Effects of peroxynitrite on plasma components of the reverse cholesterol transport pathway

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Abstract Elimination of cholesterol from arterial tissue, crucial in limiting atherogenesis, may be achieved via high-density lipoprotein (HDL)-mediated reverse cholesterol transport (RCT); components of this pathway can be modulated by oxidative stress. Here we have examined the relations between cholesterol efflux, esterification and transfer in human plasma treated with the powerfully reactive nitrogen species, peroxynitrite. Cellular cholesterol efflux to whole plasma, or to peroxynitrite-modified HDL_3, was relatively insensitive to peroxynitrite, as was the transfer of esterified cholesterol. However, plasma cholesterol esterification, via lecithin:cholesterol acyltransferase (LCAT), was markedly inhibited, both directly and indirectly, by peroxynitrite treatment, implying inefficient RCT follows HDL sequestration of cellular cholesterol.

Key words: Atherogenesis; Macrophage; High-density lipoprotein; Cholesterol efflux; Peroxynitrite

1. Introduction

Considerable epidemiological evidence has established a strong inverse relation between levels of high-density lipoprotein (HDL) cholesterol and risk of coronary artery disease [1]. Multiple mechanisms may exist by which HDL protects against premature atherosclerosis [2,3]. One proposal is that HDL transfers excess cholesterol from peripheral tissues to the liver for excretion, via the reverse cholesterol transport pathway [3,4]. This pathway involves several regulated steps, mediated by HDL apolipoproteins and two key enzymes: lecithin:cholesterol acyltransferase (LCAT) and cholesteryl ester transfer protein (CETP). Efficient cholesterol efflux from peripheral tissues requires the interaction of HDL apolipoproteins, and in particular apolipoprotein AI (apoAI), with specific cell surface receptors [4], possibly located in membrane caveolae [5]. ApoAI receptor-binding triggers mobilization of intracellular stores of cholesterol, which are channelled via the Golgi apparatus to the cell surface for efflux [6]. The free cholesterol accepted by HDL undergoes esterification, via LCAT, and most is then transferred to apolipoprotein B-containing lipoproteins, by CETP, for hepatic removal [7].

Paradoxically, however, despite efficient cellular effluxing mechanisms, deposition of excess cholesterol still occurs within arterial tissue, implying that the equilibrium between pathways of cholesterol deposition and removal can be perturbed. One possible explanation is that oxidative mechanisms thought to be associated with enhanced cholesterol deposition, via unregulated uptake of oxidized low-density lipoprotein by macrophage scavenger receptors, also exert negative influences upon reverse cholesterol transport. Evidence exists to support this contention: HDL oxidatively modified by copper [8], irradiation [9] or aldehydic compounds extracted from cigarette smoke [10], is a much less efficient mediator of cellular cholesterol efflux; similarly, plasma LCAT activity can be inhibited by incubation with mildly [11], or heavily oxidized LDL [12], copper ions [13] or extracts from cigarette smoke [10,13–15]. In contrast, however, oxidative tyrosylation of HDL results in a particle which actively promotes the mobilization of cholesterol from intracellular cholesteryl ester stores [16,17]. Further, distinct regions of apoAI mediate cholesterol efflux and LCAT activation; these can be distinguished by mutational analysis [18], and possibly by their susceptibility to oxidative stress [10,13–15]. The effects of oxidizing species on components of the plasma reverse cholesterol transport pathway are not, therefore, predictable, and may depend on the mediating radical species.

A number of oxidizing species may exert oxidative effects within the arterial wall, including myeloperoxidase [19], 15-lipoxygenase [20], nitric oxide [21] and superoxide [22]. Nitric oxide (NO) and superoxide (O_2^-) are both formed in the vascular system by endothelial cells, neutrophils and macrophages [23]. Nitric oxide exerts diverse anti-inflammatory, and anti-atherogenic effects, such as inhibition of platelet aggregation [24], monocyte adhesion to the endothelium [25], and LDL oxidation [21]; conversely, inhibition of nitric oxide synthesis enhances the degree of atheroma formed in hypercholesterolaemic rabbits [26]. However, the rapid reaction of nitric oxide with superoxide generates a potent oxidizing species, peroxynitrite ($ONOO^-$) which, when protonated ($ONOOH$), decomposes by complex reaction pathways resulting in the peroxidation of lipids [27], nitration of protein tyrosine residues [28], oxidation of thiol groups in proteins [29], depletion of antioxidants [30] and DNA oxidation and nitration [31,32]. In particular, peroxynitrite can oxidize LDL to a form which is recognized by the macrophage scavenger receptor [33], and nitrotyrosine residues have been identified in human atherosclerotic plaque [34,35]. Thus, peroxynitrite may be an important cellular mediator of biological damage in both tissue fluids, like plasma, and within the artery wall.

