Oxidative modification of HDLs (high-density lipoproteins) by MPO (myeloperoxidase) compromises its anti-atherogenic properties, which may contribute to the development of atherosclerosis. Although it has been established that HOCl (hypochlorous acid) produced by MPO targets apoA-I (apolipoprotein A-I), the major apolipoprotein of HDLs, the role of the other major oxidant generated by MPO, HOSCN (hypothiocyanous acid), in the generation of dysfunctional HDLs has not been examined. In the present study, we characterize the structural and functional modifications of lipid-free apoA-I and HDL (reconstituted discoidal HDL) containing apoA-I complexed with phospholipid, induced by HOSCN and its decomposition product, OCN\(^-\) (cyanate). Treatment of apoA-I with HOSCN resulted in the oxidation of tryptophan residues, whereas OCN\(^-\) induced carbamylation of lysine residues to yield homocitrulline. Tryptophan residues were more readily oxidized on apoA-I contained in rHDLs. Exposure of lipid-free apoA-I to HOSCN and OCN\(^-\) significantly reduced the extent of cholesterol efflux from cholesterol-loaded macrophages when compared with unmodified apoA-I. In contrast, HOSCN did not affect the anti-inflammatory properties of rHDL. The ability of HOSCN to impair apoA-I-mediated cholesterol efflux may contribute to the development of atherosclerosis, particularly in smokers who have high plasma levels of SCN\(^-\) (thiocyanate).

Key words: atherosclerosis, cholesterol efflux, hypothiocyanous acid, high-density lipoprotein, myeloperoxidase, protein oxidation.

INTRODUCTION

MPO (myeloperoxidase) is released at sites of inflammation by activated phagocytes, where it forms reactive oxidants by catalysing the reaction of halide (Cl\(^-\), Br\(^-\) and I\(^-\)) and pseudo-halide [SCN\(^-\) (thiocyanate)] ions with H\(_2\)O\(_2\), to produce the hypothaloids HOCl (hypochlorous acid), HOBBr (hypobromous acid) and HOSCN (hypothiocyanous acid) respectively (reviewed in [1]). MPO-derived oxidants play an important role in the human immune defense against bacteria; however, excessive or misplaced generation has been linked to a number of diseases, including asthma, chronic inflammation and atherosclerosis [1]. The evidence linking MPO to cardiovascular disease is particularly compelling, with MPO recognised as both a significant independent risk factor for the development of coronary artery disease [2,3], and a prognostic agent for patients with cardiac symptoms (e.g. [4]). In addition, enzymatically active MPO, together with elevated levels of the HOCl biomarker 3-Cl-Tyr (3-chloro-tyrosine), and HOCl-modified proteins, have been detected in human atherosclerotic lesions [5,6].

Human population studies have established that elevated levels of HDLs (high-density lipoproteins) reduce cardiovascular risk. This benefit has been attributed to the ability of HDLs to remove excess cholesterol from macrophages in the artery wall and to modulate various inflammatory processes [7]. However, an increasing body of evidence shows that HDLs isolated from atheroma, and the plasma of patients with established coronary artery disease, lack these anti-atherogenic properties [8,9]. Although the mechanisms responsible for the formation of this dysfunctional HDL are not completely characterized, there is strong evidence to support a role for MPO-derived chlorinating and nitrating oxidants [10–15]. Thus apoA-I (apolipoprotein A-I) isolated from human lesions and the plasma of patients with coronary artery disease contains elevated concentrations of the HOCl biomarker 3-Cl-Tyr [10,12–14]. Moreover, an inverse correlation has been reported between the amount of 3-Cl-Tyr and the cholesterol efflux capacity of the apoA-I, suggesting that MPO-derived oxidants can impair the functionality of HDLs [10,13]. A number of in vitro experiments have investigated the mechanisms involved in the MPO-dependent impairment of the cholesterol efflux capacity of apoA-I. Evidence has been presented for a combined role of methionine oxidation and tyrosine chlorination, particularly at Tyr\(^192\), for the HOCl-induced loss of apoA-I function [12,16–19]. HOCl can also target the tryptophan residues of apoA-I [20–22]. On the basis of site-directed mutagenesis studies, it has been suggested that tryptophan modification, rather than the oxidation of methionine or chlorination of tyrosine, is the major pathway responsible for impaired cholesterol efflux capacity on exposure of apoA-I to HOCl [22].

**Abstract**

Myeloperoxidase-derived oxidants modify apolipoprotein A-I and generate dysfunctional high-density lipoproteins: comparison of hypothiocyanous acid (HOSCN) with hypochlorous acid (HOCl)

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Oxidative modification of HDLs (high-density lipoproteins) by MPO (myeloperoxidase) compromises its anti-atherogenic properties, which may contribute to the development of atherosclerosis. Although it has been established that HOCl (hypochlorous acid) produced by MPO targets apoA-I (apolipoprotein A-I), the major apolipoprotein of HDLs, the role of the other major oxidant generated by MPO, HOSCN (hypothiocyanous acid), in the generation of dysfunctional HDLs has not been examined. In the present study, we characterize the structural and functional modifications of lipid-free apoA-I and HDL (reconstituted discoidal HDL) containing apoA-I complexed with phospholipid, induced by HOSCN and its decomposition product, OCN\(^-\) (cyanate). Treatment of apoA-I with HOSCN resulted in the oxidation of tryptophan residues, whereas OCN\(^-\) induced carbamylation of lysine residues to yield homocitrulline. Tryptophan residues were more readily oxidized on apoA-I contained in rHDLs. Exposure of lipid-free apoA-I to HOSCN and OCN\(^-\) significantly reduced the extent of cholesterol efflux from cholesterol-loaded macrophages when compared with unmodified apoA-I. In contrast, HOSCN did not affect the anti-inflammatory properties of rHDL. The ability of HOSCN to impair apoA-I-mediated cholesterol efflux may contribute to the development of atherosclerosis, particularly in smokers who have high plasma levels of SCN\(^-\) (thiocyanate).

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**INTRODUCTION**

MPO (myeloperoxidase) is released at sites of inflammation by activated phagocytes, where it forms reactive oxidants by catalysing the reaction of halide (Cl\(^-\), Br\(^-\) and I\(^-\)) and pseudo-halide [SCN\(^-\) (thiocyanate)] ions with H\(_2\)O\(_2\), to produce the hypothaloids HOCl (hypochlorous acid), HOBBr (hypobromous acid) and HOSCN (hypothiocyanous acid) respectively (reviewed in [1]). MPO-derived oxidants play an important role in the human immune defense against bacteria; however, excessive or misplaced generation has been linked to a number of diseases, including asthma, chronic inflammation and atherosclerosis [1]. The evidence linking MPO to cardiovascular disease is particularly compelling, with MPO recognised as both a significant independent risk factor for the development of coronary artery disease [2,3], and a prognostic agent for patients with cardiac symptoms (e.g. [4]). In addition, enzymatically active MPO, together with elevated levels of the HOCl biomarker 3-Cl-Tyr (3-chloro-tyrosine), and HOCl-modified proteins, have been detected in human atherosclerotic lesions [5,6].

