15 min (Fig. 1C). Interestingly, the changes in the total cell thiol concentration in experiments with 25 and 50 μM HOSCN were less extensive on exposure of cells to this oxidant for 1 h compared to that seen after 15 min, though the changes were similar with higher oxidant concentrations (Fig. 3C). This may reflect a greater extent of reversible modification with shorter incubation time (Supplementary Fig. S4). Therefore additional experiments were performed with cells re-incubated in media following the initial treatment.

A dose-dependent loss in thiols was also observed on exposure of HCASMC to HOCl for 15 min, before washing and 3 and 24 h re-incubation in media. However, with HOCl concentrations >50 μM, the loss of thiols although significant compared to the control, was not as extensive as that seen immediately following treatment (Fig. 3A). In contrast, there was no significant change in the concentration of cellular thiols, compared to the non-treated control cells, in analogous experiments with HOSCN following re-incubation of cells exposed to the oxidant for 15 min (Fig. 3B). The increase in cellular thiols seen in the HOSCN-treated cells following re-incubation for 3 and 24 h was significant at all oxidant concentrations (Fig. 3B). Thus, following re-incubation for 3 and 24 h, the concentration of cellular thiols was significantly lower in HOCl-treated cells compared to those treated with HOSCN for 15 min (Supplementary Fig. S3). Similar, reversible, thiol oxidation was also observed on exposure of the HCASMC to ≥100 μM HOSCN for 1 h before re-incubation for 3 and 24 h (Fig. 3C). However, the difference in the thiol concentration between the cells analyzed immediately following treatment, and after re-incubation, was only significant with 24 h re-incubation in this case (Fig. 3C). In addition, the extent of thiol loss observed on treating the cells with 200 μM HOSCN for 1 h before re-incubation (Fig. 3C), remained significantly lower when compared to the non-treated, control cells, in contrast to experiments.
with 15 min HOSCN treatment (Fig. 3B).

These studies were extended to examine the effect of HOCl and HOSCN on the concentration of GSH in the HCASM C. The intracellular GSH was measured with monobromobimane derivatization and HPLC separation and was normalized to the cell protein concentration. Exposure of HCASM C to HOCl (for 15 min) and HOSCN (for 1 h), resulted in a decrease in the concentration of GSH compared to the non-treated controls (Fig. 4A and B). With HOCl, similar results were obtained following re-incubation for 24 h in growth media after the initial oxidant treatment (Fig. 4A). In contrast, with HOSCN the decrease in GSH concentration was less extensive following re-incubation, consistent with some recovery in the GSH levels (Fig. 4B). Exposure of the HCASM C to HOCl and HOSCN also resulted in a decrease in the ratio of GSH:GSSG compared to that seen in the non-treated control cells (Fig. 4C and D). With HOSCN, but not HOCl, an increase in the relative amount of GSH compared to GSSG was apparent on re-incubation of the cells for 24 h (Fig. 4C and D). The results are expressed as a fold change to account for differences in the initial GSH and GSSG concentrations between individual HCASM C donors (data not shown).