In this study we examine the effects of peroxynitrite treatment on plasma components of the reverse cholesterol transport pathway, using a cellular model of cholesterol efflux. Our results indicate these components exhibit differential sensitiv-

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Abbreviations: apoAI, apolipoprotein AI; apoAII, apolipoprotein AII; CETP, cholesteryl ester transfer protein; HDL, high-density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; Lp, lipoprotein; LDL, low-density lipoprotein; $ONOO^-$, peroxynitrite; RCT, reverse cholesterol transport

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ities to peroxynitrite; in particular, plasma cholesterol esterification, by LCAT, appears especially susceptible to peroxynitrite treatment.

2. Materials and methods

2.1. Materials

Tissue culture medium, reagents, and laboratory chemicals were purchased from Sigma Chemical Co. (Dorset, UK), and tissue culture plastics from Falcon (Marathon, London, UK). Purified peroxynitrite was supplied by Alexis Co. (Nottingham, UK); immunoelectrophoretic and agarose gels by Sebia (YSI Clandon, Hampshire, UK).

2.2. Preparation of plasma, HDL_3 and LCAT

Blood samples were taken from six healthy volunteer subjects, by collection into EDTA (0.27%, w/v), and centrifuged at $2500 \times g$ for 30 min to remove cellular material; plasma samples were stored at −70°C, in 1 ml aliquots, and subjected to only one freeze-thaw just prior to peroxynitrite treatment, and subsequent use. This procedure does not affect either the cholesterol effluxing potential, or the LCAT activity, of the plasma [36]. Plasma samples were treated with peroxynitrite, the peroxynitrite vehicle (0.3 M NaOH) or with peroxynitrite which had undergone decay at physiological pH; all additions were made rapidly in a single bolus dose while vortexing the sample, as described previously [33].

Differential ultracentrifugation flotation was used to isolate HDL_3 (1.125–1.26 g/ml) from freshly isolated plasma [37]. Recombinant human LCAT was isolated from the serum-free culture medium of Chinese hamster ovary (CHO) cells [38], by phenyl-Sepharose chromatography [39,40].

2.3. Analysis of lipids and apolipoproteins

Plasma total cholesterol, triacylglycerols and phospholipids were measured using commercially available kits and standards (Sigma) [37]; HDL cholesterol was measured following precipitation of LDL and VLDL with phosphotungistic acid/$MgCl_2$ [36]. Low-density lipoprotein (LDL) cholesterol was calculated according to the formula described by Friedewald et al. [41]. Total apoAI was measured by electroimmunoassay, using commercial gels (Sebia). Lipoproteins containing only apoAI (LpAI) were quantified by differential electroimmunoassay (Sebia) [36]; the concentration of lipoproteins containing both apoAI and apoAII (LpAI/AII) was calculated as the difference between total apoAI and LpAI [36].

2.4. Cell culture, lipid radiolabelling and efflux assay

Human THP-1 macrophages were maintained exactly as described [37]; for experiments cells were seeded at 1×10^6/well into 12-well plates, containing RPMI 1640 medium supplemented with glutamine (4 mM), penicillin (20 IU/ml) and streptomycin (20 μg/ml), bovine fetal calf serum (10%, v/v) and phorbol 12-myristate 13-acetate (PMA) (100 ng/ml). Cells were maintained for 5 days; media containing PMA were replaced at 2 day intervals.

Cholesterol efflux from THP-1 macrophages was performed exactly as described [36]; this method has been fully characterized for Fu5AH cells [36], and is increasingly being employed to measure cholesterol efflux from other cell types [42,43]. Briefly, [^{3}H]cholesterol (1 μCi/well; final concentration 0.1% ethanol) was added to macrophages in RPMI medium containing fetal calf serum (5%); macrophages were incubated in this medium for 24 h. Cells were then incubated in RPMI medium containing bovine serum albumin (1%) for a further 18 h, prior to measuring cholesterol efflux.

The efflux potential of each plasma sample was assayed by incubating human plasma (5% by vol.) with triplicate wells of labelled macrophages, at 37°C for 4 h. Alternatively, where isolated HDL_3 was used as the cholesterol acceptor, the cells were incubated with RPMI medium containing bovine serum albumin (1%), and the appropriate concentrations of HDL_3. Medium was removed from the cells, and centrifuged at $10\,000 \times g$ to remove any floating cells; released cholesterol was measured in an aliquot of the medium by scintillation counting. Residual medium was stored at −70°C for further analysis; macrophages were washed three times with cold phosphate-buffered saline (PBS), and cellular lipids extracted by addition of isopropanol, overnight at room temperature. The fractional cholesterol efflux was calculated as the amount of label released to the medium, divided by the total label in each well [36].

