Comparative reactivity of the myeloperoxidase-derived oxidants
HOCl and HOSCN with low-density lipoprotein (LDL) : implications for foam cell formation in atherosclerosis.

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Short (page heading) title: Modification of LDL by HOSCN

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ABSTRACT
Atherosclerosis is characterised by the accumulation of lipids within macrophages in the artery wall. Low-density lipoprotein (LDL) is the source of this lipid, owing to the uptake of oxidised LDL by scavenger receptors. Myeloperoxidase (MPO) released by leukocytes during inflammation produces oxidants that are implicated in atherosclerosis. Modification of LDL by the MPO oxidant hypochlorous acid (HOCl), results in extensive lipid accumulation by macrophages. However, the reactivity of the other major MPO oxidant, hypothiocyanous acid (HOSCN) with LDL is poorly characterised, which is significant given that thiocyanate is the favoured substrate for MPO. In this study, we comprehensively compare the reactivity of HOCl and HOSCN with LDL, and show key differences in the profile of oxidative damage observed. HOSCN selectively modifies Cys residues on apolipoprotein B100, and oxidises cholesteryl esters resulting in formation of lipid hydroperoxides, 9-hydroxy-10,12-octadecadienoic acid (9-HODE) and F2-isoprostanes. The modification of LDL by HOSCN results macrophage lipid accumulation, though generally to a lesser extent than HOCl-modified LDL. This suggests that a change in the ratio of HOSCN : HOCl formation by MPO from variations in plasma thiocyanate levels, will influence the nature of LDL oxidation in vivo, and has implications for the progression of atherosclerosis.

KEYWORDS
Myeloperoxidase
Low-density lipoprotein
Thiocyanate
Cholesterol
Oxidation
Atherosclerosis
HIGHLIGHTS

- HOSCN induces modification of both the protein and lipid components of LDL
- LDL protein oxidation with HOSCN is less extensive but more specific than HOCl
- Greater oxidation of LDL lipids was observed with HOSCN compared to HOCl
- HOSCN formed modified LDL particles that are taken up by macrophages
- SCN\(^-\) modulates the extent of LDL oxidation and foam cell formation by MPO oxidants

ABBREVIATIONS

Ac-LDL, acetylated low-density lipoprotein; ApoB100, apolipoprotein B100; BCA, bicinchoninic acid; 3-Cl-Tyr, 3-chloro-tyrosine; DFO, desferrioxamine; DTT, dithiothreitol; HMDM, human monocyte-derived macrophages; HCit, homocitrulline; HDL, high-density lipoproteins; HOCl, hypochlorous acid; 9-HODE, 9-hydroxy-10,12-octadecadienoic acid; HOSCN, hypothiocyanous acid; LDL, low-density lipoprotein; LDH, lactate dehydrogenase; LPO, lactoperoxidase; MPO, myeloperoxidase; OCN\(^-\), cyanate; OPA, o-phthaldialdehyde; oxLDL, oxidised LDL; PPAR\(\gamma\), peroxisome proliferator-activated receptor \(\gamma\); REM, relative electrophoretic mobility; SCN\(^-\), thiocyanate; TCA, trichloroacetic acid; TNB, 5-thio-2-nitrobenzoic acid
INTRODUCTION

During atherosclerosis, the arterial wall progressively thickens as a result of cellular accumulation of cholesterol and cholesteryl esters, primarily from low-density lipoprotein (LDL). The retention of LDL in the arterial wall triggers an immune response, resulting in inflammation and the production of a battery of oxidants [1]. These oxidants can modify LDL forming oxidised LDL (oxLDL), which results in the uncontrolled uptake of cholesterol and cholesteryl esters, by macrophages [2]. The term oxLDL is commonly used to describe a variety of LDL preparations that have been modified ex vivo or isolated from biological samples [3]. In vitro studies show that several types of oxLDL can induce detrimental changes to cell function, which promote inflammation and accelerate the development of atherosclerosis [2]. However, the pattern of reactivity of oxLDL is dependent on the type of oxidising system and hence the specific fingerprint of the resulting oxidative modifications [3].

Human lesions are enriched in myeloperoxidase (MPO) [4], an enzyme released by activated neutrophils and monocytes that produces a number of potent oxidants, including hypochlorous acid (HOCl) [5]. High circulating MPO levels are also a significant independent risk factor for the development of coronary artery disease [6], and a prognostic agent for patients with cardiac symptoms (e.g. [7]). MPO interacts with LDL in plasma, with complexes containing LDL and MPO reported in the plasma of a subset of patients with atherosclerosis and high plasma MPO levels [8]. HOCl reacts with LDL in vivo, as evidenced by the presence of elevated levels of LDL modified by this oxidant in human lesions [9, 10] and in patients undergoing hemodialysis [11]. There is growing evidence that HOCl-modified LDL (HOCl-LDL) promotes atherogenesis [12]. Exposure of macrophages to HOCl-LDL results in the accumulation of cholesterol and cholesteryl esters and the formation of lipid-laden foam cells [13]. This is attributed primarily to interaction with the class B scavenger receptors CD36 and SR-B1 [14]. HOCl-LDL may also promote the development of atherosclerosis by stimulating macrophage adhesion to endothelial cells [15] and the induction of macrophage apoptosis (e.g. [16]), a factor known to destabilise atherosclerotic
lesions [17]. Exposure of macrophages to MPO-modified LDL is also reported to induce increased formation of reactive oxygen species, which triggers activation of NF-E2-related factor 2 (Nrf2), and an increase in the expression of antioxidant genes, including heme oxygenase 1 [18].

However, MPO also produces significant amounts of hypothiocyanous acid (HOSCN) under physiological conditions [19]. SCN⁻ is the favoured substrate for MPO, and it has been estimated that up to half of the hydrogen peroxide (H₂O₂) consumed by MPO is used to generate HOSCN under physiological conditions [19]. It is predicted that the generation of HOSCN by MPO may be of greater significance in smokers, owing to their elevated plasma levels of SCN⁻ [20]. The role of HOSCN in lipoprotein modification and lesion formation is not clear, mainly because there is a lack of a specific biomarker for this oxidant in vivo [21]. However, the magnitude of deposits of oxidised LDL and fatty streaks in the aortae of young people have been shown to correlate with serum SCN⁻ levels [22]. Similarly, smokers with high SCN⁻ levels have greater macrophage foam cell populations compared to non-smokers [23]. Protein carbamylation from decomposition of HOSCN to cyanate (OCN⁻) has also been linked to atherogenesis [24], with carbamylated LDL also shown to be pro-atherogenic [25], though OCN⁻ can also be elevated by non-MPO dependent pathways, particularly uraemia [26].

