



## Research Paper

# Low-density lipoprotein modified by myeloperoxidase oxidants induces endothelial dysfunction



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## ABSTRACT

Low-density lipoprotein (LDL) modified by hypochlorous acid (HOCl) produced by myeloperoxidase (MPO) is present in atherosclerotic lesions, where it is implicated in the propagation of inflammation and acceleration of lesion development by multiple pathways, including the induction of endothelial dysfunction. Thiocyanate (SCN<sup>-</sup>) ions are utilised by MPO to produce the oxidant hypothiocyanous acid (HOSCN), which reacts with LDL in a different manner to HOCl. Whilst the reactivity of HOCl-modified LDL has been previously studied, the role of HOSCN in the modification of LDL *in vivo* is poorly defined, although emerging evidence suggests that these particles have distinct biological properties. This is important because elevated plasma SCN<sup>-</sup> is linked with both the propagation and prevention of atherosclerosis. In this study, we demonstrate that both HOSCN- and HOCl-modified LDL inhibit endothelium-mediated vasorelaxation *ex vivo* in rat aortic ring segments. *In vitro* experiments with human coronary artery endothelial cells show that HOSCN-modified LDL decreases in the production of nitric oxide (NO<sup>•</sup>) and induces the loss of endothelial nitric oxide synthase (eNOS) activity. This occurs to a similar extent to that seen with HOCl-modified LDL. In each case, these effects are related to eNOS uncoupling, rather than altered expression, phosphorylation or cellular localisation. Together, these data provide new insights into role of MPO and LDL modification in the induction of endothelial dysfunction, which has implications for both the therapeutic use of SCN<sup>-</sup> within the setting of atherosclerosis and for smokers, who have elevated plasma levels of SCN<sup>-</sup>, and are more at risk of developing cardiovascular disease.

## 1. Introduction

Atherosclerosis is the primary underlying pathology of most cardiovascular diseases. The initial stages of the disease are characterised by loss of endothelial function [1] and chronic inflammation [2], which is coupled with the accumulation of low density lipoprotein (LDL) in the arterial intima [3]. It is well documented that LDL oxidation plays a key role in promoting lesion development in atherosclerosis *via* a number of pathways (reviewed [3,4]). In addition to the well-defined recognition of oxidised LDL (oxLDL) by various scavenger receptors, which leads to the formation of lipid-laden “foam cells”, there is strong evidence to

show a role of oxLDL as a mediator of endothelial dysfunction *via* its action on endothelial nitric oxide synthase (eNOS), which perturbs nitric oxide (NO<sup>•</sup>) bioavailability both *in vitro* [5–7] and *ex vivo* [8–10].

The formation of NO<sup>•</sup> by eNOS is dependent on both the dimeric structure of the enzyme and the binding of multiple co-factors and subunits, including zinc, tetrahydrobiopterin (BH<sub>4</sub>), and calcium-bound calmodulin (CaM) [11]. Uncoupling and disruption to the structure of eNOS therefore leads to a depletion in bioavailable NO<sup>•</sup> and subsequent endothelial dysfunction [12]. This has been implicated as a key pathway to impair arterial distensibility in atherosclerosis, given the potent vasodilatory properties of NO<sup>•</sup> [13]. In addition to uncoupling,

**Abbreviations:** ACh, acetylcholine; ApoB100, apolipoprotein B100; B2M, β2-microglobulin; BH<sub>4</sub>, tetrahydrobiopterin; CaM, calmodulin; DHE, dihydroxyethidine; E<sup>+</sup>, ethidium; eNOS, endothelial nitric oxide synthase; EPR, electron paramagnetic resonance; FAD, flavin adenine dinucleotide; FMN, Flavin mononucleotide; HOCl, hypochlorous acid; HOSCN, hypothiocyanous acid; HCAEC, human coronary artery endothelial cells; HUVEC, human umbilical vein endothelial cells; LDH, lactate dehydrogenase; LDL, low-density lipoprotein; L-NIO, N<sup>5</sup>-(1-iminoethyl)-L-ornithine; MGD, N-methyl-D-glucamine diithiocarbamate; MPO, myeloperoxidase; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NADPH, nicotinamide adenine dinucleotide phosphate; NE, norepinephrine; NO<sup>•</sup>, nitric oxide; 2-OH-E<sup>+</sup>, 2-hydroxyethidium; O<sub>2</sub><sup>-</sup>, superoxide; oxLDL, oxidised low-density lipoprotein; PKC, protein kinase C; RIPA, radioimmunoprecipitation assay; RT-PCR, real-time polymerase chain reaction; SCN<sup>-</sup>, thiocyanate ions; SNP, sodium nitroprusside; TBS, tris-buffered saline; TNB, 5-thio-2-nitrobenzoic acid; 13-HPODE, 13-hydroperoxyoctadecadienoate; 18S, 18S ribosomal RNA

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eNOS activity can be influenced by disruption of CaM binding by several factors, including caveolin-1, which binds to eNOS and prevents L-Arg catalysis to L-citrulline and hence NO<sup>•</sup> production [14]. The production of NO<sup>•</sup> by eNOS is also regulated by a series of complex post-translational modifications, including phosphorylation, acylation, S-nitrosylation, and acetylation [11,15]. Similarly, alterations in the sub-cellular localisation of eNOS can perturb the production of NO<sup>•</sup> by endothelial cells *in vitro* [5,6].

Although it is well accepted that oxLDL can perturb endothelial NO<sup>•</sup> production, the specific pathways responsible are defined by the nature of the oxidising system responsible for the modification of LDL, and the type of endothelial cell under study (e.g. [5,6,16–18]). This may be related, at least in part, to the pattern of oxidation on the LDL, which can differ widely between oxidants [3,4]. There is some debate as to the most relevant type of oxLDL to use as a model in studies relating to atherosclerosis. The most commonly studied oxLDL is generated *via* incubation of native LDL with Cu<sup>2+</sup> ions, which forms a highly-modified particle with extensive lipid oxidation. The relevance of this type of LDL to atherosclerosis has been questioned, largely due to the marked difference in magnitude between the concentration of Cu<sup>2+</sup> used to modify LDL *in vitro*, compared to that measured in diseased tissue [3,19].

Human atherosclerotic lesions contain elevated amounts of proteins that have been modified by the oxidant hypochlorous acid (HOCl) [20], including LDL [21]. This oxidant is produced by the enzyme myeloperoxidase (MPO), which is also elevated in diseased tissue [22] and is recognised as both a risk factor for the development of coronary artery disease and prognostic agent for patient outcome following different cardiac events (reviewed [23]). It is also reported that the presence of HOCl-modified proteins correlates with intimal thickening in atherosclerotic plaques and apolipoprotein content [24]. Additionally, the cationic MPO has an affinity for non-covalent binding to the predominantly anionic LDL to form a complex [25], which has also been shown to augment the chlorinating activity of MPO *in vitro* [26]. Importantly, MPO-LDL complexes are present in the circulation of patients with atherosclerosis, further supporting the *in vivo* relevance of this type of modified LDL [27].

There is significant evidence that the modification of LDL by HOCl promotes atherogenesis (reviewed [28,29]). There are *in vitro* studies that provide support for the induction of endothelial dysfunction by HOCl-modified LDL, with evidence for reduced NO<sup>•</sup> production by delocalisation of the eNOS in human umbilical vein endothelial cells (HUVEC) [5]. Similarly, the ability of RAW 264.7 murine macrophages to produce NO<sup>•</sup> following stimulation with LPS was compromised following exposure to HOCl-LDL [30]. In contrast, there are rather limited data regarding the biological reactivity of LDL modified by hypothiocyanous acid (HOSCN), which is the other major MPO-derived oxidant formed under normal physiological conditions [31]. This is significant because there are conflicting data regarding the role of thiocyanate (SCN<sup>-</sup>), which is the precursor to HOSCN, in the development of atherosclerosis [32].