2.5. Measurement of cholesterol esterification

Cholesterol esterification, in the efflux medium containing test samples of human plasma, was assayed by measuring the proportion of labelled cellular cholesterol which became esterified in the medium during the efflux phase. Medium lipids were extracted by the method of Bligh and Dyer [44], the extract dried under N_2, and free cholesterol and cholesteryl esters separated by thin layer chromatography (t.l.c.), using hexane:diethyl ether:glacial acetic acid (90/20/1, by vol.) as the mobile phase. Preliminary experiments confirmed that, as in Fu5AH cells [36], the bulk of cellular cholesterol in the macrophages was unesterified, and only free cholesterol was released to acceptors in the culture medium.

Plasma LCAT activity was determined using an exogenous proteoliposome substrate of [^{14}C]cholesterol, egg lecithin and apoAI, as described previously [38,45]. Incubation time was 1 h at 37°C, or as appropriate, and the production of [^{14}C]cholesteryl ester measured by t.l.c., as above. Alternatively, in some experiments the effect of peroxynitrite on endogenous LCAT substrate was investigated using human recombinant LCAT as the enzyme source. In this case, heat-inactivated plasma was incubated with [^{3}H]cholesterol/human serum albumin (HSA) complex, exactly as described [46], before the addition of human recombinant LCAT, and incubation for a further 1 h at 37°C. Percentage conversions of substrate to radiolabelled cholesteryl ester were determined by t.l.c., as before.

2.6. Transfer of effluxed cellular cholesterol to LDL/VLDL

The distribution of [^{3}H]cholesteryl esters between HDL and apoB100-containing lipoproteins was used as an estimate of cholesteryl ester transfer protein (CETP) activity in the efflux medium. ApoB lipoproteins were precipitated from the efflux medium by treatment with phosphotungistic acid (0.4%) and $MgCl_2$ (0.2 M) (final concentrations); samples were vortexed, and placed on ice for 10 min, before centrifugation at $10\,000 \times g$ [36]. Pellets were washed with PBS containing phosphotungistic acid/$MgCl_2$ mixture (as above) and lipids extracted by the method of Bligh and Dyer [44]. Cholesterol

Table 1
Average parameters of test plasma samples

Parameter	Mean ± S.D.	(Range)
Total cholesterol, mmol/l	4.48 ± 1.45	(2.51–6.71)
Triacylglycerol, mmol/l	1.48 ± 1.09	(0.46–3.34)
Phospholipids, mmol/l	2.72 ± 0.47	(1.94–3.32)
HDL cholesterol, mmol/l	0.86 ± 0.25	(0.50–1.27)
LDL cholesterol, mmol/l	2.95 ± 1.09	(1.26–4.40)
Apolipoprotein AI, g/l	1.13 ± 0.20	(0.82–1.34)
LpAI, g/l	0.55 ± 0.16	(0.38–0.81)
LpAII, g/l	0.58 ± 0.20	(0.42–0.86)
Fractional cholesterol efflux/h (%)	6.27 ± 2.04%	(4.11–9.46)
Cholesterol esterification (%)	2.49 ± 0.37%	(1.96–3.06)
Cholesterol ester transfer (%)	0.51 ± 0.17%	(0.30–0.81)

Sample measurements were performed as outlined in Section 2; values given are means ± S.D. ($n = 6$).

and cholesteryl esters were separated by t.l.c., as described above; values were expressed as a percentage of the total cholesterol effluxed, and of the total cholesterol esterified in the medium.

2.7. Statistics

All results are expressed as means ± S.D., or as means ± ranges; statistical differences ($P < 0.05$) were determined by Student's two-tailed *t*-test.

3. Results

3.1. Cholesterol efflux from human (THP-1) macrophages

Measurement of cholesterol efflux and subsequent esterification and transfer reactions, were performed using human (THP-1) macrophages, radiolabelled with [^{3}H]cholesterol (Section 2) and incubated with samples of plasma (5%) isolated, and characterized, from six healthy volunteers (Table 1). Although the efflux of cholesterol is lower in macrophages than other cell types [36], our values for fractional esterification, and subsequent transfer to LDL, of cell-derived cholesterol are in good agreement with published values [36].