There are a lack of detailed studies relating to the ability of HOSCN to modify LDL, and current published data are conflicting. Evidence has been presented for the MPO-mediated oxidation of the lipid moiety of LDL by a radical pathway catalysed by SCN⁻ [27], which supports an earlier study showing that SCN⁻ is a major endogenous substrate in plasma for MPO to promote the peroxidation of plasma lipids [28]. In contrast, SCN⁻ is also reported to protect LDL from oxidation by MPO/H₂O₂/(Cl⁻ or Br⁻), which prevents macrophage accumulation of cholesterol and cholesteryl esters [29]. Thus, in this study, we comprehensively characterised the reactivity of HOSCN with LDL, and compared the nature and extent of oxidative modification observed with the fingerprint of oxidation induced by HOCl. We also examined the resulting functional consequences of HOSCN-induced LDL modification on macrophage cholesterol and cholesteryl ester
MATERIALS AND METHODS

Materials and Reagents – All aqueous reagents were prepared using nanopure water, filtered through a four stage Milli-Q system. All reagents were from Sigma Aldrich (Castle Hill, NSW, Australia) unless stated otherwise. HOSCN was enzymatically prepared using lactoperoxidase (LPO; from bovine milk; Calbiochem, Kilsyth, VIC, Australia), and used immediately after quantification with 5-thio-2-nitrobenzoic acid (TNB; Sigma-Aldrich) [30, 31], using a molar absorption coefficient of 14,150 M$^{-1}$ cm$^{-1}$ at 412 nm [32]. HOCl was prepared by dilution of a concentrated stock solution of NaOCl (BDH, Poole, Dorset, UK) into PBS pre-treated with Chelex resin (BioRad, Hercules, CA, USA) [31].

Isolation of low-density lipoprotein (LDL) – Plasma was isolated from healthy adult donors with informed consent and local ethical approval (Sydney South West Area Health Service, protocols X09-0013 and X12-0375) in accordance with the Declaration of Helsinki (2000) of the World Medical Association. LDL (density 1.019-1.050) was isolated from plasma using sequential density gradient ultracentrifugation (L-80 Optima; Beckman, Palo Alto, CA, USA) in a vertical rotor (VTi50; Beckman) at 10 °C for 2.5 h at 206,000 g as previously described [33]. The LDL fraction was collected and subsequently washed by ultracentrifugation, density 1.064, in a fixed-angle rotor (Ti70; Beckman) for 20 h at 206,000 g at 10 °C before overnight dialysis at 4 °C into PBS containing 0.1 mg mL$^{-1}$ chloramphenicol and 1 mg mL$^{-1}$ EDTA. The protein concentration of the isolated LDL was assessed using the bicinchonic acid (BCA) assay.

Cell culture – The J774A.1 murine macrophage-like cells (ATCC: TIB-67) were grown in Dulbecco’s modified Eagle’s medium (DMEM – JRH Biosciences, Lenexa, Kansas, USA)
supplemented with 10 % (v/v) Fetal Bovine Serum (Invitrogen, Eugene, Oregon, USA), 2 mM L-glutamine (Thermostrace, Melbourne, Victoria, Australia), 100 U/mL penicillin (Invitrogen) and 0.1 mg mL⁻¹ streptomycin (Invitrogen) in 175 cm² tissue culture flasks at 37 °C in a humidified atmosphere of 5 % CO₂. Prior to all experiments, confluent J774A.1 cells were seeded overnight at a density of 0.5 x 10⁶ cells per well in 12-well culture plates (Costar, Corning, NY, USA). Human monocytes were isolated by countercurrent elutriation, plated in 12-well plates at a density of 1 x 10⁶ cells per well, before washing with RPMI (containing 10% v/v heat-inactivated human serum, 4 mM glutamine and 1% v/v PenStrep) as described previously [33]. The monocytes were incubated (5% CO₂, 37 °C) for 9–11 days, with the medium being changed every 3 days, to enable maturation into macrophages prior to exposure to modified LDL.

**Preparation of modified LDL** – Stock solutions of LDL were purified immediately before treatment using a PD-10 column and diluted to the required concentration (1 mg mL⁻¹ based on apoB100) into PBS pre-treated with Chelex resin to remove contaminating trace metal ions. LDL was exposed to HOSCN or HOCl (0 – 500 µM) for either 30 min or 24 h at 37 °C or KOCN (0 – 2.5 mM) for 24 h at 37 °C. LDL (in PBS) was also treated with MPO (100 nM), H₂O₂ (200 µM) and increasing concentrations of NaSCN (0, 50, 100, 200 µM) for 24 h at 37 °C. Any residual, unreacted excess oxidant was removed using a PD-10 column prior to LDL characterisation or addition to macrophages.

**Determination of LDL relative electrophoretic mobility** – ApoB100 charge modification was determined by measuring the relative electrophoretic mobility (REM) of LDL upon separation by agarose gel electrophoresis [33]. LDL samples (10 µg) were transferred to individual wells of pre-cast 1% w/v agarose gels (Helena Laboratories, Mount Waverley, VIC, Australia) before running in a Ciba Corning system containing barbitone buffer (pH 8.6) at 90 V for 45 min. The gel was fixed in 100% v/v methanol for 30 s and stained with Fat Red stain (2.8 g L⁻¹ Fat Red 7B in 95% v/v
methanol) for 5 to 10 min. The gel was de-stained using 70% v/v methanol and dried at 60 °C for 30 min. REM of each LDL was measured by comparing the distance migrated by modified LDL samples to migration of native/control LDL.

**Separation of LDL by SDS-PAGE** – ApoB100 fragmentation and aggregation were assessed using SDS-PAGE. LDL samples (5 µg) were mixed with loading buffer (10% w/v SDS, 10% v/v glycerol, 2.5% v/v saturated bromophenol blue and 5% v/v 2-mercaptoethanol in 300 mM pH 6.8 Tris-HCl buffer) and reducing agent (500 mM dithiothreitol; DTT). Samples were incubated at 70 °C for 10 min. Non-reduced samples were prepared without the addition of the reducing agent. Both reduced and non-reduced samples (20 µL) were transferred to individual wells of a precast 3-8% w/v Tris-acetate gel (Invitrogen) and run at 150 V for 1 h. Protein bands were stained using Coomassie Blue dye (25% w/v Coomassie blue, 5% v/v acetic acid) for 1-2 h, and de-stained using 12.5% v/v isopropanol containing 10% v/v acetic acid for 24 h.