Smoking results in elevated blood SCN<sup>-</sup> levels and a higher incidence of cardiovascular disease [33]. Similarly, serum SCN<sup>-</sup> levels in smokers correlate with a higher deposition of oxidised LDL and fatty streak formation in the arteries [34,35]. However, supplementation of transgenic, atherosclerosis-prone, mice that over-express human MPO, with SCN<sup>-</sup> resulted in a decreased extent of lesion formation [36]. The reactivity of HOCl and HOSCN are strikingly different [37], which results in the formation of modified LDL particles that have distinct cellular effects [27,38,39]. In light of these conflicting data, and given the importance of endothelial dysfunction in the pathogenesis of atherosclerosis, we performed experiments to assess the effect of HOCl- and HOSCN-modified LDL on endothelium-dependent vasorelaxation *ex vivo*, using pre-constricted rat aortic segments and on the production of NO<sup>•</sup> and eNOS functionality *in vitro* using human coronary artery endothelial cells (HCAEC).

## 2. Materials and methods

### 2.1. Materials and reagents

All Chemicals were purchased from Sigma-Aldrich unless stated otherwise. All aqueous reagents were prepared with nanopure water from a four stage-filtered Milli-Q water system. PBS (Amresco) was treated with Chelex-100 (BioRad) for 2 h to remove trace transition metal ions, prior to filtration and re-adjusting the pH to 7.4 with HCl. HOCl was prepared in PBS by dilution of a NaOCl stock solution (BDH), quantified at 292 nm ( $\epsilon_{292} = 350 \text{ M}^{-1} \text{ cm}^{-1}$ ) at pH 11 [40]. HOSCN was synthesised enzymatically using lactoperoxidase (LPO; from bovine milk; Calbiochem) as described previously [41] and used immediately following quantification with 5-thio-2-nitrobenzoic acid (TNB) at 412 nm ( $\epsilon_{412} = 14150 \text{ M}^{-1} \text{ cm}^{-1}$ ) [42,43].

### 2.2. Isolation and oxidation of human LDL from plasma

Human blood was taken by a qualified phlebotomist with the donor's consent and under ethics approval (Sydney Local Health District, protocol X12-0375) in accordance with the Declaration of Helsinki, 2000 of the World Medical Association. LDL ( $\rho$  1.019–1.050 kg L<sup>-1</sup>) was isolated from plasma using sequential density gradient ultracentrifugation (Optima™ XPN; Beckman) as previously described [44] before four changes of dialysis into PBS containing 0.1 mg mL<sup>-1</sup> chloramphenicol and 1 mg mL<sup>-1</sup> EDTA (Astral Scientific) and stored at 4 °C in the dark. LDL was adjusted to a concentration of 1.0 mg mL<sup>-1</sup> apoB100 following desalting through a PD-10 column (GE Healthcare) to remove chloramphenicol and EDTA and incubation with HOCl or HOSCN (100–250  $\mu\text{M}$ ) at 37 °C for 24 h. A control LDL sample was incubated under the same conditions without oxidant (control LDL). A minimum of 3 independent LDL donors were used in all experiments.

### 2.3. Ex vivo rat aortic ring studies of vasodilation

The thoracic aortae were harvested from 5 Sprague Dawley male rats (~ 150–250 g) and rapidly cleaned and stripped of fat and adhered tissue in Hanks balanced salt solution (HBSS) with Royal Prince Alfred Hospital Animal Ethics Committee approval (protocols # 2014-020, 2014-030 and 2014-040). The thoracic aorta was cut into 4 mm lengths before mounting in 7 mL myograph chambers (Danish Myo Technology, Aarhus, Denmark) containing HBSS and maintained at 37 °C and pH 7.4 by continuous bubbling with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Aortic segments were washed with HBSS before treatment with each preparation of LDL (0.1 mg mL<sup>-1</sup>) for 1 h at 37 °C. The segments were washed with HBSS before measurement of the vasoconstrictive dose-response to norepinephrine (NE; 10<sup>-9</sup>–10<sup>-5</sup> M). The dilatory response of 80% norepinephrine pre-constricted rings to incremental doses of acetylcholine (ACh; 10<sup>-8</sup>–10<sup>-3</sup> M) or endothelium-independent sodium nitroprusside (SNP; 10<sup>-9</sup>–10<sup>-5</sup> M) was determined for each LDL treatment.

### 2.4. Cell culture and viability

Human coronary artery endothelial cells (HCAEC) from 3 independent donors were cultured using MesoEndo Endothelial Cell Growth Media (Cell Applications) and used between passages 4–6. HCAEC were dissociated with 0.1% (w/v) trypsin-EDTA for 1 min at 37 °C before deactivation of trypsin with growth media. Cells were pelleted by centrifugation (5 min at ~ 232g), aspirated, resuspended in media before seeding overnight at a density of 0.5 × 10<sup>5</sup> cells mL<sup>-1</sup> in tissue culture plates. Cells were washed twice with PBS before incubation with the different LDL preparations at a concentration of 0.1 mg mL<sup>-1</sup> apoB100 protein in serum-free defined media supplemented with 5% foetal bovine serum. Cell viability and metabolic activity were measured by the lactate dehydrogenase (LDH) assay and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

assay respectively, as previously [44,45].

## 2.5. Quantification of NO' production

The release of NO' from HCAEC following treatment with each LDL preparation was assessed by electron paramagnetic resonance (EPR) spectroscopy using the Fe(II) chelate spin trap *N*-methyl-D-glucamine dithiocarbamate (MGD) [46]. HCAEC were treated with each LDL preparation for 24 h before washing and incubation at 37 °C for 36 min with Fe(II)-MGD, which was prepared immediately before adding to cells by mixing degassed solutions of FeSO<sub>4</sub> and MGD (both 0.95 mM). The release of NO' into the cell media was then assessed using an X-band EMXplus EPR spectrometer (Bruker) and Xenon software with the following parameters: frequency: 9.8 GHz; centre field: 342 mT; modulation amplitude: 0.6 mT; sweep width: 10 mT; power: 25 mW. Signal intensity was quantified by double-integration of the signal from 338.7 to 345.2 mT. Cells were treated with N<sup>5</sup>-(1-iminoethyl)-L-ornithine (L-NIO, 100 μM) for 1 h prior to the addition of Fe(II)-MGD to assess eNOS independent NO' production.

## 2.6. Measurement of eNOS activity using <sup>3</sup>H citrulline

HCAEC eNOS activity was measured using the NOS activity assay (Cayman Chemicals) following treatment of the HCAEC in 60 mm petri dishes for 24 h prior to dissociation in PBS containing 1 mM EDTA for 5 min at 37 °C. The cell suspension was then pelleted by centrifugation, before lysis with homogenisation buffer and normalisation of the protein concentration to 0.5 mg mL<sup>-1</sup> following BCA assay [44]. 5 μL of each sample was added to a 25 mM Tris-HCl reaction buffer containing 1 μL <sup>3</sup>H-arginine (1 μCi μL<sup>-1</sup>), 750 μM CaCl<sub>2</sub>, 3 μM tetrahydrobiopterin, 1 μM flavin adenine mononucleotide and dinucleotide (FAD and FMN), 2 mM NADPH and 0.2 μM calmodulin, then incubated for 1 h at 21 °C. Reaction stopping buffer (400 μL, 50 mM HEPES, 5 mM EDTA, pH 5.5) was added before separating unreacted <sup>3</sup>H-arginine from <sup>3</sup>H-citrulline product using an equilibrated resin and eluting <sup>3</sup>H-citrulline through a spin cup (22,000g for 30 s). Eluate (200 μL) was transferred to a scintillation vial, mixed with 5 mL scintillation fluid and scintillation was measured using a liquid scintillation counter (Perkin Elmer). Total and background scintillation controls were also performed to calculate the percentage <sup>3</sup>H-citrulline conversion.