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**Abbreviations used:** ABCA1, ATP-binding cassette transporter A1; acLDL, acetylated low-density lipoprotein; apoA-I, apolipoprotein A-I; BCA, bicinchoninic acid; 3-Cl-Tyr, 3-chloro-tyrosine; Cy5, indodicarbocyanine; FBS, fetal bovine serum; HCAEC, human coronary artery endothelial cell; HClt, homocitrulline; HDL, high-density lipoprotein; HOSCN, hypothiocyanous acid; HOCl, hypochlorous acid; HT-PBS, heat-treated PBS; ICAM-1, intercellular adhesion molecule 1; LC-MS, liquid chromatography MS; LDL, low-density lipoprotein; LPO, lactoperoxidase; MPO, myeloperoxidase; MS/MS, tandem MS; NF-κB, nuclear factor κB; OCN\(^-\), cyanate; PE, phycoerythrin; PLPC, 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; rHDL, reconstituted discoidal high-density lipoprotein; SCN\(^-\), thiocyanate; SR-B1, scavenger receptor B1; TBS, Tris-buffered saline; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TNF\(_α\), tumour necrosis factor α; VCAM-1, vascular cellular adhesion molecule 1.

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In addition to compromising its cholesterol efflux ability, modification of HDLs by HOCl also reduces the anti-inflammatory properties of the particles, as shown by a significant loss in the ability of HDLs to inhibit TNFα (tumour necrosis factor α)-induced VCAM-1 (vascular cellular adhesion molecule 1) expression in endothelial cells [23,24]. This is attributed to a decreased ability of modified HDLs to bind to SR-B1 (scavenger receptor B1), which appears to occur independently of methionine, tyrosine or tryptophan oxidation [23]. MPO-modified HDLs are also reported to be pro-inflammatory, as exposure of endothelial cells to the modified particle results in activation of dysfunctional particles, the role of the other major MPO-derived oxidant HOSCN remains to be established. Importantly, SCN− is a favoured substrate for MPO, with estimates that up to 50% of the H2O2 consumed by MPO oxidizes SCN− under physiological conditions [25]. The production of HOSCN by MPO is likely to be of major significance in smokers, as a result of their elevated plasma levels of SCN− resulting from the detoxification of hydrogen cyanide present in cigarette smoke [26]. This results in elevated levels of thiol oxidation [26] and decreased formation of 3-Cl-Tyr [27,28]. Indirect evidence supports a role of SCN− in the development of atherosclerosis, as early markers of disease (fatty streaks and lipid-laden macrophages) correlate with serum SCN− levels in the aortae of young people [29,30]. Furthermore, SCN− (cyanate) production via MPO-catalysed oxidation of SCN− has been implicated as an important pathway responsible for the carbamylation of lysine residues and the elevated HCit (homocitrulline) detected as an important pathway responsible for the carbamylation of α-linoleoyl-sn-glycero-3-phosphocholine) (Avanti Polar Lipids) (initial PLPC/apoA-I molar ratio, 100:1) were prepared using the colcholate dialysis method as described previously [40]. Buffer exchange from TBS to endotoxin-free HT-PBS was carried out using Amicon Ultra-2 centrifugal filters (Millipore) and treated with oxidants. The concentration of lipid-free apoA-I was quantified using the BCA (bicinchoninic acid) assay (Pierce) using USA standards.

**Preparation of rHDLs containing apoA-I**

rHDLs containing apoA-I complexed to PLPC (1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine) (Avanti Polar Lipids) (initial PLPC/apoA-I molar ratio, 100:1) were prepared using the cholate dialysis method as described previously [40]. Buffer exchange from TBS to endotoxin-free HT-PBS was carried out using Amicon Ultra-2 centrifugal filters, with three washes at 4000 g for 10 min at 4°C. The resulting apoA-I concentration in the rHDL preparations was measured using the BCA assay as described above.

**Isolation of LDLs (low-density lipoproteins)**

Plasma was isolated from blood collected from healthy donors with informed consent and local ethical approval (Sydney South West Area Health Service, protocol X09–0013 & HREC/09/RPAH/19) in accordance with the Declaration of Helsinki (2000) of the World Medical Association. LDLs (density 1.019–1.050) were isolated from plasma using sequential density gradient ultracentrifugation (L–80 Optima; Beckman).
in a vertical rotor (VTi50; Beckman) at 10°C for 2.5 h at 206,000 g as previously described [41]. LDLs were extracted and subsequently washed by ultracentrifugation, density 1.064, in a fixed-angle rotor (T170; Beckman) for 20 h at 206,000 g at 10°C before overnight dialysis at 4°C into PBS containing 0.1 mg/ml chloramphenicol and 1 g/ml EDTA. The protein concentration of the isolated LDLs was assessed using the BCA assay.

### Preparation of acLDLs (acylated LDLs)

LDLs were acetylated using saturated sodium acetate and acetic anhydride (6 μg/ml of LDL protein) as described previously [41]. Excess reagent was removed by elution through two successive PD10 columns (GE Healthcare) equilibrated with PBS, before using the BCA assay to determine the resulting protein concentration. Modification was confirmed by determining the relative electrophoretic mobility on agarose gels as described previously [41]. The acLDLs were sterile filtered with a 0.45 μm syringe filter (Millipore) before incubation with THP-1 cells for 24 h as described below.

### Protein hydrolysis and amino acid analysis by HPLC

ApoA-I or rHDL (1 mg/ml) were incubated with HOSCN (0–125 μM), HOCl (0–125 μM) or KOCN (0–25 mM) for either 30 min at 22°C or 24 h at 37°C, before precipitation, washing to remove any residual oxidant and hydrolysis with 4 M methanesulphonic acid containing 0.2% trypsin as described previously [35,42]. Amino acid analysis was performed with pre-column derivatization with o-phthalaldehyde reagent (with 2-mercaptoethanol added) and HPLC separation with fluorescence detection at λex = 340 nm and λem = 440 nm as described previously [35,42].