**Quantification of ApoB100 amino acids** – LDL was treated as described above before delipidation, precipitation and hydrolysis with 4 M methanesulfonic acid containing 0.2% w/v tryptamine as previously described [30, 31]. Amino acid analysis was performed with pre-column derivatisation with o-phthalaldehyde reagent (with 2-mercaptoethanol added) and HPLC separation with fluorescence detection at λ_ex 340 nm and λ_em 440 nm as previously [30, 31].

**Quantification of ApoB100 Cys residues** – The concentration of LDL Cys residues was assessed using ThioGlo 1, as described [31]. The Cys concentration was quantified based on a standard curve constructed with GSH (0 – 5 µM) with fluorescence recorded at λ_ex 384 nm and λ_em 513 nm.

**Quantification of LDL cholesterol and cholesteryl esters** – LDL samples (200 µL) were mixed with 10 µL of EDTA (200 mM) and 10 µL BHT (0.2 mM) and extracted with 200 µL of H_2O, 2.5
mL of methanol and 5 mL of hexane. Samples were centrifuged at 1000 g for 5 min at 10 °C before removal of the hexane layer (4 mL) for drying under vacuum. Samples were redissolved in HPLC mobile phase containing isopropanol (55% v/v) and acetonitrile (45% v/v). Cholesterol and cholesteryl esters were quantified by HPLC as previously [33].

**Measurement of lipid hydroperoxides, 9-hydroxy-10,12-octadecadienoic acid (9-HODE) and F2-isoprostanes** – LDL-derived lipid hydroperoxides (LOOH) were measured using a commercially available lipid peroxide kit (Cayman, MI, USA) as per the manufacturers instructions. 9-HODE was quantified after treatment of LDL with tin (II) chloride (SnCl₂, 1 mM) for 30 min to yield a stable and detectable product before lipid extraction and separation by HPLC as described in [28]. F₂-isoprostanes (F₂-IsoP) were measured using a modification of a previously reported method [34]. Briefly, LDL samples (400 µL) were spiked with 2 ng internal standard 15-F₂-IsoP-d₄ (8-iso-PGF₂α-d₄; Cayman Chemicals, Ann Arbor, MI, USA), then flushed with nitrogen and hydrolysed with 1 M KOH in methanol at 40 °C for 30 min, prior to acidification to pH 4.6. Following centrifugation, the supernatants were applied to prewashed Certify II cartridges (Varian, Lake Forrest, CA, USA), washed with methanol/water (1:1) and hexane/ethyl acetate (75:25), with F₂-isoprostanes eluted with ethyl acetate/methanol (90:10) and dried under vacuum. F₂-isoprostanes were measured by gas chromatography mass spectrometry (GC-MS) using electron capture negative ionisation after derivatisation and silylation as described previously [34]. Ions monitored were m/z 569 and 573 for F₂-IsoP and 15 F₂-IsoP-d₄ respectively.

**Assessment of cholesterol and cholesteryl ester accumulation in macrophages** – Macrophages were washed twice with heat-treated PBS prior to addition of control or each modified LDL (100 µg mL⁻¹) in cell media containing 10% (v/v) lipoprotein deficient serum for 24 h (J774A.1) or 48 h (HMDM). LDL-containing media was removed and cells were washed with heat-treated PBS before determination of cell viability using the lactate dehydrogenase (LDH) assay [33]. Cholesterol
and cholesteryl esters were extracted from cell lysates using methanol and hexane as described previously [33, 35]. Total cholesterol and cholesteryl ester accumulation was quantified by HPLC and expressed relative to cell protein levels, assessed in cell lysates by the BCA assay [33, 35].

**Statistical analyses** – Statistical analyses were performed using GraphPad Prism software 5.0 (GraphPad Software, San Diego, USA) using either 1- or 2-way ANOVA with p < 0.05 taken as significant. Details of specific tests are outlined in the Figure Legends.
RESULTS

Modification of apoB100 on exposure of LDL to MPO-derived oxidants

Exposure of LDL (1 mg protein mL⁻¹) to increasing concentrations of HOSCN or HOCl (100 – 750 µM) for 30 min or 24 h at 37 °C resulted in modification of apoB100 as indicated by amino acid loss, and changes in particle charge, aggregation and fragmentation. The reactivity of HOSCN and HOCl with Cys (thiol) residues on LDL (for 30 min) was investigated using ThioGlo-1. A significant decrease in Cys was observed on exposure of LDL to very low amounts of HOSCN (0.5 µM), which was more significant than the loss of Cys seen in the corresponding experiments with HOCl (Fig. 1A). Loss of apoB100 Cys residues was also observed on exposure of LDL to MPO (5 nM), H₂O₂ (10 µM), Cl⁻ (100 mM) and SCN⁻ (50 – 200 µM) (Fig. S1). In this case, a low concentration of MPO (5 nM) was employed, as higher concentrations resulted in significant loss of the apoB100 Cys residues in the absence of H₂O₂ (data not shown). The presence of SCN⁻ significantly increased the oxidation of Cys residues on exposure of LDL to the MPO/H₂O₂/Cl⁻ system in a dose-dependent manner (Fig. S1). This is attributed to the greater selectivity of HOSCN formed in the presence of SCN⁻ for Cys residues [21] compared to HOCl, which reacts readily with other amino acid residues [36]. Some reduction in apoB100 free Cys residues was also seen in the absence of SCN⁻, though this was not significant compared to non-treated LDL, and was similar to that observed with MPO in the absence of H₂O₂ (Fig. S1).

The susceptibility of other apoB100 amino acid residues to modification by MPO-derived oxidants was examined using an amino acid analysis approach, with methanesulfonic acid hydrolysis to preserve Met and Trp residues [30, 31]. Reaction of LDL with HOSCN (for 30 min) resulted in loss of Trp residues (Fig. 1B), though this is likely to be related to the low pH (< 2) resulting from the hydrolysis conditions [37]. No evidence obtained for the loss of any other amino acid residues including Met, Lys and Tyr, which were sensitive to oxidation by HOCl (Fig. 1C-F).