## 2.7. RNA isolation and RT-PCR

HCAEC were plated in 12-well plates overnight prior to the addition of LDL or modified LDL and further incubated for 3 or 24 h. RNA extraction was performed using the Promega Reliaprep RNA Miniprep Kit using the manufacturer's protocol with a 45 min DNase step. The RNA concentration of each sample was measured using a Nanodrop 2000C spectrophotometer (Thermo Scientific) before normalizing to 5–10 μg mL<sup>-1</sup>. Reverse transcription was performed using the iScript cDNA synthesis kit and the Eppendorf Mastercycler 2 per manufacturer's instructions (BioRad). The gene expression of eNOS was measured by quantitative real-time polymerase chain reaction (qRT-PCR) and normalised to 18S ribosomal RNA (1818S) and β2-microglobulin (B2M) housekeeping genes with the following primer sequences (5' to 3'): eNOS, forward: GGATCCAGTGGGGGAAACTG, reverse: TGGCTGAACGAAGATTGCCT, 18S, forward: GAGGATGAGGTGGAACGTGT, reverse: TCTTCAGTCGCTCCAGGTCT and B2M, forward: TGTCTC AGTTCACCCACCT, reverse: ATTACATGTCTCGGTCCCAGG. All primers were designed using NCIB software and synthesised by GeneWorks. Quantitative RT-PCR was performed with the iQ SYBR Green supermix as per the manufacturers' instructions (BioRad). Gene expression was measured using the Pfaffl method [47].

## 2.8. Western blot and eNOS protein-protein interactions by co-immunoprecipitation

For immunoprecipitation of eNOS, 1 μg of eNOS antibody (BD Bioscience; cat. # 610297) was incubated with 30 mg of Dynabeads<sup>®</sup> protein G (cat. # 10003D; Life Technologies) for 20 min at 21 °C. Whole cell pellets were lysed in tris-buffered saline (TBS) and 0.1% v/v Triton X-100, then sonicated to dislodge eNOS from the plasma membrane. Immunoprecipitation was then carried out per manufacturer's instruction. HCAEC eNOS-immunoprecipitate and the immunoprecipitate supernatant were stored on ice until separation of whole cell lysate or supernatant, and total eNOS immunoprecipitated (10 μg) by SDS-PAGE. For eNOS uncoupling, HCAEC were treated as previously described for 24 h before being lysed with radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitor cocktail (cat. # P8340; Sigma-Aldrich) and phosphatase inhibitors (PhosStop; Roche). Samples (20 μg protein) were separated by SDS-PAGE at 120 V for approximately 1.5 h in an ice bath to minimise thermal uncoupling of eNOS. The proteins were transferred to a PVDF membrane using an iBlot 2 system (Life Technologies). Membranes were blocked with 5% (w/v) bovine serum albumin in TBS with 0.1% v/v Tween-20 overnight at 4 °C. Blots were incubated with 1:1000 anti-eNOS, anti-(pS1177)-eNOS, (BD Bioscience; cat. # 612393), or (1:2000) β-actin (Santa Cruz; cat # sc-47778) for 2 h at 21 °C. The secondary IgG-HRP-conjugated antibody (Cell Signalling Technologies; cat. # 7076) was added (dilution 1:2000) for 1 h at 21 °C. Chemiluminescence was induced with the enhanced chemiluminescence substrate (Perkin Elmer) and imaged with a ChemiDoc MP (BioRad) imaging system.

## 2.9. Quantification O<sub>2</sub><sup>-</sup> using dihydroethidium by HPLC

After treatment, freshly prepared dihydroethidium (DHE, 50 μM) was added to the HCAEC for 30 min at 37 °C. Cells were removed from the plate after washing by addition of cold PBS and gentle scraping, before precipitation of the proteins and preparation of samples for HPLC analysis as described previously [48]. Separation of 2-hydroxyethidium (2-OH-E<sup>+</sup>) and ethidium (E<sup>+</sup>) from parent DHE was performed using a Synergi Polar RP C18 column (250 × 4.6 mm, 4 μM, 80 Å; Phenomenex), equilibrated with 60% mobile phase A (10% CH<sub>3</sub>CN in 50 mM potassium phosphate buffer pH 2.6) and 40% mobile phase B (60% CH<sub>3</sub>CN in 50 mM potassium phosphate buffer pH 2.6) with a linear increase to 100% mobile phase B over 63 min at a flow rate of 0.5 mL min<sup>-1</sup> using a Shimadzu LC-10AT liquid chromatograph system [48]. Products were quantified by fluorescence (DHE; λ<sub>ex</sub> 358 nm, λ<sub>em</sub> 440 nm, and 2-OH-E<sup>+</sup> and E<sup>+</sup>; λ<sub>ex</sub> 490 nm, λ<sub>em</sub> 565 nm). The concentration of 2-OH-E<sup>+</sup> was normalised to cell protein concentration.

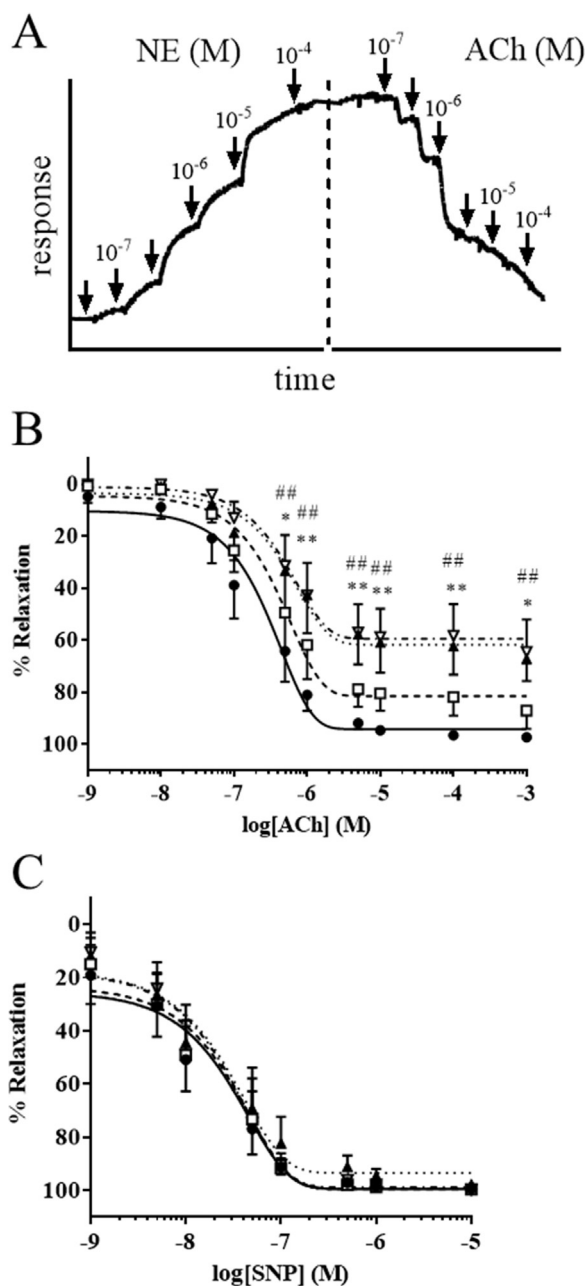
## 2.10. Statistical analyses

Data are expressed as mean ± standard error of the mean (SEM) from at least 3 independent experiments, with statistical analyses performed using either one-way or two-way ANOVA with appropriate post-hoc tests as detailed in the Figure Legends (GraphPad Prism 7, GraphPad Software, San Diego, USA), with p < 0.05 taken as significant.