3.3. HOCl and HOSCN alter Ca\(^{2+}\) homeostasis and decrease mitochondrial respiration in HCASM C

The pathways involved in the HOCl- and HOSCN-induced decrease in metabolic activity and cell death was examined further by measuring changes in the distribution of intracellular Ca\(^{2+}\) and examining mitochondrial function and ATP production. HCASM C were exposed to HOCl (25–100 μM, 15 min) or HOSCN (50–200 μM, 15 min and 1 h), followed by assessment of cytosolic Ca\(^{2+}\) by flow cytometry with Fluo-4AM staining. Treatment of the cells with HOCl in HBSS containing Ca\(^{2+}\), resulted in an increased level of cytosolic Ca\(^{2+}\) with oxidant concentrations ≥50 μM (Fig. 5A). The increase in Ca\(^{2+}\) was dependent on the presence of extracellular Ca\(^{2+}\), and was not observed if the cells were treated with HOCl in HBSS lacking Ca\(^{2+}\) (data not shown). The pathway responsible for the HOCl-induced change in cytosolic Ca\(^{2+}\) was examined further by treating the cells with thapsigargin (3 μM), an inhibitor of the sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA), nisoldipine (4 μM), a blocker of L-type Ca\(^{2+}\) channels, or Ru360 (4 μM), an inhibitor of the mitochondrial Ca\(^{2+}\) uniporter, before the addition of HOCl (100 μM). Pre-treatment of the cells with each inhibitor had no effect on the distribution of intracellular Ca\(^{2+}\) in the non-treated control cells. However, in each case, there was significant attenuation of the increased cytosolic Ca\(^{2+}\) seen in response to HOCl (Fig. 5B). Taken together, these data suggest that HOCl induces an influx of Ca\(^{2+}\) from the extracellular environment, and also alters the extent of Ca\(^{2+}\) re-uptake into cellular stores. In contrast, there was no change in cytosolic Ca\(^{2+}\) levels on exposure of HCASM C to HOSCN for 15 min (Fig. 5C), and only a small increase in cytosolic Ca\(^{2+}\) was observed on treatment of the cells with 100 μM HOSCN for 1 h.

Mitochondrial function was assessed by examination of the effects of HOCl and HOSCN on mitochondrial respiration using an Oxygraph – 2k, to record the changes in oxygen flux in HCASM C, after exposure to the oxidants. HCASM C were treated with HOCl (50 μM) for 15 min and HOSCN (100 μM) for 1 h, where a significant decrease in metabolic activity was observed in the absence of a marked loss in viability (Fig. 1). Non-treated control and oxidant-treated cells were added to the two oxygraph chambers, before sealing, and measurement of the routine respiration (R). Addition of oligomycin (2.5 μM) to inhibit ATP synthase, enabled measurement of the leak respiration (L), whereas addition of CCCP (0.5–2.5 μM), an uncoupler of mitochondria phosphorylation, was...
titrated to measure the maximum electron transfer – pathway capacity (ETS capacity, E). Finally, inhibitors of Complexes I and III, rotenone (0.5 μM) and antimycin A (2.5 μM), respectively, were added to record the residual oxygen consumption (ROX). The results were corrected for the non-mitochondrial respiration (ROX) and expressed as flux control ratios to the ETS capacity (E).

HOCl and HOSCN significantly decreased the routine control ratio (R/E) compared to the untreated cells, whereas no significant changes were observed in the leak control ratio (L/E), for either oxidant (Fig. 6 A and B). The difference between the routine and leak control ratio (netR) is a measure of the phosphorylation-related respiration or ATP production, as a function of ETS capacity (E). The netR/E was significantly decreased on exposure of the HCASMC to HOCl or HOSCN, suggesting the ATP production in these cells will be compromised (Fig. 6 A and B). Therefore, the concentration of ATP was determined following exposure of HCASMC to HOCl (0 – 100 μM, 15 min) and HOSCN (0 – 200 μM, 1 h). For both oxidants, a dose-dependent decrease in HCASMC ATP concentration was observed, with HOCl reaching significance at concentrations ≥50 μM (Fig. 6C), and HOSCN at concentrations ≥100 μM (Fig. 6D). To assess whether HOCl and HOSCN could also affect ATP production by perturbation of glycolysis, experiments were extended to measure the release of lactate, the end-product of glycolysis. A dose-dependent decrease in the concentration of extracellular lactate was observed on re-incubation of the cells for 24 h in basal media following an initial exposure of the HCASMC to HOCl (0 – 100 μM, 15 min) or HOSCN (0 – 200 μM, 1 h) (Fig. 6E and F), consistent with a decrease in glycolysis in each case.