### Tandem MS peptide mass mapping

ApoA-I (1 mg/ml) was incubated with HOSCN (100 or 250 μM) for 30 min at 22°C prior to precipitation with TCA (trichloroacetic acid; 5%, v/v), was washed to remove any residual oxidant and pelleted by centrifugation (5 min at 7500 g, 4°C). Pellets were solubilized in 8 M urea/0.4 M NH₄HCO₃ after washing twice with ice-cold acetone. Proteins were reduced with dithiothreitol (45 mM, 15 min, 50°C) and alkylated with iodoacetamide (100 mM, 15 min, 21°C) prior to 4-fold dilution with H₂O and digestion with either trypsin (4 μg, sequencing grade from Promega) or endoproteinase Glu-C (5 μg, sequencing grade from Roche) for 18 h at 37°C or 22°C respectively. The digestion was stopped by the addition of freshly prepared TCA (0.01%) before filtration of samples through 0.22 μm nanopore filters (Pall) prior to injection.

Peptides were analysed by LC-MS (liquid chromatography MS) in the positive ion mode with a Finnigan LCQ Deca XP ion-trap mass spectrometer equipped to a Finnigan Surveyor HPLC system. Peptides were separated on a Zorbax ODS column (3.0 mm × 250 mm, 5 μm particle size; Agilent Technologies) at 30°C with a flow rate of 0.4 ml/min using the following gradients: 5–20% B over 15 min, 20–50% B over 20 min, then 50–100% B over 5 min with a 15 min wash at 100% B before returning to 5% B over 5 min and re-equilibration for 10 min. Solvent A consisted of 0.1% TFA (trifluoroacetic acid) in H₂O and solvent B was 0.1% TFA in acetonitrile. The electrospray needle was held at 4500 V. Nitrogen, the sheath and sweep gas, was set to 80 and 10 units respectively. The collision gas was helium. The temperature of the heated capillary was 250°C.

### Cholesterol efflux

THP-1 cells were seeded at a density of 1.25 × 10⁶ cells/ml in 12-well plates (Corning) and differentiated using PMA (100 nM) for 72 h before incubation with 150 μg/ml acLDLs in RPMI-1640, 2 mM L-glutamine and 10% lipoprotein-deficient serum (prepared as described previously [41]) for 24 h. Cells were equilibrated in media containing 0.2% BSA (fatty-acid-free), PMA (100 nM), and the nuclear receptors LXR (liver X receptor)/RXR (retinoid X receptor) agonists 9-cis-retinoic acid and TO-901317 (both 5 μM) for 18 h to up-regulate ABCA1 (ATP-binding cassette transporter A1) [43]. Cells were then treated with media containing 0.2% BSA and PMA (100 nM) in the presence and absence of 10 μg/ml native or oxidant-treated lipid-free apoA-I for 4 h. Medium was collected from all wells and cells were washed twice in ice-cold PBS prior to being lysed in 1 ml of nanopure H₂O at 4°C for 30 min. Cell viability was measured by the LDH (lactate dehydrogenase) assay [41] and the protein concentration was measured using the BCA assay.

### Analysis of cholesterol and cholesteryl esters by HPLC

Media and cell lysate samples (800 μl each) were mixed with 10 μl of EDTA (200 mM) and 10 μl of BHT (butylated hydroxytoluene; 0.2 mM) and extracted with 200 μl of nanopure water, 2.5 ml of methanol and 5 ml of hexane. Samples were centrifuged at 2060 g for 5 min at 10°C before removal of the hexane layer (4 ml) and drying under vacuum. Samples were redissolved in HPLC mobile phase containing propan-1-ol and acetonitrile at a 7:3 ratio respectively. Cholesterol and cholesteryl esters were quantified by HPLC as described previously [41]. Total cholesterol mass (cholesterol and cholesteryl esters) was calculated for all media and cell lysate samples and used to measure the percentage of efflux to apoA-I by calculating the cholesterol levels in the media as a percentage of the combined cellular and media cholesterol.

### Quantification of cellular adhesion molecules using flow cytometry

HCAECs were plated overnight at a density of 1 × 10⁵ cells/ml in 24-well plates (Corning) before incubation with native or oxidant-treated rHDL (24 μM apoA-I) for 16 h [44,45]. rHDL was washed after oxidant treatment as described above to remove any residual oxidant. Cells were then stimulated with TNFα (0.2 ng/ml) for 5 h, washed twice with PBS and incubated in PBS containing 10% FBS with fluorochrome-conjugated antibodies, VCAM-1 FITC, E-selectin PE (phycoerythrin) and ICAM-1 (intercellular adhesion molecule 1) PE–Cy5 (indodicarbocyanine), and the appropriate isotype controls (BD Biosciences) for 30 min at 4°C in the dark. Antibody concentrations were previously titrated to ensure that the appropriate amount of antibody was used and that single-, double- and triple-stained compensation controls were included for both stimulated and non-stimulated cells. After antibody incubation, cells were washed twice with PBS and removed from the plate with 2 mM EDTA in PBS. Cells were then pelleted at 500 g for 5 min and resuspended in 500 μl PBS before analysis using a FC500 flow cytometer (Beckman Coulter).

### Statistical analyses

Statistical analyses were performed using GraphPad Prism software 5.0 (GraphPad Software) using either 1- or 2-way ANOVA with P < 0.05 taken as significant. Details of specific tests are outlined in the Figure legends.
Figure 1  Exposure of lipid-free apoA-I to HOSCN results in the loss of tryptophan residues

Lipid-free apoA-I (1 mg/ml, 36 μM) was treated with HOSCN (25–125 μM, white bars) or HOCl (25–125 μM, grey bars) for 30 min at 22°C prior to protein precipitation, hydrolysis and amino acid analysis. Graphs show changes in (a) tryptophan, (b) methionine and (c) methionine sulfoxide (MetSO), with control non-treated apoA-I shown as the black bar in each case. The values shown represent the means ± S.E.M. (n≥6). Data were analysed by one-way ANOVA with Dunnett’s post-hoc test, with *P < 0.05, **P < 0.01 and ***P < 0.001, a significant change compared with non-treated, incubation control apoA-I.

RESULTS

Reactivity of lipid-free apoA-I with HOSCN and OCN−

Initial experiments investigated the susceptibility of lipid-free apoA-I amino acid residues to modification by HOSCN using an amino acid analysis approach, with methanesulphonic acid hydrolysis to preserve methionine and tryptophan residues [35,42]. Treatment of lipid-free apoA-I (1 mg/ml; 36 μM) with various concentrations of HOSCN (25–125 μM) for 30 min at 22°C resulted in a significant dose-dependent loss of tryptophan residues (Figure 1a; white bars). The extent of tryptophan loss was greater with HOSCN than HOCl with oxidant concentrations <100 μM (Figure 1a; grey bars). There was no evidence for the loss of any other amino acid residues upon treatment with HOSCN, including methionine (Figure 1b) and tyrosine (results not shown). In contrast, HOCl induced loss of methionine and formation of the oxidation product methionine sulfoxide (Figures 1b and 1c), although no loss of tyrosine was observed. This may reflect the relatively low molar excess (≤4-fold) of HOCl to protein used in the present study compared with previously published data [12,16–19].