With HOCl, a significant, dose-dependent, loss of apoB100 Met and Trp residues was observed (Fig. 1B-C). With 100 µM HOCl, formation of the oxidation product, methionine
sulfoxide (MetSO) was observed (Fig. 1D). However, the concentration of MetSO decreased with increasing concentrations of HOCl, consistent with further oxidation, possibly to methionine sulfone [38]. In experiments with high concentrations of HOCl (≥ 500 µM), evidence was also obtained for a significant decrease in Lys and Tyr residues (Fig. 1E-F). No further decrease in Trp loss was observed on longer incubation (24 h) of LDL with either HOSCN or HOCl, though in this case, extensive oxidation of Trp was apparent in the incubation control LDL (Fig. S2). However, exposure of LDL to HOCl for 24 h did result in a greater extent of Lys and Tyr loss (Fig. S2). This is consistent with previous observations, and is attributed to secondary oxidation reactions mediated by apoB100 chloramines, which decompose over 24 h [39, 40].

The reactivity of the HOSCN decomposition product OCN⁻ with LDL was also examined. No changes in the concentration of any amino acid residues were apparent on reaction of LDL (1 mg protein mL⁻¹) with OCN⁻ (500 – 2500 µM) for either 30 min or 24 h (data not shown). However, extended incubation of LDL with OCN⁻ (24 h) resulted in the formation of low levels of homocitrulline (HCit) (Fig. S2F, black bars), which was not reflected by any significant loss of the parent Lys residues (Fig. S2F, white bars). Loss of Trp and Met residues was also observed on exposure of LDL to the MPO system, though significant oxidation of these residues was also observed in the non-treated LDL samples, owing to the incubation conditions employed (24 h at 37 °C; Fig. S3). In this case, the presence of increasing concentrations of SCN⁻ resulted in a dose-dependent reduction in the extent of Met oxidation observed. These results are consistent with the formation of increasing concentrations of HOSCN, which displays a lower reactivity with Met compared to HOCl [36].

The reaction of HOSCN with LDL also resulted in an increase in REM, consistent with apoB100 charge modification, which was slightly greater on 24 h compared to 30 min incubation (Fig. 2A). The change in LDL particle charge was more marked with HOCl treatment, particularly after 24 h incubation (Fig. 2B). Change in REM was seen with OCN⁻ treatment, though this change appeared to be independent of dose or incubation time (Fig. 2C). A significant increase in REM
consistent with charge modification, was also seen on exposure of LDL to the MPO (100 nM) /H₂O₂ (50 µM) /Cl⁻ (100 mM) system (Fig. 2D). The presence of increasing concentrations of SCN⁻ (0 – 200 µM) had no significant additional effect on LDL particle charge (Fig. 2D).

Treatment of LDL with HOSCN (30 min at 37 °C) also resulted in some loss of staining of the parent apoB100 band (500 kD) and an increase in protein fragmentation as assessed by SDS-PAGE performed under reducing conditions (Fig. 3A). The fragmentation of apoB100 on exposure of LDL to HOSCN resulted in the production of a new low-molecular mass protein band at ca. 97 kD (Fig. 3A). In contrast, exposure of LDL to HOCl under identical conditions prior to SDS-PAGE, resulted in a more dramatic loss of the parent protein band, with smearing of the protein bands observed (Fig. 3B). These results are consistent with both non-specific protein fragmentation and extensive aggregation, as reported previously [12, 39]. Similar results were obtained in experiments where LDL was exposed to HOSCN or HOCl for 24 h (data not shown). No change in LDL structural integrity was seen in the analogous experiments with OCN⁻ (Fig. 3A).

Experiments were performed to examine the extent of fragmentation and aggregation of apoB100 observed on exposure of LDL to the MPO/H₂O₂/Cl⁻/SCN⁻ system, with incubation for 24 h. In the absence of SCN⁻, evidence was obtained for the loss of the parent apoB100 band, and the generation of both low- and high-molecular mass bands, consistent with fragmentation and aggregation, respectively (Fig. 3C). Some protection in the integrity of the apoB100 parent band was seen on reaction of LDL with the enzymatic MPO system in the presence of 50 µM SCN⁻, though protein aggregation was still apparent (Fig. 3C). No change in the apoB100 band compared to the incubation control LDL was seen in the presence of 100 µM SCN⁻. However, greater loss of the parent apoB100 band was observed in the presence of 200 µM SCN⁻, though not to the extent seen in the absence of SCN⁻ (Fig. 3C).

**Modification of cholesterol and cholesteryl esters on exposure of LDL to MPO-derived oxidants**

Initial studies examined the reaction of LDL (1 mg protein mL⁻¹) with HOSCN (100 - 750
µM) over a 24 h treatment period at 37 °C. Under these conditions, a significant loss of cholesteryl linoleate and cholesteryl arachidonate was observed compared to the incubation control LDL (Fig. 4A-B), with no changes apparent in the concentration of free cholesterol or other cholesteryl esters, including oleate and palmitate (data not shown). Some loss of cholesteryl linoleate and arachidonate was also apparent on reaction of LDL with HOCl (Fig. 4A-B). However, in this case, a higher concentration of oxidant was required for the loss in each cholesteryl ester to be significant compared to data obtained for HOSCN (Fig. 4A-B). Similarly, no evidence was obtained for the loss of free cholesterol or other cholesteryl esters on reaction of LDL with HOCl (data not shown).

With the MPO system, no loss in cholesterol or any of the cholesteryl esters was observed in the absence of SCN⁻ (Fig. 4C). Addition of SCN⁻ (50 – 200 µM) resulted in a loss of cholesteryl linoleate (Fig. 4C) and cholesterol arachidonate (Fig. 4D), which was significant in the case of linoleate, but not arachidonate.

The loss in cholesteryl linoleate and arachidonate observed on exposure of LDL to HOSCN was accompanied by a corresponding dose-dependent increase in the formation of lipid hydroperoxides, 9-HODE and F₂-isoprostanes (Fig. 5, white bars). These products were not observed to any significant extent in the corresponding experiments with HOCl (Fig. 5, black bars). An increase in the formation of lipid hydroperoxides and 9-HODE was also observed on incubation of LDL with the complete MPO/H₂O₂/Cl⁻ system in the presence, but not absence, of SCN⁻ (Fig. 6). This is consistent with HOSCN-mediated oxidation of cholesteryl linoleate. F₂-isoprostanes and other arachidonate-derived oxidation products were not measured in this case.