3.4. HOCl and HOSCN modulate the expression of inflammatory and phenotypic genes in HCASMC

The mRNA expression of a range of inflammatory genes (Table S2) was examined by qPCR, 24 h post exposure to HOCl (50 μM, for 15 min) or HOSCN (100 μM, for 1 h), where altered metabolic activity was observed in the absence of extensive cell death (Fig. 1). However, there was significant variation in the extent of mRNA expression of the inflammatory genes examined between the individual HCASMC donors, in experiments with HOCl and HOSCN (Fig. 7). With HCASMC from donor 1596, there was a significant increase in the mRNA expression of monocyte chemotactic protein 1 (MCP-1), interleukin 6 (IL-6), early growth response protein 1 (Egr-1), and activator protein 1 (AP-1) (Fig. 7 A). A significant increase in the expression of IL-6 was also seen with this donor on exposure of the cells to HOSCN (Fig. 7 B). However, no significant increase in IL-6 expression was seen in the corresponding experiments with the other cell donors (1522 and 1559; Fig. 7 C – F).

Exposure of HCASMC from donor 1559 to HOCl resulted in a significant increase in the mRNA expression of matrix metalloproteinase 9 (MMP9), but this was not observed in experiments with cells from donors 1596 and 1522. The reason for this difference is not certain, as the sex, age and race of the donors is at least partially matched and the initial cell viability and doubling rate is comparable between the cell donors (Table S2). However, the HCASMC from donor 1596 come from a non-plaque region of a diseased artery, rather than from a non-diseased artery, as is the case for donors 1522 and 1559. Therefore, these studies were extended to examine differences in SMC phenotypic markers, which is also relevant to the pathogenesis of atherosclerosis [43]. There was also significant variation in the mRNA expression of phenotypic genes on exposure of
HCASMC from each individual donor to HOCl or HOSCN (Fig. 8). With HOCl, a significant increase in the expression of osteopontin (OPN) was observed in experiments with donors 1522 and 1559, but not donor 1596 (Fig. 8A–C). However, there were no changes in the expression of SMCα-actin, S100A4, Cx43, type 1 collagen or calponin for any of the donors (Fig. 8A–C). There was no significant change in the expression of any of the phenotypic genes in experiments with HOSCN (Fig. 8D–F).

In light of the evidence for increased expression of IL-6 (donor 1596) and OPN (donors 1552 and 1559) with HOCl, and IL-6 (donor 1596) with HOSCN, further experiments were performed to examine expression of each protein using an ELISA approach. Experiments were performed under identical treatment conditions to those used for the gene expression studies, using cell donors 1596 and 1559, which showed an increase in the expression of IL-6 and OPN, respectively. However, there was no change in the concentration of IL-6 in the cellular supernatant following treatment of the HCASMC with either HOCl (50 μM) or HOSCN (100 μM), consistent with no alteration in secretion of this cytokine (Supplementary Fig. S5). Exposure of the HCASMC to HOCl (50 μM) resulted in a significant decrease in the intracellular concentration of OPN, which was not seen on treating the cells with HOSCN (100 μM) (Supplementary Fig. S5).