ApoA-I was also incubated with HOSCN, HOCl or OCN− for 24 h at 37°C, to examine the occurrence of secondary oxidation reactions and the effect of oxidant decomposition products, such as OCN−. Under these conditions, exposure of the protein to HOSCN (white bars) or HOCl (grey bar) resulted in a similar extent of tryptophan loss (Figure 2a). The loss of tryptophan observed under these conditions was not as marked as that observed after incubation for 30 min. This is partly related to a greater extent of tryptophan oxidation observed in the controls in the absence of oxidant after prolonged incubation.
Modification of apolipoprotein A-I by HOSCN

Figure 3 Oxidation of amino acid residues on apoA-I in rHDLs on treatment with HOSCN, HOCl and OCN^-

Treatment of rHDLs containing apoA-I (1 mg/ml) with HOSCN (25–125 μM, white bars), HOCl (25–125 μM, grey bars) or OCN^- (5–25 mM, striped bars) for either 30 min at 22°C (a and b) or 24 h at 37°C (c-f) prior to protein precipitation, hydrolysis and amino acid analysis. Graphs show changes in (a and c) tryptophan, (b and d) methionine, (e) lysine and (f) HCit formation, with control non-treated rHDLs shown by a black bar in each case. The values shown represent the means ± S.E.M. (n⩾3). Data were analysed by one-way ANOVA with Dunnett’s post-hoc test. *P < 0.05, **P < 0.01 and ***P < 0.001, a significant change compared with control non-treated rHDLs.

at 37°C. This difference may also reflect the formation of reversible tryptophan-derived products, which decompose under the extended incubation conditions.

Treatment of apoA-I with HOCl, but not HOSCN or OCN^-, resulted in a significant loss of methionine (Figure 2b, grey bar). Exposure of the apoA-I to OCN^- resulted in a decrease in lysine and the formation of HCit, consistent with the carbamylation of these residues in apoA-I (Figures 2c and 2d, striped bars), with no change in the concentration of any other amino acid residue (trypotphan and methionine data shown in Figures 2a and 2b). A significant formation of HCit was only observed with concentrations of OCN^- > 1 mM (Figure 2d); although urea concentrations of up to 100 mM have been reported in patients with chronic renal failure [28]. In contrast, there was no evidence for loss of lysine (Figure 2c) or the formation of HCit (results not shown) after incubation of apoA-I with HOSCN or HOCl. This suggests that the level of OCN^- formed upon decomposition of HOSCN under the conditions employed in the present study is too low to facilitate detectable protein carbamylation at lysine residues.

Reactivity of rHDLs containing apoA-I with HOSCN and OCN^-.

Treatment of rHDLs containing apoA-I complexed with PLPC (1 mg/ml apoA-I) with increasing HOSCN (25–125 μM) for 30 min at 22°C resulted in a dose-dependent decrease in apoA-I tryptophan residues (Figure 3a, white bars). In this case, a similar extent of tryptophan loss was seen in the analogous experiments with HOCl (Figure 3a, grey bars). Exposure of rHDLs to HOCl, but not HOSCN, also resulted in a dose-dependent decrease in the concentration of methionine (Figure 3b). As with lipid-free apoA-I, no significant changes in the levels of any of the other amino acid residues examined were seen with either HOCl or HOSCN (results not shown). A similar pattern of reactivity was observed upon incubation of rHDLs with HOSCN or HOCl for 24 h at 37°C (Figures 3c and 3d). In this case, a detectable level of HCit could also be seen in rHDLs treated with HOSCN (125 μM, Figure 3f; white bar), although no significant decrease in lysine was observed. Loss of lysine and a much more extensive increase in HCit was seen upon treatment of rHDLs with OCN^-, albeit at much higher concentrations (Figure 3e and 3f; striped bars).

LC-MS peptide mass mapping studies with HOSCN-treated apoA-I

The susceptibility of each individual apoA-I tryptophan residue (Trp^5, Trp^50, Trp^72 and Trp^108) to modification by HOSCN, and the nature of the resulting products formed, was examined using a LC-MS/MS (tandem MS) peptide mass mapping approach. Reaction of apoA-I (1 mg/ml) with HOSCN (50 or 125 μM) for 30 min at 22°C before digestion with trypsin resulted in a dose-dependent decrease in the concentration of the four peptides...
Figure 4 Exposure of apoA-I to HOSCN results in the oxidation of all tryptophan residues and the formation of mono- and di-oxygenated products

ApoA-I (1 mg/ml) was treated with HOSCN (50 and 125 μM) for 30 min at 22 °C prior to digestion with either trypsin (a–c) or endoproteinase Glu-C (d–f) and the resulting peptides were analyzed using LC-MS/MS. (a) and (d) show the parent, unmodified tryptophan-containing peptides, (b) and (e) show the mono-oxygenated product peptides with m/z + 16, (c) and (f) show the di-oxygenated product peptides with m/z + 32, resulting from trypsin and endoproteinase Glu-C digestion with Trp8 (black bars), Trp50 (white bars) and Trp108 (striped bars). The recovery of the tryptophan-containing peptides was normalized to QGLLPVLESFK for the tryptic peptides, and DLRQGLLPVLE for the endoproteinase Glu-C digested peptides, as these peptides are not affected by exposure of the protein to HOSCN (Supplementary Figures S4 and S5 respectively at http://www.biochemj.org/bj/449/bj4490531add.htm). The values shown represent the means ± S.E.M. (n≥3). Data were analysed by one-way ANOVA with Dunnett’s post-hoc test, with *P < 0.05 and **P < 0.01, a significant decrease compared with non-treated apoA-I.

that contain tryptophan residues (Figure 4 and Table 1). The loss of the unmodified Trp8-, Trp50- and Trp72-containing peptides corresponded to the detection of six new related peptides (Figure 4 and Table 1). In each case, the mass-to-charge ratios of the product peptides corresponded to the addition of 16 or 32 atomic mass units relative to the native parent peptides, consistent with the addition of one or two oxygen atoms. The results for the Trp108-containing peptide (KWQEMELYR) were complicated by the presence of a methionine residue which was partially oxidized in the oxidant-free samples. Upon treatment with HOSCN, five new related peptides were observed with mass-to-charge ratios that could be assigned to the addition of one, two or three oxygen atoms to the peptide.