The mechanism involved in HOSCN-induced oxidation of LDL cholesteryl esters was investigated by performing the incubations of LDL with HOSCN in the presence of either the radical scavenger butylated hydroxytoluene (BHT) or the iron chelator, desferrioxamine (DFO). Reaction of LDL (1 mg protein mL⁻¹) with HOSCN (100 – 250 µM) for 24 h in the presence of BHT (200 µM) resulted in a significant decrease in the concentration of lipid hydroperoxides (Fig. 7A), 9-HODE (Fig. 7B) and F₂-isoprostanes (Fig. 7C), consistent with the formation of these
products via a radical-mediated pathway. Incubation of LDL with HOSCN in the presence of DFO (50 µM) also resulted in a decrease in the formation of the lipid-derived oxidation products, though the extent of inhibition of product formation was not as marked as that seen with BHT, particularly in the case of 9-HODE (Fig. 7). As these results were consistent with a potential role of iron in the HOSCN-induced lipid peroxidation of LDL, the role of LPO degradation and heme release as a source of exogenous iron during the preparation of HOSCN was examined. Changes in the absorbance of the LPO Soret band was examined by UV-Vis spectroscopy in the presence and absence of H₂O₂, under the conditions used to generate HOSCN, which was isolated prior to reaction with LDL. The spectral changes observed are consistent with the formation of OSCN⁻, shown by an increasing absorbance at 376 nm [41], with a small decrease in the intensity of the LPO Soret band at 412 apparent after 15 min incubation of LPO/H₂O₂/SCN⁻ (Fig. S4); this may reflect some heme degradation, resulting in the release of iron.

**Cellular cholesterol and cholesteryl accumulation in macrophages on exposure to modified LDL**

The functional consequences of LDL oxidation by HOSCN were examined by assessing cholesterol and cholesteryl ester accumulation within macrophage cells. Cholesterol and cholesteryl ester accumulation was quantified by mass using HPLC after exposure of either J774A.1 murine macrophage-like cells or human monocyte-derived macrophage cells (HMDM) to HOSCN-LDL or HOCl-LDL (100 µg protein mL⁻¹). For these experiments, LDL (1 mg protein mL⁻¹) was pre-treated with either HOSCN or HOCl (250 µM) for 30 min or 24 h at 37 °C. With J774A.1 cells, an increase in the cellular accumulation of cholesterol and cholesteryl esters was seen over a 24 h incubation period with LDL pre-treated with HOSCN, but not HOCl, for 30 min (Fig. 8A-B). The extent of cholesterol and cholesteryl ester accumulation was greater on incubation of the J774A.1 cells with LDL pre-treated with HOSCN or HOCl, for 24 h prior to exposure to the cells (Fig. 8C-D), consistent with more extensive modification of LDL after 24 h compared to 30 min incubation. In contrast, only low levels of cholesterol and cholesteryl ester accumulation were seen with LDL pre-
treated with OCN⁻ (1 mM) for 24 h (Fig. 8C-D), which may reflect the low extent of modification observed under these conditions, as quantified by HCit formation (Fig. S2).

Similar results were obtained in experiments with HMDM, which were exposed to each type of modified LDL (100 µg protein mL⁻¹) for 48 h prior to analysis of cholesterol and cholesteryl ester accumulation by HPLC. Evidence for increased cellular lipid accumulation was obtained in experiments with both HOCl and HOSCN-modified LDL on exposure to HMDM (Fig. 9). In this case, HOCl-modified LDL induced greater total cholesterol accumulation, particularly on treatment of HMDM with LDL pre-treated for 24 h (Fig. 9C-D) rather than 30 min (Fig. 9A-B). With HMDM, the extent of total cholesterol accumulation seen with LDL modified by OCN⁻ was similar to that observed with LDL exposed to HOSCN (Fig. 9C-D).

**DISCUSSION**

In this study, we compared the reactivity of the major MPO-derived oxidants HOCl and HOSCN with LDL, to assess the potential role of oxidants derived from SCN⁻ in generating a pro-atherogenic LDL particle under inflammatory conditions. This is significant as SCN⁻ ions are both the favoured substrate for MPO, and elevated in the plasma of smokers, who are more likely to develop atherosclerosis and cardiovascular complications. Exposure of LDL to both HOSCN and HOCl resulted in protein modification leading to alterations to particle charge and the structural integrity of apoB100. HOCl induced a more significant change to LDL charge compared to HOSCN, and induced a greater extent of fragmentation and aggregation, particularly on 24 h incubation. These data agree well with previous studies, and are consistent with secondary oxidation reactions mediated by decomposition of N-chloramines [39, 42]. The greater extent of protein modification observed with HOCl, is also reflected by the amino acid analysis, where modification of apoB100 Cys, Met, Trp, Tyr and Lys residues is observed. This pattern of reactivity agrees well with previous studies [11, 12, 39].

HOSCN treatment also resulted in changes to LDL particle charge and induced apoB100
protein fragmentation, as assessed by SDS-PAGE, though to a lesser extent than HOCl. This is consistent with the more specific pattern of oxidation, with targeting of Cys residues, which has less impact on overall charge. With HOSCN, there was evidence for the formation of a specific protein fragment, with molecular-mass ca. 97 kDa, which does not appear to be formed with HOCl. Although exposure of proteins to HOSCN has been linked to protein unfolding and aggregation under non-reducing conditions via the formation of disulfide bonds, there are a lack of previous data showing fragmentation [21, 30]. It is not certain if this apoB100 fragment is related to the selectivity of HOSCN for Cys (thiol) residues, though evidence for HOSCN-induced protein fragmentation was obtained under both reducing and non-reducing conditions.