4. Discussion

Although it is known that MPO-derived oxidants modify the function of different cell types relevant to the development of atherosclerosis (reviewed [2,12]), there are few studies that have examined the effects of HOCl and HOSCN on primary human vascular cells, particularly VSMC, which maintain the structure and function of blood vessels. In the present study, the mechanisms responsible for HOCl- and HOSCN-induced cellular damage and death of HCASMC from different cell donors have been examined. We show that exposure of HCASMC to HOCl and HOSCN results in cell death by both necrotic and apoptotic pathways, which is dependent on exposure time, and is more extensive with HOCl. Both oxidants cause a rapid depletion of intracellular thiols and GSH, which can be reversible with HOSCN, in a manner dependent on the oxidant concentration and exposure time. Exposure of HCASMC to HOCl and HOSCN decreases mitochondrial respiration, glycolysis and ATP production. Moreover, in experiments with some cell donors, particularly the cells isolated from a diseased artery, HOCl could increase the expression of multiple inflammatory and phenotypic genes. Exposure of HCASMC to HOCl and HOSCN resulted in a significant loss of metabolic activity and release of LDH, consistent with a loss of viability. HOCl was more potent than HOSCN, with the latter only giving rise to decreased cell viability in experiments with higher oxidant doses (≥100 μM) and after longer incubation time. This is attributed to the slower consumption or uptake of HOSCN by the cells compared to HOCl, and is consistent with previous studies in other cell types [30,31,42,44]. HOCl is known to induce both necrotic and apoptotic cell death in a manner dependent on cell type, oxidant concentration and incubation...
conditions (reviewed [12]). With HCASMC, a significant increase in the cell population stained with PI was seen on analysis immediately following exposure of the cells to HOCl or HOSCN, consistent with cell death occurring mainly by necrosis. Re-incubation of the HCASMC for 3 h or 24 h following the initial treatment had no significant effect on the extent of loss in metabolic activity observed on treating the cells with either HOCl or HOSCN. However, a greater proportion of the cell population stained positively with Annexin V, consistent with apoptotic cell death, on analysis 24 h after treatment with HOCl. In this case, there was only a low percentage of necrotic cells, unlike the analyses performed immediately following treatment. This could reflect the washing step performed to remove any residual oxidant before re-incubation with cell media, which may also remove any non-adherent, necrotic cells, thereby excluding them from subsequent analysis following re-incubation.

This induction of apoptotic cell death by HOCl is believed to reflect the induction of mitochondrial dysfunction and/or pro-apoptotic signaling cascades, as reported in other cell types under comparable conditions [45–48]. In contrast, apoptotic cell death was not as prevalent in the cells exposed to HOSCN, on analysis immediately after treatment, or following 24 h re-incubation. The reason for this low level of apoptosis is not certain, but could be related to the ability of HOSCN to inhibit the thiol-dependent caspases involved in apoptosis, which occurs on exposure of human umbilical vein endothelial cells to this oxidant [31]. However, exposure of other cell types, including macrophages and human coronary artery endothelial cells, to HOSCN can result in apoptotic cell death, though this is reported to occur by caspase-independent pathways [30,42].

As the differential reactivity of cells with HOCl and HOSCN is often associated with a greater selectivity of HOSCN for thiols [28], the ability of each oxidant to perturb redox status was examined. A significant loss of thiols was observed immediately following treatment of HCASMC with HOCl or HOSCN. However, a greater proportion of the cell population stained positively with Annexin V, consistent with apoptotic cell death, on analysis 24 h after treatment with HOCl. In this case, there was only a low percentage of necrotic cells, unlike the analyses performed immediately following treatment. This could reflect the washing step performed to remove any residual oxidant before re-incubation with cell media, which may also remove any non-adherent, necrotic cells, thereby excluding them from subsequent analysis following re-incubation.

This induction of apoptotic cell death by HOCl is believed to reflect the induction of mitochondrial dysfunction and/or pro-apoptotic signaling cascades, as reported in other cell types under comparable conditions [45–48]. In contrast, apoptotic cell death was not as prevalent in the cells exposed to HOSCN, on analysis immediately after treatment, or following 24 h re-incubation. The reason for this low level of apoptosis is not certain, but could be related to the ability of HOSCN to inhibit the thiol-dependent caspases involved in apoptosis, which occurs on exposure of human umbilical vein endothelial cells to this oxidant [31]. However, exposure of other cell types, including macrophages and human coronary artery endothelial cells, to HOSCN can result in apoptotic cell death, though this is reported to occur by caspase-independent pathways [30,42].