Analysis of the MS/MS fragmentation spectra for the product peptides containing Trp8, Trp50 and Trp72 revealed that the oxygen atom incorporation was exclusively at the tryptophan residue (Table 1), as demonstrated for the tryptic peptides of Trp50 (LLDNWDSVTSTFSK; Figure 5) and Trp72 (EQLGPVTQEFWQDNLEK; Supplementary Figure S1 at http://www.biochemj.org/bj/449/bj4490531add.htm). For the Trp108-containing peptide product, oxygen incorporation was observed at both the tryptophan and methionine residues (Table 1). In this case, oxidation of methionine is unlikely to be mediated by HOSCN as no loss of methionine is seen in the amino acid analysis experiments (Figures 1b and 2b), and methionine oxidation occurs during isolation of the apoA-I (compare with the moderate background levels of methionine sulfoxide in Figure 1c).

As the results from the tryptic peptide containing Trp108 were complicated by the presence of the Met112 residue, analogous experiments using endoproteinase Glu-C to digest the apoA-I were also undertaken (Figure 4 and Table 2). As endoproteinase Glu-C exhibits different cleavage selectivity to trypsin it was possible to obtain peptides for all four tryptophan residues that did not contain any residues that were susceptible to reaction with HOSCN. These results confirmed the incorporation of one or two oxygen atoms at each of the apoA-I tryptophan residues (Table 2 and Figure 4). The MS/MS fragmentation spectra for the endoproteinase Glu-C peptides containing Trp8 (DEPPQSPWDRVKD) and Trp108 (VKAKVQPYLDDFQKQWQE), together with the spectral assignments are shown.
Modification of apolipoprotein A-I by HOSCN

Table 1  The specific apoA-I peptides probed on digestion of native and HOSCN-treated ApoA-I with trypsin

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Peptide mass</th>
<th>m/z (charge)</th>
<th>RT (min)</th>
<th>Assignment</th>
<th>Xcorr value</th>
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<tr>
<td>DEPPQSPWDR</td>
<td>1227.3</td>
<td>614.0 (+2)</td>
<td>23.2</td>
<td>Trp^6 (Parent)</td>
<td>1.9 ± 0.2</td>
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<tr>
<td>DEPPQSPW^DR</td>
<td>1243.3</td>
<td>622.0 (+2)</td>
<td>20.6</td>
<td>Trp^6 + 16</td>
<td>1.9 ± 0.3</td>
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<tr>
<td>DEPPQSPW^DR</td>
<td>1259.3</td>
<td>630.0 (+2)</td>
<td>17.2</td>
<td>Trp^6 + 32</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>LLDNW^DSVTSTFSK</td>
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<td>807.4 (+2)</td>
<td>29.3</td>
<td>Trp^20 (Parent)</td>
<td>3.6 ± 0.6</td>
</tr>
<tr>
<td>LLDNW^DSVTSTFSK</td>
<td>1629.8</td>
<td>815.4 (+2)</td>
<td>27.7</td>
<td>Met^16 + 16</td>
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<td>714.6 (+2)</td>
<td>26.5</td>
<td>Met^2 + 16</td>
<td>2.8 ± 0.3</td>
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<td>2.1 ± 0.5</td>
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in Supplementary Figures S2 and S3 (at http://www.biochemj.org/bj/449/bj4490531add.htm).

Effect of HOSCN and OCN− treatment on the ability of apoA-I to efflux cholesterol from lipid-laden macrophages

The effect of HOSCN-induced oxidation on the function of apoA-I was examined by assessing the ability of the modified protein to efflux cholesterol from lipid-loaded macrophages. ApoA-I (1 mg/ml) was exposed to HOSCN (25–125 μM) for 30 min at 22 °C, or 24 h at 37 °C, prior to addition to THP-1 cells pre-loaded with acLDLs and further incubation for 4 h in cell media as described in the Experimental section. The extent of cholesterol efflux was determined by measuring (by HPLC) the concentration of cholesterol in the medium and cells after lysis. No significant change in cell viability was observed upon exposure of the THP-1 cells to either non-treated or modified apoA-I. A significant reduction in the extent of apoA-I-dependent cholesterol efflux from THP-1 cells was seen upon pre-treatment of the apoA-I with HOSCN, under both incubation conditions, compared with the non-treated control protein (Figures 6a and 6b). With 30 min of pre-treatment of apoA-I, a significant loss in efflux was observed upon treatment of the protein with 25 μM HOSCN (Figure 6a), compared with 125 μM HOSCN in experiments with 24 h pre-treatment (Figure 6b). This may be related to the more extensive reduction in cholesterol efflux capacity observed in the non-treated controls upon incubation of apoA-I for 24 h compared with 30 min (8% compared with 12% efflux respectively); this is attributed to a greater extent of oxidation and/or aggregation of the protein in the non-treated controls upon prolonged incubation.

In contrast, no change in cholesterol efflux was observed upon treatment of apoA-I with HOCl (125 μM) for 30 min at 22 °C (Figure 6a), despite the evidence for significant methionine and tryptophan oxidation under these conditions (Figure 1b). However, a significant decrease in cholesterol efflux capacity was observed upon extended incubation (24 h at 37 °C) of the apoA-I with HOCl prior to addition to the THP-1 cells (Figure 6b). This likely reflects more extensive modification of apoA-I induced by lysine-derived chloramines, in accord with previous results [18].

A decrease in cholesterol efflux to apoA-I was also observed upon treatment of the protein with OCN− (Figure 6b), under conditions where significant lysine loss and protein carbamylation was observed (Figures 2c and 2d), in agreement with previous studies [32,47]. Although pre-treatment of apoA-I with OCN− reduced the cholesterol efflux capacity, a significant decrease was only observed with a supraphathological concentration of OCN− (5 mM) compared with HOSCN (125 μM) under identical treatment conditions (Figure 6b). This suggests that decomposition of HOSCN to OCN− is not the major pathway responsible for the formation of dysfunctional apoA-I in the present study. This is supported by the lack of HCl formation observed upon prolonged incubation of HOSCN with lipid-free apoA-I.

Effect of HOSCN on the anti-inflammatory properties of rHDL-containing apoA-I

The effect of HOSCN-induced modifications to apoA-I on the anti-inflammatory properties of HDLs was examined by assessing the ability of rHDL-containing apoA-I to reduce the expression of the cellular adhesion molecules ICAM-1, VCAM-1 and E-selectin in HCAECs stimulated with TNF-α. rHDLs (1 mg/ml apoA-I) were treated with HOSCN (125 μM) for 30 min at 22 °C prior to incubation with HCAECs (at a concentration of 24 μM apoA-I for 16 h). After incubation in the absence or presence of rHDLs, the cells were stimulated with TNF-α (0.2 ng/ml for 5 h), and the expression of cellular adhesion markers was assessed by flow cytometry. Incubation of HCAECs with TNF-α alone (no rHDLs, Figure 7, hatched bars) resulted in the increased expression of ICAM-1, VCAM-1 and E-selectin compared with unstimulated cells (Figure 7, striped bars). Incubation of HCAECs with rHDLs prior to stimulation with TNF-α resulted in a significant decrease in the expression of ICAM-1, VCAM-1 and E-selectin, with the reduction in VCAM-1 particularly pronounced (Figure 7, black bars).