## 3. Results

### 3.1. Effect of modified LDL on endothelium-dependent vasorelaxation *ex vivo*

The reduction of eNOS activity and subsequent decreased production of NO' *in vivo* is a key pathway to endothelial dysfunction in atherosclerosis, therefore initial studies were performed to examine the effect of HOCl- and HOSCN-modified LDL on vasorelaxation using pre-constricted



**Fig. 1. Modified LDL compromises vasorelaxation in rat aortic rings.** Vasorelaxation dose-response curves of rat aortic ring after incubation with control LDL, HOSCN- or HOCl-modified LDL for 1 h. (A) Representative example of a myographic trace of rat aortic ring vasoconstricted with increasing doses of NE ( $5 \times 10^{-7}$ – $1 \times 10^{-4}$  M) and vasodilated with increasing doses of ACh ( $1 \times 10^{-7}$ – $1 \times 10^{-4}$  M). (B, C) Rat aortic segments pre-constricted with NE and relaxation assessed by accumulative dose of (B) ACh ( $10^{-9}$ – $10^{-3}$  M) and (C) SNP ( $10^{-9}$ – $10^{-5}$  M). Symbols represent (●) control, (□) control LDL, (▽) LDL modified by HOSCN (250  $\mu$ M, 24 h) and (▲) LDL modified by HOCl (250  $\mu$ M, 24 h). Non-linear dose-response curves; (solid line) control, (dashed line) control LDL, (dotted line) HOSCN-LDL and (dashed and dotted line) HOCl-LDL. Data represent the mean  $\pm$  SEM (n = 5). Two-way ANOVA with Dunnett's multiple comparison test. \* and \*\* represents significant difference ( $p < 0.05$  and  $0.01$ , respectively) between HOSCN-modified LDL compared to the control. ## represents significant difference ( $p < 0.01$ ) between HOCl-modified LDL compared to the control.

rat aortic segments. Aortic rings were constricted with norepinephrine ( $10^{-9}$ – $10^{-5}$  M) and dilated with increasing concentrations of acetylcholine ( $10^{-8}$ – $10^{-3}$  M), to enable assessment of the return to baseline tension, measured by myography (Fig. 1A). Treatment of the aortic rings with

HOSCN- or HOCl-modified LDL ( $0.1 \text{ mg mL}^{-1}$ ) for 1 h resulted in a significant decrease in pre-constricted vessel relaxation in response to acetylcholine ( $E_{\text{max}}$  60%) compared to the non-treated aortic rings ( $E_{\text{max}}$  94%) in each case (Fig. 1B).

A decrease in vessel relaxation was also observed in experiments with the control LDL, but in this case, the decrease was less pronounced ( $E_{\text{max}}$  80%), and not statistically significant compared control aortic rings (Fig. 1B). In contrast, the endothelium-independent vasorelaxation responses as assessed by addition of sodium nitroprusside ( $10^{-9}$ – $10^{-5}$  M) to pre-constricted rings were not significantly different between the control and all LDL treatments (control: 99.4%, control LDL: 99.5%, HOSCN-modified LDL: 93.3% and HOCl-modified LDL: 98.8%; Fig. 1C). This suggests that the effect of HOCl and HOSCN-modified LDL on the aortic ring relaxation is related to its effects on the endothelium, therefore further *in vitro* experiments were performed with HCAEC to examine the cellular mechanism involved.

### 3.2. Effect of modified LDL on NO<sup>•</sup> production and eNOS activity in HCAEC

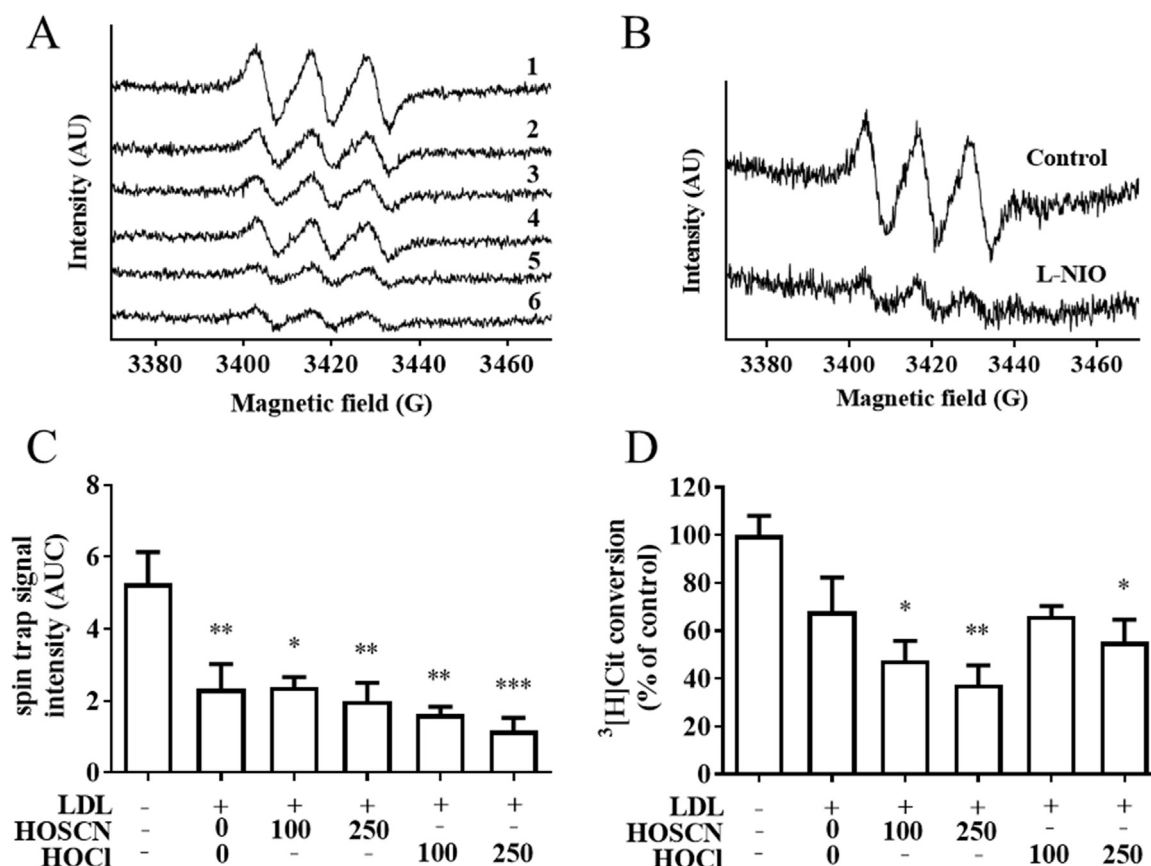
HCAEC ( $0.5 \times 10^5$  cells  $\text{mL}^{-1}$ ) were treated with each modified LDL ( $0.1 \text{ mg mL}^{-1}$ ) for 24 h before addition of the Fe(II) chelate spin trap MGD and assessment of NO<sup>•</sup> production by EPR spectroscopy. Exposure of HCAEC to LDL modified by either HOCl or HOSCN (100–250  $\mu$ M) resulted in a significant decrease in the intensity of the Fe-MGD-NO adduct compared to the non-treated cells (Figs. 2A, C). This is consistent with a decrease in NO<sup>•</sup> production by the cells. A loss in EPR signal intensity was also observed in the analogous experiments with the incubation control LDL, which could be associated with oxidation of the LDL during the incubation conditions either before or after addition to the HCAEC. The contribution of eNOS activity in HCAEC to the formation of the Fe-MGD-NO adduct was verified by pre-treatment of the HCAEC with the eNOS inhibitor with L-NIO (100  $\mu$ M), which almost completely ablated the EPR signal (Fig. 2B).