Alteration of particle charge was also observed in experiments with the MPO/H₂O₂/Cl⁻ system. In this case, the presence of increasing SCN⁻ did not significantly affect the extent of charge modification, though some protection of protein structural integrity was apparent. This is in contrast to a previous study where a protective effect of SCN⁻ on HOCl-induced LDL charge modification has been observed [27]. However, this difference may be related to both the higher concentrations of SCN⁻ (up to 1 mM) employed, and the inclusion of the chelator DTPA and antioxidant BHT in the former study [27], which influence the reactivity of HOSCN with LDL (Fig. 7). The decrease in apoB100 fragmentation and aggregation seen on exposure of LDL to the MPO/H₂O₂/Cl⁻ system in the presence of increasing amounts of SCN⁻ is attributed to the more specific pattern of amino acid modification observed with HOSCN compared to HOCl, where greater Cys oxidation and reduced loss of Met and Trp residues is observed. Under the conditions used in this study, extensive Lys and Tyr oxidation was not apparent on exposure of LDL to the MPO system, either in the presence or absence of SCN⁻, though other studies have provided evidence for reduced HOCl-mediated Tyr chlorination in the presence of increasing SCN⁻ [43].

Reaction of LDL with HOSCN resulted in the loss of cholesteryl linoleate and arachidonate, and the formation of a number of lipid-derived oxidation products, including lipid hydroperoxides, and specific products of linoleate and arachidonate oxidation, including 9-HODE and F₂-
isoprostanes, respectively. SCN\(^-\) also induced a greater loss of cholesteryl linoleate and increased the formation of lipid hydroperoxides and 9-HODE on incubation of LDL with the MPO/H\(_2\)O\(_2\)/Cl\(-\) system. In this case, the level of lipid hydroperoxides formed appears to be most significant with the lowest concentration of SCN\(^-\) (50 µM; Fig. 6a); this may reflect further oxidation of the hydroperoxides in the presence of higher concentrations of HOCl. Similarly, the formation of lipid hydroperoxides (and 9-HODE) is seen to plateau on exposure of LDL to increasing amounts of isolated HOCl. With the MPO system, no significant lipid oxidation was observed with HOCl, in contrast to other studies [12, 39, 42]. This may be related to the lower concentrations of HOCl employed (≤ 250 fold molar excess HOCl) compared to previous studies (400 - 800-fold molar excess HOCl [39, 42]). The mechanism involved in the HOCl-induced lipid peroxidation of LDL is not clear. With the MPO/H\(_2\)O\(_2\)/SCN\(^-\) system, it has been postulated that lipid peroxidation may be initiated by thiocyanyl radicals (SCN\(^-\)), formed by SCN\(^-\) acting as a substrate for the MPO peroxidase cycle [27]. However, the extent that SCN\(^-\) partitions between the halogenation (two-electron) and peroxidase (one-electron) pathways is not certain, owing to a lack of kinetic data relating to the rate of the one-electron oxidation reactions. Thus, the two-electron reaction of SCN\(^-\) with MPO Compound I is thermodynamically more favourable than the one-electron oxidation of SCN\(^-\) to SCN\(^-\) with consequent formation of Compound II [44, 45]. An alternative hypothesis was suggested by Zhang and colleagues, with MPO-derived protein radicals postulated to promote SCN\(^-\) -induced lipid peroxidation [28]. In our study, lipid peroxidation was not dependent on the presence of MPO, precluding a role of peroxidase-derived radicals in this case.

The antioxidant BHT prevented cholesteryl linoleate and arachidonate loss and the formation of lipid oxidation products on reaction of LDL with HOCl in the absence of MPO, which is consistent with a 1-electron, radical pathway. It is possible in this case, that lipid peroxidation is initiated via the decomposition of apoB100-derived radicals, rather than MPO intermediates. This is supported by the detection of protein modifications prior to lipid oxidation and previous mass spectrometry studies showing a lack of reactivity of HOCl with isolated
phospholipids [46]. LDL lipid oxidation may occur via a mechanism analogous to that reported for HOCl, where radicals formed on decomposition of N-chloramines act as initiators of lipid oxidation [42]. However, the formation of amino thiocyanate (RN-SCN) species is reported to be disfavoured at physiological pH [47], though these species are formed on exposure of poly-Lys to HOSCN [30]. Similarly, other unstable intermediates including thiosulfenyl thiocyanate (RS-SCN) and sulfenic acid (RS-OH) species have been reported on treatment of proteins with HOSCN (e.g. [48]). It is not certain whether decomposition of RS-SCN generates thyl radicals (RS•), or whether this process could be catalysed by the presence of transition metal ions, similarly to the decomposition of analogous sulfenyl chloride intermediates [49]. That partial prevention of lipid peroxidation was observed on treating LDL with HOSCN in the presence of the iron chelator DFO, is consistent with a role of iron. Indeed, loss of the LPO Soret band during the enzymatic preparation of HOSCN used in these studies is supportive of some heme degradation, which may result in iron release, as reported for HOCl [50]. However, further studies are required to elucidate the mechanisms responsible for HOSCN-induced lipid peroxidation, both in the presence and absence of protein, which are beyond the scope of this current study.

The functional consequences of HOSCN-induced LDL modification was examined by assessing the uptake and accumulation of cholesterol and cholesteryl esters by macrophages, as it is well established that HOCl-modified LDL is recognised by scavenger receptors (reviewed [12]). Evidence for increased lipid uptake was obtained with both the macrophage-like cell line J774A.1 and primary HMDM on incubation with HOSCN-modified LDL. However, the extent of cholesterol and cholesteryl ester accumulation was greater on exposure of the macrophages to HOCl-modified LDL, rather than HOSCN-modified LDL. These results are consistent with a previous report that the presence of SCN− (200 µM) prevented uptake of LDL exposed to MPO/H2O2/Cl⁻ [29], though evidence for the accumulation of cholesterol and cholesteryl esters was seen in our experiments with HOSCN-treated LDL. This may reflect either differences in the extent of LDL modification or the method used to quantify cellular cholesterol and cholesterol esters.
Treatment of LDL with OCN also resulted in apoB100 modification, with evidence for the formation of HCit, from carbamylation of Lys residues. The extent of lipid uptake observed on exposure of cells to carbamylated LDL, was similar to that seen with HOSCN-LDL, although LDL was pre-treated with higher amounts of OCN (1 mM) compared to HOSCN (250 µM). This suggests that carbamylation is unlikely to be a major pathway for MPO-dependent macrophage lipid accumulation, particularly as OCN is a relatively minor product of MPO/H₂O₂/SCN⁻ [24].