Treatment of rHDLs with HOSCN did not alter the ability of rHDLs to suppress adhesion molecule expression in HCAECs after TNF-α treatment (Figure 7, white bars). In contrast, treatment of rHDLs with HOCl under identical conditions significantly increased ICAM-1, VCAM-1 and E-selectin expression in HCAECs stimulated with TNF-α when compared with both control non-treated rHDLs and
Figure 5  MS fragmentation spectra of LLDNWDSVTSTFSK and related peptides from tryptic digests of HOSCN-treated apoA-I

ApoA-I (1 mg/ml) was treated with HOSCN (125 μM) for 30 min at 22°C prior to digestion with trypsin and data-dependent MS/MS analyses. The results shown are the fragment ions for the doubly charged (a) parent peptide LLDNWDSVTSTFSK m/z 807.4, (b) Trp50 +16 product peptide LLDNW*DSVTSTFSK m/z 815.4 and (c) Trp50 +32 product peptide LLDNW#DSVTSTFSK m/z 823.4. Underlined fragment ion labels indicate fragment ions that incorporate the +16 or +32 modification. The results are representative of at least three independent experiments.

HOSCN-treated rHDLs (Figure 7, grey bars). With ICAM-1 and E-selectin, treatment of rHDLs with HOCl ameliorated any inhibitory effect of rHDLs on expression, with the levels of the adhesion molecules increasing to that observed in the absence of rHDLs (Figure 7). No stimulation of cellular adhesion molecule expression was observed upon incubation of HCAECs with either HOSCN- or HOCl-treated rHDLs in control experiments performed in the absence of TNF-α (results not shown). Experiments were not performed with apoA-I exposed to OCN−, owing to cellular toxicity, which was attributed to residual KOCN remaining after protein purification.

DISCUSSION

Although it is well established that MPO can impair the cardioprotective properties of HDLs by inducing oxidative damage to apoA-I [7,48], the role of SCN−-derived oxidants, particularly HOSCN, in this process is poorly characterized. This is significant given that SCN− is the favoured substrate for MPO, particularly in smokers, where plasma levels of this pseudo-halide ion can be up to 200 μM [26]. In the present study, the reaction of apoA-I with HOSCN was investigated and compared with HOCl, to determine the type and extent of protein modification and the functional affects of these changes, as these oxidants have differential biological effects [49]. HOCl is a potent oxidant, which reacts rapidly with most biological substrates, causing extensive modification [1]. In contrast, HOSCN reacts rapidly with free cysteine (thiol) and selenol residues and to a lesser extent with tryptophan residues [35,50–52].

We show that HOSCN specifically targets apoA-I tryptophan residues, with no detectable loss of other amino acid residues observed, including residues known to be sensitive to oxidation by HOCl. The modification of apoA-I tryptophan residues by HOSCN results in the formation of various mono- and dioxygenated species, which have been attributed to mono- and
Table 2 The specific apoA-I peptides probed on digestion of native and HOSCN-treated ApoA-I with endoproteinase Glu-C

<table>
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<tr>
<th>Sequence</th>
<th>Peptide mass</th>
<th>m/z (charge)</th>
<th>RT (min)</th>
<th>Assignment</th>
<th>Xcorr value</th>
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<tr>
<td>DEPPQSPWDRVKD</td>
<td>1569.7</td>
<td>785.2 (+2)</td>
<td>24.8</td>
<td>Trp² (Parent)</td>
<td>2.4 ± 0.4</td>
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<td>793.2 (+2)</td>
<td>23.4</td>
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<td>2.4 ± 0.3</td>
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<tr>
<td>DEPPQSPWDRVKD</td>
<td>1601.7</td>
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<td>20.5</td>
<td>Trp² + 32</td>
<td>1.5 ± 0.3</td>
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<td>1046.7 (+3)</td>
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<td>Trp² (Parent)</td>
<td>6.7 ± 0.5</td>
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<td>34.8</td>
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<td>Trp² + 32</td>
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<td>26.0</td>
<td>Trp² (Parent)</td>
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<td>24.3</td>
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<td>Trp² (Parent)</td>
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<td>Trp² + 32</td>
<td>2.3 ± 0.6</td>
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<td>DLRGLLPVLE</td>
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<td>627.1 (+2)</td>
<td>31.6</td>
<td>Internal reference</td>
<td>2.4 ± 0.2</td>
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![Figure 6](image.png)  **Figure 6** Effect of HOSCN and OCN− on the cholesterol efflux capacity of apoA-I

ApoA-I (1 mg/ml) was treated with HOSCN (25–125 μM), HOCl (125 μM) or OCN− (5–25 mM) for either (a) 30 min at 22°C or (b) 24 h at 37°C before incubation with THP-1 cells (1.25 × 10⁵ cells exposed to 10 μg/ml apoA-I for 4 h) and measurement of cholesterol efflux with control non-treated apoA-I (black bars), HOSCN-treated apoA-I (white bars), HOCl-treated apoA-I (grey bars) and OCN−–treated apoA-I (striped bars). Values represent the means ± S.E.M. (n ≥ 3).

The oxidation of these residues impairs the cholesterol efflux capacity of lipid-free apoA-I, but does not appear to perturb the anti-inflammatory properties of rHDLs containing apoA-I, as assessed by cellular expression of the adhesion molecules VCAM-1, ICAM-1 and E-selectin in HCAECs stimulated with TNF-α.