The effect of each modified LDL on the enzymatic activity of eNOS within the HCAEC was measured by quantification of the conversion of  $^3\text{H}$ -arginine to  $^3\text{H}$ -citrulline. Incubation of the HCAEC with HOCl- or HOSCN-modified LDL resulted in a decrease in  $^3\text{H}$ -citrulline formation, consistent with reduced eNOS activity (Fig. 2D). The decrease in activity was dependent on the concentration of oxidant used to modify the LDL and was more marked with HOSCN-modified LDL compared to HOCl-modified LDL (Fig. 2D). In this case, the decrease in eNOS activity was greater with the modified LDL compared to that seen in the corresponding experiments with the incubation control LDL (Fig. 2D).

To confirm that the decrease in NO<sup>•</sup> production and eNOS activity was not related to cytotoxic effects of each modified LDL, we examined the viability of the HCAEC following exposure to each LDL for 24 h as described above. No change in viability was observed by assessing either LDH leakage (black bars) or alterations in metabolic activity by MTT assay (white bars) (Fig. 3). Taken together, these data are consistent with reduced capability for eNOS to synthesise NO<sup>•</sup> after treatment with HOSCN- and HOCl-modified LDL that is independent of perturbations in cell viability.

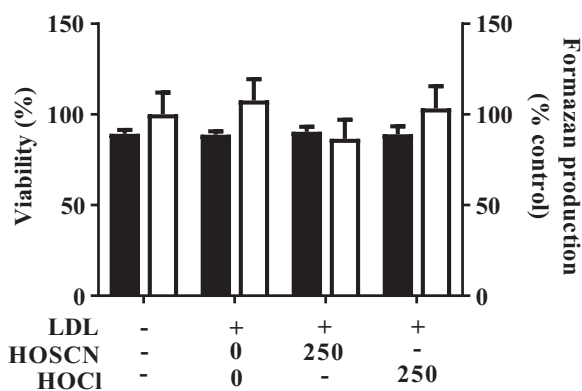
### 3.3. Effect of modified LDL on eNOS expression, post-translational modification, coupling and O<sub>2</sub><sup>•-</sup> production in HCAEC

To examine the pathways involved in the modified LDL induced loss in eNOS activity and NO<sup>•</sup> production, eNOS mRNA and protein expression were assessed by qRT-PCR and Western blotting, respectively. No change in the expression of eNOS mRNA was observed on exposure of HCAEC to control LDL or HOCl-modified LDL for 3 or 24 h (Fig. 4A). In contrast, treatment of HCAEC with HOSCN-modified LDL for 3 and 24 h resulted in an unexpected increase in the expression of eNOS mRNA (Fig. 4A). Western blotting studies were also performed to assess



**Fig. 2. Treatment of HCAEC with modified LDL decreases NO<sup>•</sup> production and eNOS activity.** (A) Representative EPR spectra of Fe-MGD-NO adducts showing production of NO<sup>•</sup> by HCAEC over 36 min at 37 °C in control cells (spectrum 1) and after treatment of the cells with either incubation control LDL (spectrum 2), HOSCNC-modified LDL (100 μM HOSCNC, spectrum 3; 250 μM HOSCNC, spectrum 4) or HOCl-modified LDL (100 μM HOCl, spectrum 5; 250 μM HOCl, spectrum 6) with cells exposed to each LDL (0.1 mg mL<sup>-1</sup>) for 24 h. (B) EPR spectra from HCAEC as in (A) but in control cells in the presence and absence of the eNOS inhibitor L-NIO (100 μM). (C) Graph showing the EPR signal area (AUC; area under the curve) assessed by double integration of the Fe-MGD-NO spectra as a measure of relative NO<sup>•</sup> production. (D) Graph showing eNOS activity in HCAEC treated with each modified LDL as for (A), measured by the conversion of <sup>3</sup>H-arginine to <sup>3</sup>H-citrulline. Data in (C) and (D) represent mean ± SEM of n ≥ 3 with \*, \*\* and \*\*\* showing significance compared to the non-treated control with p < 0.05, 0.01 and 0.001, respectively, by one-way ANOVA with Dunnett's post hoc test.

the effect of modified LDL treatment on the protein levels of eNOS and the extent of eNOS phosphorylation, which plays a key role in eNOS activation and influences NO<sup>•</sup> production [11,15]. However, no significant changes in either total eNOS protein or eNOS phosphorylation

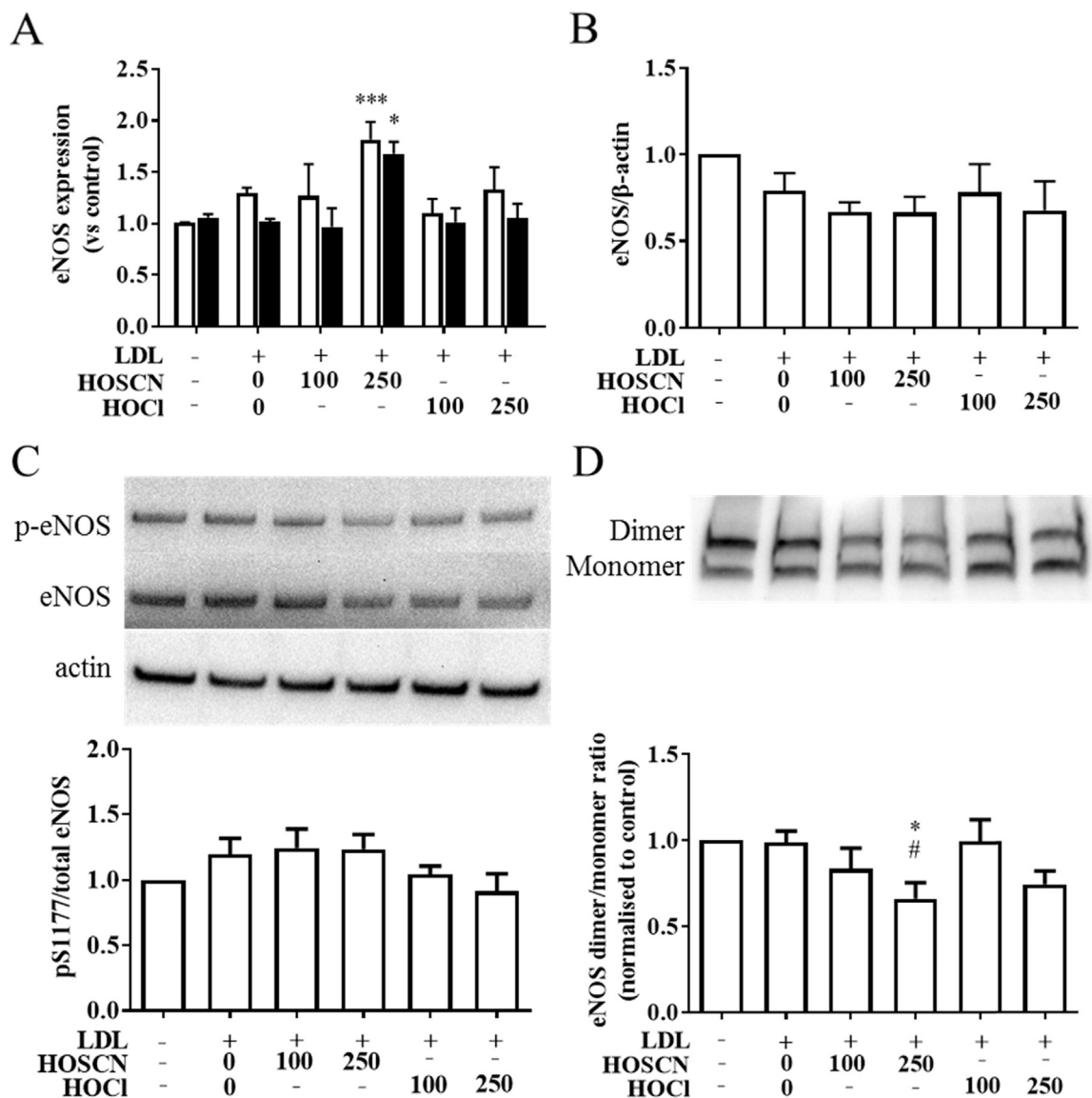


**Fig. 3. No change in viability on treatment of HCAEC with modified LDL for 24 h.** HCAEC were incubated in the absence and presence of incubation control LDL, LDL modified by HOSCNC (250 μM, 24 h) or LDL modified by HOCl (250 μM, 24 h). HCAEC viability (LDH assay; black) and metabolism (MTT assay; white) was determined after 24 h treatment with LDL (0.1 mg mL<sup>-1</sup>). Data represent mean ± SEM of n ≥ 3 with no significant alterations in viability seen between control and LDL treatments by one-way ANOVA with Dunnett's post hoc test.