As the differential reactivity of cells with HOCl and HOSCN is often associated with a greater selectivity of HOSCN for thiols [28], the ability of each oxidant to perturb redox status was examined. A significant loss of thiols was observed immediately following treatment of HCASMC with HOCl or HOSCN. Interestingly, the greatest extent of thiol loss was seen in experiments where the cells were treated with HOSCN for 15 min, where there was no loss of cell viability as assessed by the MTS assay. This reflects the formation of reversible thiol oxidation products under these conditions, as thiol loss is further attenuated following re-incubation of the cells with media for 3 and 24 h. That the extent of thiol oxidation is greater at 15 min compared to 1 h treatment, implies that the repair of thiols is more rapid than the oxidation induced by greater exposure time to HOSCN, which contrasts with experiments with macrophages [35,42]. The rapid regeneration of cellular thiols was also associated with cellular survival following exposure to HOSCN as...
indicated in experiments where the cells were treated with >100 μM HOSCN for 1 h. In this case, the level of thiols did not return to that seen in non-treated control cells, and significant toxicity was observed. This likely reflects the further oxidation of some thiols to non-reversible products such as sulfinic and sulfonic acids, as seen in experiments with high ratios of HOSCN to protein [34].

Evidence for reversible thiol oxidation was also obtained in experiments with HOCl, but this occurred to a lesser extent, and was only significant following 24 h rather than 3 h re-incubation. It is possible that HOCl also induces the formation of reversible thiol oxidation products within the cells, though this was not seen in previous experiments with macrophages, unless SCN− was added to promote the formation of HOSCN [35]. Alternatively, this could reflect the activation of stress-related, pro-survival signaling cascades, such as nuclear factor erythroid 2-related factor 2 (Nrf2) in the HOCl-treated cells, as previously [35,44,49]. However, the increase in thiols observed on re-incubation of the HOCl-treated HCASMC was not associated with an increase in GSH levels or any alteration of the GSH:GSSG ratio, which might be expected on activation of Nrf2 [49,50]. Therefore, these effects might be associated with the activation of other pro-survival signaling pathways, which could be examined in future studies.

It is well established that protein thiol oxidation and depletion of GSH is strongly associated with perturbation of cellular function, and can promote mitochondrial dysfunction, the alteration of key metabolic pathways within the cells and influence cellular survival [51,52]. With HCASMC, the non-reversible loss of thiols seen on exposure to HOCl and HOSCN correlated well with a loss of mitochondrial basal respiration, decreased glycolysis and ATP production. This is attributed to the Fig. 8. Effects of HOCl and HOSCN on phenotypic gene expression. Graphs show the mRNA expression of a range of phenotypic genes analyzed by qPCR with normalization to the expression of the housekeeping genes 18S and B2M. HCASMC were exposed to either 50 μM HOCl for 15 min (black bars) or 100 μM HOSCN for 1 h (grey bars), before re-incubation in cell media for 24 h. The data for the 3 individual donors 1596 (A,B), 1522 (C,D) and 1559 (E,F) are shown separately. Results represent the mean ± SD from at least 3 replicates and are expressed as an increase compared to (ct) the non-treated control.*p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 indicate a significant increase compared to control using one-way ANOVA with Dunnett’s multiple comparisons post hoc test.
oxidation of mitochondrial thiol-containing proteins, particularly those associated with energy production, the TCA cycle and the electron transport chain, and other cytosolic glycolytic and metabolic enzymes, such as GAPDH and creatine kinase [30,32–34,53,54]. Although the decrease in ATP levels correlates well with thiol oxidation, a significant reduction in lactate was observed on exposure of HCASMC to lower concentrations of the oxidants (25 μM HOCI and 50 μM HOSCN), and occurred in the absence of significant cell death. It is possible that lactate may be influenced by the activity of the monocarboxylate transporter (MCT-1), a lactate and pyruvate transporter localized at the plasma membrane and mitochondrial inner membrane [55]. MCT-1 plays an important role in oxidative stress under inflammatory conditions and can be a target of reactive oxygen species [56], and therefore is a possible target of HOCI and HOSCN.