From the tryptic digests, Trp¹⁰⁸ of apoA-I appears to be slightly more oxidized by the other tryptophan residues, as determined by the extent of parent peptide (WQEEMELYR) loss, and the high yield of dihydroxytryptophan (Trp¹⁰⁸ + 32 mass units) formed (Figure 4). Similarly, with the Glu-C digests, 30–40% of the peptides containing Trp¹⁰⁷ and Trp¹⁰⁸, compared with 10–15% of those containing Trp¹ and Trp¹⁰⁶, are lost on reaction of apoA-I with 50 μM HOCl (1.4-fold molar excess). However, although a greater loss of parent peptide is detected with Trp¹⁰⁷ and Trp¹⁰⁸ in this case, the formation of tryptophan di-oxyindoylalanine/hydroxytryptophan derivatives has been reported previously [35]. This reaction is likely to be favoured in the case of apoA-I, owing to the lack of free cysteine (thiol) residues on this protein, which are highly reactive with this oxidant (reviewed in [52]). LC-MS peptide mass mapping studies show that all four tryptophan residues are susceptible to oxidation by HOSCN, resulting in the formation of the corresponding mono- and di-oxyindoylalanine derivatives (+16 and +32 mass units) in each case. There are some minor differences between the susceptibility of each tryptophan to HOSCN-mediated oxidation. From the tryptic digests, Trp¹⁰⁸ of apoA-I appears to be slightly more oxidized than the other tryptophan residues, as determined by the extent of parent peptide (WQEEMELYR) loss, and the high yield of dihydroxytryptophan (Trp¹⁰⁸ + 32 mass units) formed (Figure 4). Similarly, with the Glu-C digests, 30–40% of the peptides containing Trp¹⁰⁷ and Trp¹⁰⁸, compared with 10–15% of those containing Trp¹ and Trp¹⁰⁶, are lost on reaction of apoA-I with 50 μM HOCl (1.4-fold molar excess). However, although a greater loss of parent peptide is detected with Trp¹⁰⁷ and Trp¹⁰⁸ in this case, the formation of tryptophan di-oxyindoylalanine/hydroxytryptophan derivatives has been reported previously [35].
oxidation products (peptides containing hydroxytryptophan and dihydroxytryptophan) appears to be similar or greater with Trp⁴ and Trp⁸; this may reflect differences in the ionization efficiencies of the respective peptides.

Modification of apoA-I tryptophan residues has been reported to occur under pathological conditions, with hydroxytryptophan produced at all four tryptophan sites, and dihydroxytryptophan formation at Trp⁸ [49]. It has been postulated that HOCl produced by MPO plays a key role in the induction of tryptophan oxidation in vivo, with evidence for the formation of both hydroxytryptophan and dihydroxytryptophan derivatives on exposure of lipid-free apoA-I to HOCl and a MPO/H₂O₂/Cl⁻ system [21,22,53]. However, the specificity of HOSCN for individual apoA-I tryptophan residues is different to that reported for HOCl, where a higher extent of Trp⁹⁰ and Trp⁰² oxidation is observed compared with that seen with Trp⁴ and Trp⁸ [21]. This may be related to the difference in pKₐ of HOSCN and HOCl resulting in predominantly OSCN⁻ compared with HOCl/OCI⁻ [49]. The observation of dihydroxytryptophan residues on the tryptic peptide containing Trp⁸⁰⁰ on apo-A-I from atheroma, in particular, is consistent with a role of HOSCN in vivo, as this residue appears to be more susceptible to oxidation by HOSCN than HOCl.

Previous site-directed mutagenesis studies are consistent with an essential role for tryptophan residues in the cholesterol acceptor activity of apoA-I, with substitution of each tryptophan residue for leucine significantly reducing the ability of apoA-I to efflux cholesterol [22]. Recent data highlight a key role for Trp⁷ in ABCA1-mediated cholesterol efflux, with this residue shown to be responsible for mediating approximately 50% of the efflux capacity of apoA-I [53a]. Moreover, substitution of all of the tryptophan residues with phenylalanine resulted in the formation of an oxidation-resistant, yet fully functional, apoA-I [22]. The results of the present study support these data, with HOSCN treatment of lipid-free apoA-I shown to both target tryptophan residues, including Trp²; and significantly reduce cholesterol efflux from THP-1 cells loaded with acLDLs. It is interesting to note that the extent of inhibition of cholesterol acceptor activity observed with HOSCN-treated apoA-I was greater than (30 min treatment) or similar to (24 h treatment) that seen with the HOCl-modified protein, despite the lack of reactivity of HOSCN with methionine and tyrosine residues, and the similar extent of tryptophan oxidation observed in each case. With HOSCN, the extent of inhibition correlated well with the level of tryptophan loss observed in amino acid analysis. However, with HOCl, it appears that the oxidation of other residues is important, and differences in the extent of oxidation of specific tryptophan residues may also play a role.

The relationship between tryptophan oxidation and loss of cholesterol efflux capacity seen upon treatment of apoA-I with HOSCN and HOCl may be associated with changes in the secondary and tertiary structure of the protein, which impairs the ability of the apoA-I to interact with ABCA1. Previous studies show that HOSCN-induced tryptophan oxidation correlates with the extent of protein unfolding [35]. Similarly, the decomposition of N-chloramines formed on proteins treated with HOCl is also associated with protein unfolding, with this correlating with the extent of tryptophan oxidation [54]. Alteration of the ability of apoA-I to bind with ABCA1 owing to the impairment of lipid-free apoA-I remodelling is also postulated to be (at least partly) responsible for the loss of cholesterol efflux capacity seen under conditions where Tyr⁴⁰ chlorination and methionine oxidation are observed (reviewed in [55]).

Exposure of rHDLs containing apoA-I (and PLPC) to HOSCN also resulted in the significant oxidation of tryptophan residues. However, this modification did not appear to alter the anti-inflammatory properties of rHDLs, as assessed by the suppression of cellular adhesion molecule expression in HCAECs stimulated with TNF-α. In addition, no stimulation of ICAM-1, VCAM-1 or E-selectin was seen upon treatment of the rHDLs with HOSCN prior to exposure to HCAECs in the absence of TNF-α. In contrast, treatment with HOCl impaired the anti-inflammatory properties of rHDLs, in accordance with previous studies performed with rHDLs containing apoA-I and POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) [23]. In this case, the residues responsible for the HOCl-induced loss of function have not been defined, although mutagenesis studies have suggested that methionine, tyrosine and tryptophan residues are not involved, and hence are distinct from those involved in the loss of cholesterol efflux activity [23]. This may explain the lack of efficacy of HOSCN in impairing the anti-inflammatory properties of rHDLs compared with the cholesterol efflux capacity of lipid-free apoA-I, given that this oxidant targets only tryptophan residues on this protein.

Modification of rHDLs by MPO-derived HOCl is also reported to generate a pro-inflammatory particle [23], although this was not observed in the present study. This may reflect differences in either the nature of the endothelial cells (human coronary artery compared with bovine aortic), the lipid content of the rHDLs (PLPC rather than POPC) or more likely, the ratio of oxidant/apoA-I employed (3.5-fold compared with 10-fold molar excess) and incubation conditions (30 min at 37°C compared with 1 h at 37°C). Thus exposure of the apoA-I to higher concentrations of MPO-derived chlorinating oxidants for a longer period of time induces a greater extent of protein modification (e.g. [21]), with N-chloramine formation and subsequent decomposition favoured under the latter incubation conditions. It has been postulated that N-chloramines may play a role in mediating or controlling the binding of MPO-modified HDLs to endothelial cell receptors, which results in the activation of NF-κB and the expression of VCAM-1 [23].