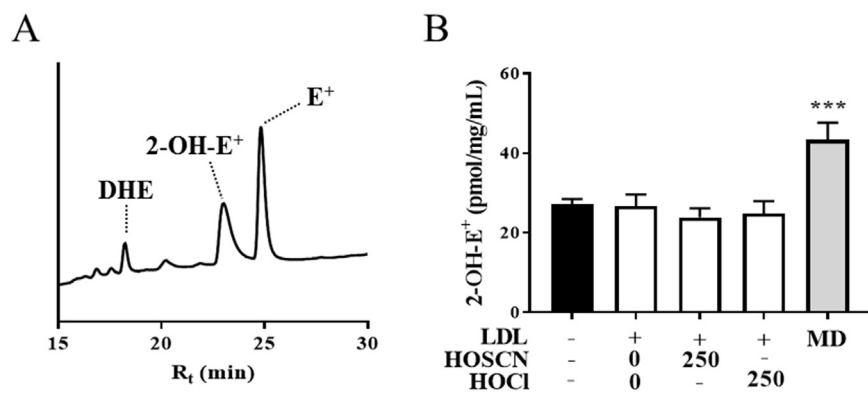
(at S1177) relative to a β-actin loading control were observed on treatment of HCAEC with any of the LDL preparations for short (< 3 h) or extended (24 h) incubation times (Figs. 4B, 4C).

To further examine eNOS functionality, the ratio of protein dimer: monomer expression was assessed. Treatment of HCAEC with HOSCNC-modified LDL resulted in a significant decrease in the dimer: monomer ratio of eNOS compared to the non-treated control cells, or cells exposed to the incubation control LDL (Fig. 4D). A decrease in the dimer: monomer was also seen in the experiments with HOCl-modified LDL, but in this case, the changes were not significant compared with either the non-treated control or incubation control LDL samples (Fig. 4D).

Uncoupling of eNOS results in the disruption of the electron transport from the reductase domain of the enzyme, which decreases NO<sup>•</sup> production owing to the direct reduction of O<sub>2</sub> to O<sub>2</sub><sup>•-</sup> and elevated formation of peroxynitrite [12]. Alterations in the production of O<sub>2</sub><sup>•-</sup> by HCAEC exposed to each modified LDL were therefore examined by measuring the formation of 2-OH-E<sup>+</sup> by HPLC following addition of DHE to the cells. However, no change in the concentration of 2-OH-E<sup>+</sup> was observed on addition of DHE to HCAEC treated with either HOCl- or HOSCNC-modified LDL for 24 h, compared to the non-treated cells (Fig. 5). In contrast, exposure of the HCAEC to menadione (50 μM) for 30 min as a positive control resulted in the detection of a significant increase in the formation of 2-OH-E<sup>+</sup>, consistent with elevated O<sub>2</sub><sup>•-</sup> production (Fig. 5). This suggests that the production of O<sub>2</sub><sup>•-</sup> by HCAEC is not influenced by treatment with modified LDL under the conditions employed in this study. The



**Fig. 4.** Effect of modified LDL on eNOS expression, phosphorylation and coupling in HCAEC. (A) eNOS gene expression after treatment of HCAEC with each modified LDL (0.1 mg mL<sup>-1</sup>) for 3 h (white bars) or 24 h (black bars) normalised to 18S and B2M housekeeping genes. (B, C) Western blot analysis of eNOS, eNOS phosphorylation (at S1177) and β-actin in HCAEC after 24 h treatment with each modified LDL. Graphs show densitometry of (B) total eNOS relative to β-actin and (C) phosphorylated eNOS relative to total eNOS. (D) Western blot analysis of the uncoupling of eNOS in HCAEC treated with modified LDL for 24 h as measured by the ratio of dimer: monomer bands assessed by densitometry. Data represent mean ± SEM of n ≥ 3 with \*, \*\* and \*\*\* showing significance compared to the non-treated control with p < 0.05, 0.01 and 0.001, respectively, with # and ## representing p < 0.05 and 0.01 compared to incubation control LDL using one-way ANOVA with Dunnett's (A) or Sidak's (B–D) post hoc test.



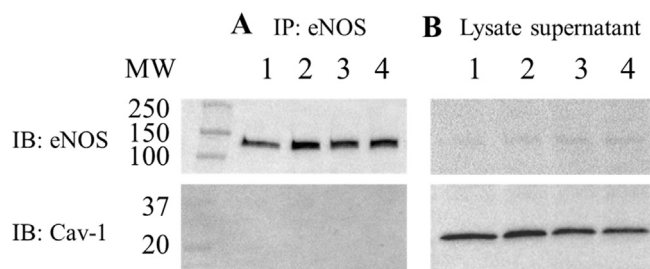
**Fig. 5.** No change in O<sub>2</sub><sup>-</sup> production in HCAEC exposed to each modified LDL. The production of O<sub>2</sub><sup>-</sup> was measured over 30 min at 37 °C following addition of DHE (50 μM) to HCAEC (0.5 × 10<sup>5</sup> cells mL<sup>-1</sup>) treated with in the absence (black bars) or presence (white bars) of each modified LDL (0.1 mg mL<sup>-1</sup> apoB100) for 24 h. Panel (A) shows HPLC trace from the fluorescence detector (DHE; λ<sub>ex</sub> 358 nm, λ<sub>em</sub> 440 nm, and 2-OH-E<sup>+</sup> and E<sup>+</sup>; λ<sub>ex</sub> 490 nm, λ<sub>em</sub> 565 nm) from control cells. Panel B shows 2-OH-E<sup>+</sup> concentration normalised to cellular protein from non-treated, incubation control LDL, HOSCN-modified LDL (250 μM HOSCN), HOCl-modified LDL (250 μM HOCl) and cells treated with menadione (MD, 50 μM). Data represent mean ± S.E.M. of n = 3. \*\*\* show p < 0.001 comparing MD treatment to the non-treated control cells by one-way ANOVA with Dunnett's post hoc test.

extent of 3-nitro-Tyr formation on HCAEC proteins following treatment of the cells with each modified LDL was also measured by Western blotting to assess alterations in the production of peroxynitrite. However, no evidence was obtained for any alteration in the extent of 3-nitro-Tyr formation, suggesting that there is no significant alteration in peroxynitrite formation in HCAEC exposed HOCl- or HOSCN-modified LDL (data not shown).