Our results are consistent with HOCl rather than HOSCN being the major driver of foam cell formation by MPO-induced LDL modification. However, atherosclerosis is a complex, multifaceted disease, and therefore it is possible that HOSCN-LDL may contribute to the disease process by other pathways. Thus, LDL-derived lipid oxidation products, including 9-HODE, which is formed on HOSCN-LDL, are endogenous activators of peroxisome proliferator-activated receptor γ (PPARγ), which is involved in the control of cellular lipid uptake [51]. Activation of PPARγ by LDL-derived 9-HODE has been linked to the transcriptional control of macrophage gene expression, resulting in increased expression of scavenger receptors [51] and decreased CCR2 expression [52], which may promote lesion development in atherosclerosis by promoting lipid uptake and retention of monocytes in the arterial wall, respectively. The effect of HOSCN-LDL on scavenger receptor expression, generation of reactive oxygen species and inflammatory cytokine expression are important areas of future study, particularly in light of recent work showing that LDL modified by MPO-derived chlorinating oxidants is responsible for activating the antioxidant Nrf2 signalling pathway in macrophages [18]. In addition, the role of HOSCN-modified LDL on endothelial cell function has not been characterised. The interaction of HOSCN itself with vascular cells may also play a role in disease (reviewed [21]).

In summary, we show that MPO-derived oxidant HOSCN induces the oxidation of both the protein and lipid components of LDL, which results in the formation of a modified particle that is taken up by macrophages, leading to cellular cholesterol accumulation. Importantly, the pattern of LDL reactivity observed with HOSCN is different to that seen with HOCl, which influences the
extent of uptake of the modified material, and the accumulation of cholesterol and cholesteryl esters within macrophages. Our data clearly demonstrate that SCN⁻ can modulate the type and extent of MPO-induced LDL damage, which affects macrophage cell behaviour, and hence has implications for the development of atherosclerosis. This has significance given that plasma SCN⁻ levels are readily modifiable by both dietary and lifestyle (e.g. smoking) choices.

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FIGURE LEGENDS

Figure 1 – Loss of apoB100 amino acid residues after exposure of LDL to HOSCN and HOCl.
LDL (1 mg protein mL\(^{-1}\) apoB100) was treated with (A) 0.5 – 10 µM HOSCN (white bars) or HOCl (black bars) for 30 min with the concentration of Cys residues quantified using ThioGlo 1 fluorescence (\(\lambda_{\text{EX}} 384\) nm and \(\lambda_{\text{EM}} 513\) nm) or (B-F) 100 – 750 µM of HOSCN (white bars) or HOCl (black bars) for 30 min prior to protein precipitation, hydrolysis and amino acid analysis. Graphs show changes in (B) Trp, (C) Met, (D) MetSO, (E) Lys and (F) Tyr. In (A) concentration of Cys in control LDL is 0.25 – 0.3 µM. Data represent mean ± S.E.M. (n=3 with different LDL donors). *, ** and *** show a significant difference (p < 0.05, 0.01 and 0.001) compared to the control LDL (0 µM) by one-way ANOVA with Dunnett’s post-hoc test. ## and ### represent a significant (p<0.01 and <0.001, respectively) difference on comparing HOSCN to HOCl treatment by two-way ANOVA with Bonferroni’s post-hoc test.

Figure 2 – Modification of LDL charge after exposure to HOSCN, HOCl and the MPO system.
LDL (1 mg protein mL\(^{-1}\)) was treated with increasing concentrations of (A) HOSCN (100 – 500 µM), (B) HOCl (100 – 500 µM), (C) OCN\(^-\) (500 – 2500 µM) or (D) the enzymatic MPO/H\(_2\)O\(_2\)/Cl\(^-\)/SCN\(^-\) system (100 nM MPO, 200 µM H\(_2\)O\(_2\), 50 – 200 µM SCN\(^-\) in PBS containing 140 mM Cl\(^-\)) for 30 min (white bars) and 24 h (black bars) at 37 °C. Treatment with the MPO system was only performed for 24 h at 37 °C. Relative electrophoretic mobility of LDL was calculated relative to the incubation control LDL. Data represent mean ± S.E.M. (n=3 with different LDL donors). *, ** and *** show a significant difference (p < 0.05, 0.01 and 0.001, respectively) compared to the control LDL (0 µM) by one-way ANOVA with Dunnett’s post-hoc test. # shows a significant difference (p < 0.05) between 30 min and 24 h incubation times by two-way ANOVA with Bonferroni’s post-hoc test.

Figure 3 – SDS-PAGE showing fragmentation and aggregation of apoB100 on exposure to
**HOSCN, HOCl and the MPO system.** LDL (1 mg protein mL⁻¹) was treated with increasing concentrations of (A) HOSCN (50 – 500 µM) or OCN⁻ (500 – 2500 µM), (B) HOCl (50 – 500 µM), or (C) the enzymatic MPO/H₂O₂/Cl⁻/SCN⁻ system (100 nM MPO, 200 µM H₂O₂, 50 – 200 µM SCN⁻ in PBS containing 140 mM Cl⁻) for 24 h at 37 °C. LDL (13 µg) was loaded onto the gel after oxidant treatment and reduction with DTT (50 mM). Protein bands were visualised by Coomassie Blue staining. Gels shown represent at least 3 independent experiments with different LDL donors.

**Figure 4 – Loss of cholesteryl linoleate and arachidonate after exposure of LDL to HOSCN, HOCl and the MPO system.** LDL (1 mg protein mL⁻¹) was treated with (A,B) HOSCN (100 - 750 µM; white bars) or HOCl (black bars) (C,D) the enzymatic MPO system containing 100 nM MPO, 200 µM H₂O₂ and 0 – 200 µM SCN⁻ in the presence of 140 mM Cl⁻ ions, for 24 h at 37 °C. The loss in (A,C) cholesteryl linoleate and (B,D) cholesteryl arachidonate was assessed by HPLC after lipid extraction. Data represent mean ± S.E.M. (n=3 with different LDL donors). * and ** show a significant difference (p < 0.05 and 0.01, respectively) compared to the incubation control LDL (0 µM, white bar in C and D).

