Redox Biology 13 (2017) 623–632

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research Paper

Low-density lipoprotein modified by myeloperoxidase oxidants induces endothelial dysfunction

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ARTICLE INFO

Keywords:
Atherosclerosis
Myeloperoxidase
Low-density lipoprotein
Endothelial dysfunction
Nitric oxide

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^-) 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^- 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 ($NO^•$) 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^- within the setting of atherosclerosis and for smokers, who have elevated plasma levels of SCN^-, 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 ($NO^•$) bioavailability both *in vitro* [5–7] and *ex vivo* [8–10].

The formation of $NO^•$ 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_4), and calcium-bound calmodulin (CaM) [11]. Uncoupling and disruption to the structure of eNOS therefore leads to a depletion in bioavailable $NO^•$ 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 $NO^•$ [13]. In addition to uncoupling,

Abbreviations: ACh, acetylcholine; ApoB100, apolipoprotein B100; B2M, β2-microglobulin; BH_4, tetrahydrobiopterin; CaM, calmodulin; DHE, dihydroxyethidine; E^+, 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^5-(1-iminoethyl)-L-ornithine; MGD, *N*-methyl-D-glucamine dithiocarbamate; 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; $NO^•$, nitric oxide; 2-OH-E^+, 2-hydroxyethidium; $O_2^{•-}$, superoxide; oxLDL, oxidised low-density lipoprotein; PKC, protein kinase C; RIPA, radioimmunoprecipitation assay; RT-PCR, real-time polymerase chain reaction; SCN^-, 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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http://dx.doi.org/10.1016/j.redox.2017.08.004
Received 3 July 2017; Accepted 4 August 2017
Available online 05 August 2017

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• production [14]. The production of NO• 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 subcellular localisation of eNOS can perturb the production of NO• by endothelial cells *in vitro* [5,6].

Although it is well accepted that oxLDL can perturb endothelial NO• 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^{2+} 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^{2+} 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• 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• 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^-), which is the precursor to HOSCN, in the development of atherosclerosis [32].

Smoking results in elevated blood SCN^- levels and a higher incidence of cardiovascular disease [33]. Similarly, serum SCN^- 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^- 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• 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 filteration 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 (ε_{292} = 350 M^{-1} 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 (ε_{412} = 14150 M^{-1} 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 (ρ 1.019–1.050 kg L^{-1}) 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^{-1} chloramphenicol and 1 mg mL^{-1} EDTA (Astral Scientific) and stored at 4 °C in the dark. LDL was adjusted to a concentration of 1.0 mg mL^{-1} apoB100 following desalting through a PD-10 column (GE Healthcare) to remove chloramphenicol and EDTA and incubation with HOCl or HOSCN (100–250 μM) 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_2 and 5% CO_2. Aortic segments were washed with HBSS before treatment with each preparation of LDL (0.1 mg mL^{-1}) for 1 h at 37 °C. The segments were washed with HBSS before measurement of the vasoconstrictive dose-response to norepinephrine (NE; 10^{-9}–10^{-5} M). The dilatory response of 80% norepinephrine pre-constricted rings to incremental doses of acetylcholine (ACh; 10^{-8}–10^{-3} M) or endothelium-independent sodium nitroprusside (SNP; 10^{-9}–10^{-5} 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 ~ 232*g*), aspirated, resuspended in media before seeding overnight at a density of 0.5 × 10^5 cells mL^{-1} 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^{-1} 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)