### 3.4. Effect of modified LDL on the colocalization of eNOS with caveolin-1

The activity of eNOS can be influenced by caveolin-1 [14], with previous studies in HUVEC showing that HOCl-modified LDL can decrease the interaction of eNOS with caveolin-1 [5]. Therefore, we assessed whether exposure of HCAEC to each modified LDL influenced caveolin-1 and eNOS colocalisation. Compared to control LDL treated HCAEC, redistribution of eNOS and caveolin-1 was apparent in HCAEC exposed to modified LDL, particularly HOCl-treated LDL, with more punctate staining of colocalised eNOS and caveolin-1 apparent within the cytosol following treatment (Fig. 6: white arrows).

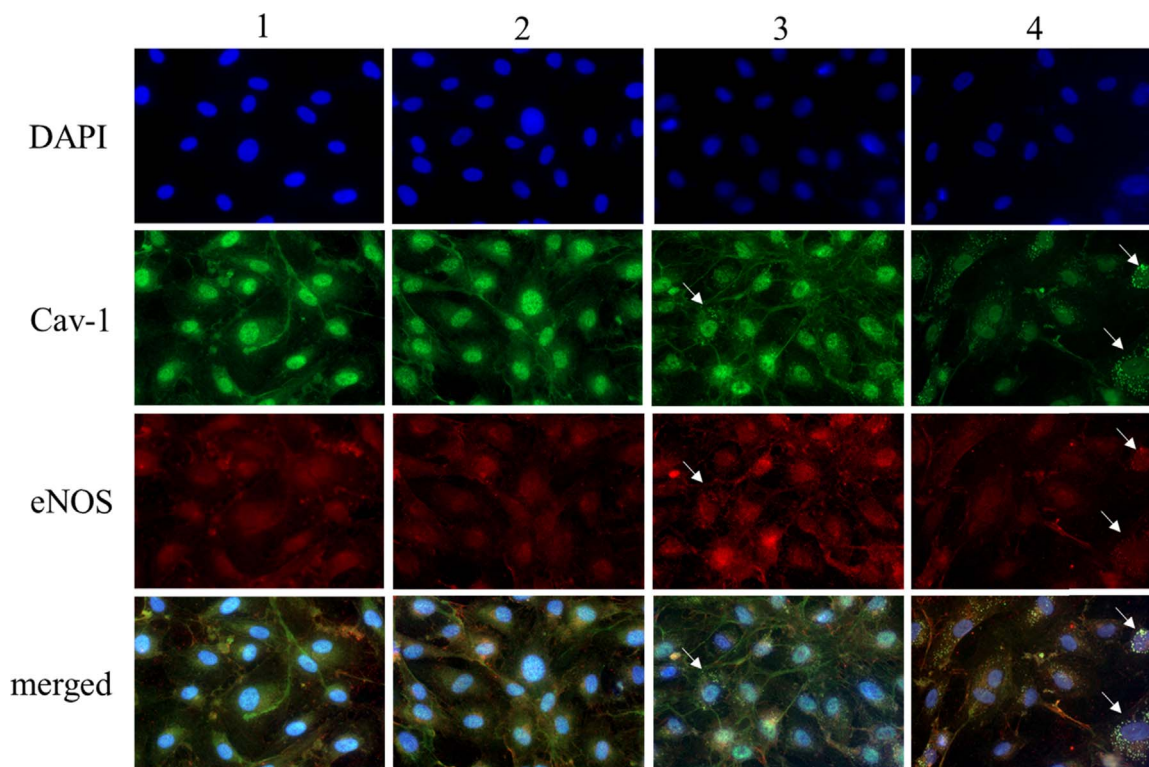
The colocalisation of eNOS and caveolin-1 in the HCAEC exposed to each modified LDL was examined further by Western blotting following immunoprecipitation using an antibody against eNOS. This immunoprecipitation resulted in the detection of similar amounts of eNOS in the lysates of HCAEC regardless of the presence of control or modified LDL (Fig. 7A). However, no evidence was obtained for coimmunoprecipitation of caveolin-1 using this technique despite the presence of caveolin-1 in cell lysates prior to the immunoprecipitation step (Fig. 7B).



**Fig. 7. No interaction of caveolin-1 and eNOS following co-immunoprecipitation.** HCAEC were treated with each modified LDL ( $0.1 \text{ mg mL}^{-1}$ ) for 24 h, before lysis and immunoprecipitation with a monoclonal antibody raised against eNOS. Immunoblotting (IB) experiments were performed with eNOS and caveolin-1 antibodies on the immunoprecipitate (IP) and lysate supernatant with lane 1: no LDL, lane 2: control LDL, lane 3: LDL modified by HOSCN ( $250 \mu\text{M}$ , 24 h), lane 4: LDL modified by HOCl ( $250 \mu\text{M}$ , 24 h). Data are representative of 3 independent experiments.

## 4. Discussion

It is well established that oxLDL can disrupt the endothelial production of  $\text{NO}^{\bullet}$ , which impairs endothelium-dependent vasodilation [8–10]. However, the specific pathways responsible for these effects *in vivo* are not well defined, as oxLDL can influence eNOS expression and functionality through a number of divergent pathways, which are dependent on the extent and nature of LDL modification and the type of endothelial cell under study. Few studies have assessed the reactivity of LDL modified by MPO and/or HOCl with endothelial cell, despite evidence for the presence of this type of oxLDL in both the diseased tissue



**Fig. 6. eNOS and caveolin-1 localisation in HCAEC following treatment with modified LDL.** Localisation of eNOS and caveolin-1 was assessed in HCAEC after treatment with each modified LDL ( $0.1 \text{ mg mL}^{-1}$ ) for 24 h by immunofluorescence. Columns represent different treatments with (1) non-treated control; (2) control LDL; (3) LDL modified with HOSCN ( $250 \mu\text{M}$ , 24 h) and (4) LDL modified with HOCl ( $250 \mu\text{M}$ , 24 h). The rows show staining for the nucleus (DAPI; blue), caveolin-1 (green), eNOS (red) and the merged image from nucleus, caveolin-1 and eNOS. White arrows indicate colocalised speckling of eNOS and caveolin-1 in HCAEC treated with LDL modified by either HOSCN or HOCl. Data are representative of 3 independent experiments.

and circulation of patients with atherosclerosis [21,27]. Similarly, the effects of LDL modified by the other major MPO-derived oxidant, HOSCN, on endothelial function remain to be established. This is significant in light of data correlating plasma SCN<sup>-</sup> (the precursor of HOSCN) with foam cell formation and other early markers of atherosclerosis in smokers [34,35]. In this study, we show for the first time that LDL modified by HOSCN alters the mRNA expression and functionality of eNOS, and induces a decrease in both the endothelial production of NO<sup>•</sup> and the extent of vasorelaxation in pre-constricted aortic rings. The effect of HOSCN-modified LDL on eNOS activity and NO<sup>•</sup> production are similar to, or greater than, that seen in the corresponding experiments with HOCl-modified LDL.

Treatment of HCAEC with HOCl- and HOSCN-modified LDL resulted in a decrease in eNOS activity and NO<sup>•</sup> production, which was associated with uncoupling of the enzyme, rather than altered expression or post-translational modification, such as phosphorylation of Ser1177, which has been shown previously to be sensitive to other types of oxLDL (e.g. [18]). Importantly, a significant reduction in aortic distensibility was observed in rat aortic rings after incubation with HOSCN- and HOCl-modified LDL *ex vivo*. HOSCN- and HOCl-modified LDL reduced endothelium-dependent (ACh-induced) signalling to impair the arterial distensibility, but did not alter aortic relaxation induced by SNP. This is consistent with impaired arterial distensibility being due to effects on the endothelium and rather than the smooth muscle, in agreement with previous studies with other types of oxLDL [9,10].