MPO-catalysed oxidation of SCN⁻ also results in the generation of OCN⁻, as a result of the formation and subsequent decomposition of HOSCN (reviewed in [52]). This may be a major pathway for protein carbamylation and HCit formation at inflammatory sites, including HDLs isolated from atherosclerotic plaques [31,32]. In the present study, HCit formation on lipid-free apoA-I by exposure to OCN⁻ resulted in a significant loss in cholesterol efflux capacity, in contrast with previous studies [32]. This may reflect a greater extent of carbamylation, as the lipid-free apoA-I was exposed to OCN⁻ for 24 h rather than 4 h [32]. It is significant that at least a 40-fold greater concentration of OCN⁻ compared with HOSCN or HOCl was required to see a significant loss of efflux capacity. This suggests that, in the present study, decomposition of HOSCN to OCN⁻ is not the major pathway responsible for loss of ABCA1-mediated cholesterol efflux. It is possible that the extent of cholesterol efflux observed with apoA-I exposed to OCN⁻ may be perturbed by competing macrophage-mediated cholesterol uptake, on the basis of previous studies showing increased binding of carbamylated HDLs to SR-B1 and enhanced lipid uptake [32].

Taken together, the protein characterization and cholesterol efflux experiments reported in the present study suggest that modification of apoA-I by HOSCN may contribute to HDL dysfunction and enhance atherogenesis, particularly in smokers where the levels of SCN⁻ are typically significantly higher than in non-smokers. The cholesterol efflux capacity of HDLs is increasingly being used as a measure of risk assessment in coronary artery disease (reviewed in [9]). Recent studies show a strong inverse association of cholesterol efflux capacity from
macrophages with markers of coronary artery disease [8]. The results from the present study rationalize the finding that smoking is a significant inverse predictor of HDL efflux capacity [8], as the higher levels of SCN− present in the plasma of smokers may contribute significantly to the MPO-induced oxidation of apoA-I; this may occur by direct HOSCN oxidation or possibly through OCN− formation and carbamylation of lysine residues, particularly as MPO selectively targets apoA-I in vivo (e.g. [12,22]).

AUTHOR CONTRIBUTION

Katrina Hadfield performed most of the research, analysed the data and contributed to the preparation of the paper. David Pattison assisted with experimental design, performed some of the research, carried out data analysis and contributed to the preparation of the paper. Bronwyn Brown assisted with the experimental design and performed some of the research. Liming Hou carried out some of the research and participated in experimental discussions. Kerry-Anne Rye assisted with experimental design and participated in experimental discussions. Michael Davies assisted with the experimental design, participated in experimental discussions and contributed to the preparation of the paper. Clare Hawkins designed the experimental plan, directed the research and wrote the paper.

ACKNOWLEDGEMENTS

We thank Dr Philip Morgan and Shirley Nakhla for technical assistance and helpful discussions.

FUNDING

This work was supported by the Australian National Health and Medical Research Council (grant number 570829), the National Heart Foundation of Australia (grant number CR 085 3959) and the Australian Research Council [grant numbers CE0561607 and DP0988311].

REFERENCES

SUPPLEMENTARY ONLINE DATA

Myeloperoxidase-derived oxidants modify apolipoprotein A-I and generate dysfunctional high-density lipoproteins: comparison of hypothiocyanous acid (HOSCN) with hypochlorous acid (HOCl)

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Supplementary Figures S1–S5 are on the following pages

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ApoA-I (1 mg/ml) was treated with HOSCN (125 μM) for 30 min at 22°C prior to digestion with trypsin and data-dependent MS/MS analyses. The results shown are the fragment ions for the doubly charged (a) parent peptide EQLGPVTQEFWDNLEK m/z 967.1, (b) Trp72 +16 product peptide EQLGPVTQEF*WDNLEK m/z 975.1 and (c) Trp72 +32 product peptide EQLGPVTQEF#WDNLEK m/z 983.1. Underlined fragment ion labels indicate fragment ions that incorporate the +16 or +32 modification. The results are representative of at least three independent experiments.
ApoA-I (1 mg/ml) was treated with HOSCN (125 μM) for 30 min at 22°C prior to digestion with endoproteinase Glu-C and data-dependent MS/MS analyses. The results shown are the fragment ions for the doubly charged (a) parent peptide DEPPQSPWDRVKD m/z 785.2, (b) Trp⁸ + 16 product peptide DEPPQSPW*DRVKD m/z 793.2 and (c) Trp⁸ + 32 product peptide DEPPQSPW#DRVKD m/z 801.2. Underlined fragment ion labels indicate fragment ions that incorporate the +16 or +32 modification. The results are representative of at least three independent experiments.

Figure S2  MS fragmentation spectra of DEPPQSPWDRVKD and related peptides from endoproteinase Glu-C digests of HOSCN-treated apoA-I
ApoA-I (1 mg/ml) was treated with HOSCN (125 μM) for 30 min at 22 °C prior to digestion with endoproteinase Glu-C and data-dependent MS/MS analyses. The results shown are the fragment ions for the doubly charged (a) parent peptide VKAKVQPYLDDFQKKWQEE m/z 1190.5, (b) Trp108 +16 product peptide VKAKVQPYLDDFQKW*QEE m/z 1198.5 and (c) Trp108 +32 product peptide VKAKVQPYLDDFQKW#QEE m/z 1206.5. Underlined fragment ion labels indicate fragment ions that incorporate the +16 or +32 modification. The results are representative of at least three independent experiments.
Modification of apolipoprotein A-I by HOSCN

ApoA-I (1 mg/ml) was treated with HOSCN (0–125 μM) for 30 min at 22 °C prior to digestion with trypsin and data-dependent MS/MS analyses. The fragmentation spectrum in (a) was obtained for the doubly charged reference peptide QGLLPVLESFK (m/z 616.0). The selective ion chromatograms in (b), (c) and (d) were obtained by selective ion monitoring (m/z 616.0) of samples of the reference peptide (35.5 min) treated with 0, 50 and 125 μM HOSCN respectively. The results are representative of at least three independent experiments.

Figure S4  MS/MS spectrum and selected ion chromatograms for the peptide (QGLLPVLESFK) used as an internal reference for normalization of tryptophan-containing peptides obtained from tryptic digests of HOSCN-treated apoA-I

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ApoA-I (1 mg/ml) was treated with HOSCN (0–125 μM) for 30 min at 22 °C prior to digestion with endoproteinase Glu-C and data-dependent MS/MS analyses. The fragmentation spectrum in (a) was obtained for the doubly charged reference peptide DLRQGLLPVLE (m/z 627.1). The selective ion chromatograms in (b), (c) and (d) were obtained by selective ion monitoring (m/z 627.1) of samples of the reference peptide (31.6 min) treated with 0, 50 and 125 μM HOSCN respectively. The results are representative of at least three independent experiments.