In addition to mitochondrial damage and loss of ATP production, which are strongly correlated with cellular survival, perturbation of Ca²⁺ homeostasis is also important, particularly in cells exposed to HOCI (reviewed [12]). Although prolonged HOSCN treatment could lead to increased cytosolic Ca²⁺ accumulation, this was more extensive in cells exposed to HOCl. This contrasts with previous studies with myoblasts [44] and human coronary artery endothelial cells [57], where exposure to both oxidants increased the intracellular Ca²⁺, in a dose-dependent manner. With HCASMC, the increase in intracellular Ca²⁺ observed with HOCI was dependent on the presence of extracellular Ca²⁺, and could be inhibited by pre-treatment of the cells with the L-type channel blocker nisoldipine. This is consistent with the influx of Ca²⁺ from the extracellular environment, and could contribute to necrotic cell death, as reported in macrophages exposed to HOCI [58]. However, HOCI also influenced the re-uptake of Ca²⁺ to the ER/SR and mitochondria, as pre-treatment with inhibitors of SERCA and the mitochondrial Ca²⁺ uniporter, prevented the oxidant-induced increase in cytosolic Ca²⁺ in HCASMC. The ability of HOCI to target SERCA and perturb Ca²⁺ homeostasis has been demonstrated in other cell types [35,57].

In macrophages, HOCl-induced alterations in Ca²⁺ homeostasis are associated with the activation of pro-inflammatory signaling and cytokine release [35]. Similarly, increases in intracellular Ca²⁺ observed in human aortic smooth muscle cells on exposure to HOCI can induce osteoblast differentiation and calcification [59]. With HCASMC, evidence was obtained for increased expression of MCP-1, IL-6, Egr-1 and AP-1 in cells from donor 1596 on exposure to HOCl. In addition, HOCl also increased expression of MMP9 in donor 1559, and OPN in donors 1522 and 1559. HOSCN had no significant effect on gene expression in HCASMC. The ability of HOCl to target SERCA and perturb Ca²⁺ homeostasis has been demonstrated in other cell types [35,57].

Overall, it is not possible to draw firm conclusions from these gene expression data, particularly as no comparable increase in protein expression of IL-6 and OPN was observed. However, these data do highlight the importance of using multiple donors in redox-related experiments with primary human cells. That the HCASMC isolated from a non-plaque region of a diseased artery appear to be more sensitive to MPO oxidant-induced perturbation of inflammatory gene expression could be important in vivo, as this could further amplify the recruitment of immune cells to the site of developing disease, and is a novel finding from this work. This, together with the necrotic and apoptotic cell death, and potential vascular remodeling mediated by OPN, could be a key pathway in lesion development and destabilization. However, further experiments should be performed with HCASMC isolated from diseased and normal arteries to confirm these preliminary results.

In summary, we have shown that exposure of HCASMC to MPO-derived oxidants results in cell death, which occurs concurrently with non-reversible thiol oxidation, loss of GSH and depletion of cellular ATP. That HCASMC are able to readily repair thiol-derived oxidation products formed by HOSCN lends further support to the use of SCN⁻ as a means to slow the development of lesions in atherosclerosis, as reported in in vivo studies with murine models [19,20]. These data also highlight a potential new role for SCN⁻ to stabilize lesions, as demonstrated by the lack of ability of HOSCN to induce increases in pro-inflammatory gene expression and phenotypic changes, which occur with HOCI, and may be particularly relevant to cells in diseased vessels.

Declaration of competing interest
None.

Acknowledgements
The authors are grateful for financial support from the Novo Nordisk Foundation (Laureate Research Grant NNFI3OC0004294 to MJD).

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2021.02.021.

References

C.L. Hawkins, P.E. Morgan, M.J. Davies, Exposure of aconitase to smoking-related oxidants results in loss of GSH and thiol enzymes in young people with high and low serum thiocyanate groups as an indication of smoking, Atherosclerosis 121 (1) (1996) 23–33.