**Figure 5 – Formation of lipid oxidation products after exposure of LDL to HOSCN and HOCl.** LDL (1 mg protein mL⁻¹) was treated with increasing concentrations (50 – 500 µM) of HOSCN (white bars) and HOCl (black bars) for 24 h at 37 °C. Graphs show the concentration of (A) lipid hydroperoxides, (B) 9-HODE and (C) F₂-isoprostanes after lipid extraction and analysis. Data represent mean ± S.E.M. (n=3 with different LDL donors). * and ** show a significant difference (p < 0.05 and 0.01) compared to the incubation control LDL (0 µM) by one-way ANOVA with Dunnett’s post-hoc test. #, ## and ### represent a significant (p <0.05, 0.01 and 0.001, respectively) difference on comparing HOSCN and HOCl treatments by two-way ANOVA with Bonferroni’s post-hoc test.
Figure 6 – Formation of lipid oxidation products after exposure of LDL to the MPO system.
LDL (1 mg protein mL⁻¹) was treated with the enzymatic MPO system containing 100 nM MPO, 200 µM H₂O₂ and 0 – 200 µM SCN⁻ in the presence of 140 mM Cl⁻ ions for 24 h at 37 °C. Graphs show formation of (A) lipid hydroperoxides and (B) 9-HODE after lipid extraction and analysis. Black bars show controls with MPO ± SCN⁻ in the absence of H₂O₂. Data represent mean ± S.E.M. (n=3 with different LDL donors). * and ** show a significant difference (p < 0.05 and 0.01, respectively) compared to the incubation control LDL (no MPO/H₂O₂(SCN⁻; white bar) by one-way ANOVA with Dunnett’s post-hoc test.

Figure 7 – Reduction of lipid oxidation products induced by HOSCN after supplementation of LDL with butylated hydroxytoluene (BHT) and desferrooxamine (DFO). LDL (1 mg protein mL⁻¹ apoB100) was treated with HOSCN (100 or 250 µM) in the presence and absence of BHT (200 µM; black bars) or DFO (50 µM; striped bars) for 24 h at 37 °C. Graphs show the concentration of (A) lipid hydroperoxides, (B) 9-HODE and (C) F₂-isoprostanes after lipid extraction and analysis. Data represent mean ± S.E.M. (n=3 with different LDL donors). *, ** and *** show a significant difference (p < 0.05, 0.01 and 0.001, respectively) compared to the respective incubation control LDL (0 µM) by one-way ANOVA with Dunnett’s post-hoc test. #, ## and ### represent a significant (p <0.05, 0.01 and 0.001, respectively) difference on comparing samples treated in the presence and absence of BHT or DFO by two-way ANOVA with Bonferroni’s post-hoc test.

Figure 8 – Cholesterol and cholesteryl ester accumulation in J774A.1 murine macrophage-like cells after treatment with HOSCN, HOCl and OCN⁻ modified LDL. J774A.1 cells (0.5 x 10⁶ cells mL⁻¹) were exposed to control LDL, HOSCN-LDL or HOCl-LDL (100 µg protein mL⁻¹) for 24 h at 37 °C. LDL (1 mg protein mL⁻¹) was pre-treated with (A,B) HOSCN or HOCl (250 µM) for 30 min or (C,D) HOSCN or HOCl (250 µM) or OCN⁻ (1 mM) for 24 h prior to addition to cells.
Cholesterol and cholesteryl esters were quantified by mass using HPLC, with results expressed as total cellular cholesterol (A,C) and cholesteryl esters as a percentage (%) of the total cellular cholesterol (B,D). Data represent the mean ± S.E.M. of at least 6 independent experiments with different LDL donors. *, ** and *** show a significant difference (p < 0.05, 0.01 and 0.001, respectively) compared to the incubation control LDL. #, ##, ### represent significant differences (p < 0.05, 0.01 and 0.001, respectively) between different types of modified LDL by two-way ANOVA with Bonferroni’s post-hoc test.

**Figure 9 – Cholesterol and cholesteryl ester accumulation in HMDM after treatment with HOSCN, HOCl and OCN⁻ modified LDL.** HMDM (0.5 x 10⁶ cells mL⁻¹) were exposed to control LDL, HOSCN-LDL or HOCl-LDL (100 µg protein mL⁻¹) for 48 h at 37 °C. LDL (1 mg protein mL⁻¹ apoB100) was pre-treated with (A,B) HOSCN or HOCl (250 µM) for 30 min or (C,D) HOSCN or HOCl (250 µM) or OCN⁻ (1 mM) for 24 h prior to addition to cells. Cholesterol and cholesteryl esters were quantified by mass using HPLC, with results expressed as total cellular cholesterol (A,C) and cholesteryl esters as a percentage (%) of the total cellular cholesterol (B,D). Data represent the mean ± S.E.M. of at least 6 independent experiments with different LDL donors. *, ** and *** show a significant difference (p < 0.05, 0.01 and 0.001, respectively) compared to the incubation control LDL. ## represents a significant difference between different types of modified LDL by two-way ANOVA with Bonferroni’s post-hoc test.
Figure 2
Figure 3

(A) HOSCN (μM) and OCN (μM) treatment shows the presence of Parent ApoB100 and ApoB100 fragment.

(B) HOCl (μM) treatment indicates the formation of Aggregates, Parent ApoB100, and Fragments.

(C) MPO (100 nM), H2O2 (200 μM), and SCN (μM) treatment demonstrates the aggregation of Parent ApoB100 and formation of Fragments.
Figure 4

(A) Linoleate (nmol/mg protein) vs. [Oxidant] (μM)

(B) Arachidonate (nmol/mg protein) vs. [Oxidant] (μM)

(C) Linoleate (nmol/mg protein) with different treatments

(D) Arachidonate (nmol/mg protein) with different treatments
Figure 5

(A) Lipid Hydroperoxide (nM)

(B) 9-HODE (nmol/mg protein)

(C) F2-isoprostanes (nmol/mg protein)
Figure 6

(A) Lipid Hydroperoxides (nM)

(B) 9-HODE (nmol/mg protein)
Figure 7
Figure 8

(A) Total Cholesterol (m mole/mg protein)

(B) % Cholesteryl Ester

(C) Total Cholesterol (m mole/mg protein)

(D) % Cholesteryl Ester

Legend:
- Control-LDL
- HOSCN-LDL
- HOCl-LDL
- OCN-LDL
- No LDL

Statistical Symbols:
- * p < 0.05
- ** p < 0.01
- *** p < 0.001
- ## p < 0.001 (compared to control-LDL)
Figure 9

(A) Total Cholesterol (nmol/mg protein)

(B) % Cholesterol Ester

(C) Total Cholesterol (nmol/mg protein)

(D) % Cholesterol Ester