Treatment of HCAEC with HOSCN-modified LDL, but not HOCl-modified LDL, resulted in an increase in eNOS mRNA expression, though this was not reflected in the protein levels, when measured by Western blotting. This may be associated with an increased extent of LDL lipid oxidation, and the formation of linoleate-derived oxidation products, which is observed on exposure of LDL to HOSCN but not HOCl under the conditions employed in this study [38]. Thus, it has been shown previously that oxidised linoleate products, including 13-hydroperoxyoctadecadienoate (13-HPODE) cause a dose-dependent increase in eNOS mRNA levels following exposure to bovine arterial endothelial cells over 24 h [49]. However, 13-HPODE also increased protein expression and enzyme activity [49], which was not observed with HOSCN-modified LDL. This may reflect parallel post-transcriptional regulatory mechanisms, such as eNOS antisense RNA, which could prevent the translation of eNOS protein by compromising eNOS mRNA stability [50]. Alternatively, a number of microRNAs are reported to post-transcriptionally regulate eNOS expression, such as miRNA-24 [51] and 27nt-miRNA [52], which has not been examined in the current study.

With HOSCN- and HOCl-modified LDL, the changes in eNOS activity in HCAEC were independent of alterations to viability, when measured by LDH release or metabolic activity, but correlated with the extent of eNOS uncoupling, assessed by measuring the eNOS monomer:dimer ratio using Western blotting. Although the extent of uncoupling agreed well with the decreased eNOS activity and NO<sup>•</sup> production, no change in O<sub>2</sub><sup>-•</sup> production was apparent on measuring the formation of 2-OH-E<sup>+</sup> by addition of DHE to the HCAEC and HPLC analysis. The reason for this is not certain, but it does not appear to be related to consumption of the O<sub>2</sub><sup>-•</sup> by NO<sup>•</sup> to form the potent oxidant ONOO<sup>-</sup>, as no evidence was obtained for any alteration in the formation of 3-nitroTyr (a biomarker of ONOO<sup>-</sup> reactivity) in HCAEC under these experimental conditions.

That there is no change in the HCAEC production of O<sub>2</sub><sup>-•</sup> on incubation with the modified LDLs, suggests that stimulation of O<sub>2</sub><sup>-•</sup> production may not be the cause of eNOS uncoupling, in contrast to studies with other types of oxLDL (e.g. [17,53]). Perturbations in O<sub>2</sub><sup>-•</sup> production in cells exposed to oxLDL leading to eNOS uncoupling have been attributed to scavenger receptor binding, particularly to LOX-1 (reviewed [54]), and it is well established that various types of oxLDL can trigger activation of mitogen activated protein kinase (MAPK) and protein kinase C (PKC) pathways, which can influence eNOS

phosphorylation as well as altering O<sub>2</sub><sup>-•</sup> production [17,18]. Whether these pathways play a role in the altered NO<sup>•</sup> production seen in HCAEC exposed to HOSCN- and HOCl-modified LDL is not certain, though no changes in eNOS phosphorylation were apparent. Similarly, no changes are seen in scavenger receptor expression or modified LDL uptake on comparison to control LDL (data not shown).

The enzymatic activity of eNOS relies on tetrahydrobiopterin (BH<sub>4</sub>) to bind haem and L-Arg to produce NO<sup>•</sup> [55]. Similarly, O<sub>2</sub><sup>-•</sup> production by uncoupled eNOS can be dependent on the BH<sub>4</sub>/BH<sub>3</sub><sup>-•</sup> ratio, independently of the L-Arg concentration [56]. In the catalytic process, BH<sub>4</sub> is oxidised to BH<sub>3</sub><sup>-•</sup> radical, which can be recovered to BH<sub>4</sub> with ascorbate and eNOS itself in a BH<sub>4</sub>/BH<sub>3</sub><sup>-•</sup> redox cycle [57]. However, if eNOS coupling is impaired as we have shown by HOSCN-modified LDL and to a lesser extent with HOCl-modified LDL, depletion or displacement of BH<sub>4</sub> may be an important factor in modulating eNOS activity in MPO-oxidant modified LDL. Levels of BH<sub>4</sub> and BH<sub>3</sub><sup>-•</sup> in the loss of eNOS activity seen in the HCAEC exposed to modified LDL was not assessed, however this was not involved in the loss of eNOS activity seen in HUVEC exposed to HOCl-modified LDL [5].

It has been demonstrated in experiments with Cu<sup>2+</sup>-modified LDL that decreased availability of L-Arg by an acute increase in arginase II activity and expression also contributes to decreased eNOS activity in HCAEC, as L-Arg catabolism by arginase II is almost 200-fold greater than that by eNOS [58]. This pathway was also shown to influence NO<sup>•</sup> production in isolated aortic segments [58]. It is not clear if HOSCN- or HOCl-modified LDL perturb arginase expression in HCAEC, but it is worth noting that the nature and extent of modification seen on exposure of LDL to Cu<sup>2+</sup> is very different to that seen with MPO oxidant modified LDL. Moreover, it is not clear as to what effect exposure of HCAEC to native LDL has on arginase activity [58].

An increase in caveolin-1 expression has been implicated as a pathway to eNOS activity impairment, with the ability of HOCl-modified LDL to perturb eNOS activity in HUVECs also associated with the translocation of the eNOS from the plasma membrane and Golgi to other cellular compartments rather than uncoupling [5]. This redistribution of eNOS was attributed to the induction of changes to the physicochemical properties of the membranes, which occurred independently of eNOS myristoylation or palmitoylation [5], which is also observed with Cu<sup>2+</sup>-modified LDL [6,59]. In the latter case, the internalisation of eNOS is related to depletion of cholesterol in the caveolae, which could be prevented by blocking oxLDL binding to the CD36 scavenger receptor [59], which could be important with HOCl-modified LDL that is known to bind to this receptor, in HUVEC at least [60]. Similarly, high-density lipoprotein (HDL) was able to recover eNOS localisation at the caveolae following delocalisation by oxLDL [59]. This was attributed to HDL donating cholesterol to the caveolae membrane [59].

In HCAEC, our data support some alteration to the localisation of eNOS, which was more pronounced on exposure of the cells to HOCl-modified LDL. By immunofluorescence, the eNOS appeared to colocalise with caveolin-1, in contrast to data from HUVEC, where there was no alteration in caveolin-1 localisation [5]. However, caveolin-1 did not colocalise with eNOS in immunoprecipitation experiments. This may reflect a disruption in the interaction between the two proteins resulting from the experimental conditions used to precipitate and resububilise the cell lysates for analysis. Further experiments are required to fully assess the effects of HOSCN- and HOCl-modified LDL on both the scavenger receptor binding and membrane cholesterol content, and define the cellular pathway responsible for uncoupling. A limitation of the current study is that O<sub>2</sub><sup>-•</sup> production was measured for a defined period of time (30 min) following 24 h treatment of HCAEC with modified LDL, rather than during treatment. Thus, it is possible that increased O<sub>2</sub><sup>-•</sup> production could play a role in the eNOS uncoupling observed here, particularly as altered ROS fluxes can occur very rapidly after exposure of endothelial cells to other types of oxLDL [54].

Overall, these data show that both HOSCN- and HOCl-modified LDL



decrease NO<sup>•</sup> production, which directly affects arterial distensibility in functioning tissue by inducing endothelial dysfunction. That the extent of decreased NO<sup>•</sup> production and vasorelaxation was comparable on modification of LDL by HOSCN and HOCl is significant, as it has been suggested that SCN<sup>-</sup> may slow lesion development in atherosclerosis, as HOSCN-LDL is not taken up by macrophages to the same extent as HOCl-LDL [27,38]. This has potential implications for smokers who have elevated plasma levels of SCN<sup>-</sup>, and show evidence of increased endothelial dysfunction. This study also highlights the importance of additional mechanistic studies to better understand how supplementation with SCN<sup>-</sup> to promote HOSCN rather than HOCl formation influences disease progression, including atherosclerosis, during chronic inflammation.

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