Addition of peroxynitrite (1 mM) to human plasma did not significantly alter the total efflux of [^{3}H]cholesterol from THP-1 macrophages; higher levels (5 mM) resulted in only a 30% reduction in efflux ($P < 0.05$) (Fig. 1A). This insensitivity to peroxynitrite implied either (a) an inherent resistance of HDL to modification by peroxynitrite, or (b) that plasma constituents may be protecting against peroxynitrite modification. To distinguish between these possibilities, we incubated radiolabelled THP-1 macrophages with isolated HDL_3, following the direct modification of this lipoprotein with peroxynitrite. Increases in HDL_3 relative electrophoretic mobility on agarose gels (1.25 ± 0.05, $n = 3$, mean ± S.D.) and in thiobarbituric acid reactive substances (6.97 ± 0.63 nmol/mg HDL_3, $n = 2$, mean ± range) were observed for HDL_3 (1 mg/ml) treated with peroxynitrite (1 mM). Modification of HDL by peroxynitrite (0.5–5 mM) is associated with polymerization of apoAI and nitration of tyrosine residues [47]. These evident changes in HDL structure and composition were accompanied by decreased cholesterol efflux from THP-1 macrophages (Fig. 1B); the maximal decrease achieved was around 40% (1 mM peroxynitrite). Therefore, it seems likely that plasma proteins and antioxidants protect HDL against modification by peroxynitrite [30]; in support of this, no changes in the electrophoretic mobility of plasma lipoproteins were observed following peroxynitrite treatment (data not shown).

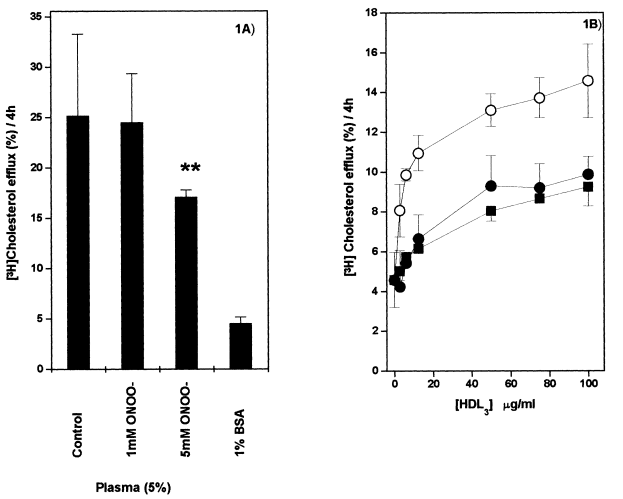

Fig. 1. A: Cellular efflux of cholesterol to plasma acceptors. Macrophages were pre-labelled with [^{3}H]cholesterol in the absence (1% BSA) or presence of plasma samples (5%), treated with the indicated concentrations of peroxynitrite. Values are means ± S.D. of six plasma samples; ** indicates values significantly different ($P < 0.05$) from both the control incubation, and from plasma treated with 1 mM peroxynitrite. Results were confirmed in a second independent experiment. B: Cellular cholesterol efflux to peroxynitrite-modified HDL_3. Macrophages were incubated (Section 2) in the presence of isolated HDL_3 (○), modified by the addition of peroxynitrite (0.5 mM (●); 1 mM (■)). Values are means ± S.D. of triplicate wells in a single representative experiment; values were confirmed in three further independent experiments.

3.2. Cholesterol esterification in human plasma

Cholesterol released from human (THP-1) macrophages to acceptors in human plasma (5%), undergoes transesterification via LCAT. In contrast to cholesterol efflux, esterification in plasma-containing tissue culture medium was markedly inhibited (> 65%, $P < 0.005$) by the addition of peroxynitrite (1 mM or higher) (Fig. 2A). No inhibition was noted with peroxynitrite which had been allowed to decay at physiological pH, before addition to the plasma sample at a concentration equivalent to 5 mM peroxynitrite. As the esterification of effluxed [^{3}H]cholesterol was strikingly reduced, we pursued this finding by measuring LCAT activity in plasma treated with peroxynitrite (Fig. 2B, C). This involves the addition of plasma (7.5 μl) to an artificial proteoliposome substrate (0.24 ml) [38,45] labelled with [^{14}C]cholesterol; essentially, therefore, plasma LCAT activity is assayed under optimized conditions, in the presence of an intact, non-oxidized, substrate. Results from the cellular cholesterol efflux studies were confirmed: plasma LCAT activity is dose-dependently inhibited by peroxynitrite ($IC_{50} = 0.75$ mM) (Fig. 2B) at all time points assayed (Fig. 2C), in two independent experiments using different plasma samples. Additional controls monitored the effects of adding either the peroxynitrite vehicle (0.3 M NaOH), or decayed peroxynitrite (Fig. 2B): non-specific effects on LCAT activity were not detected.

These experiments indicate that peroxynitrite treatment of plasma results in direct inhibition of LCAT activity. We confirmed this finding by treating human recombinant LCAT [38–40] directly with peroxynitrite, and measuring activity using the proteoliposome substrate; total inhibition of LCAT activity was achieved at 0.5 mM (data not shown). Nevertheless, we examined the effect of peroxynitrite on the ability of plasma to sustain exogenous LCAT activity. Heat-inactivated human plasma was equilibrated with [^{3}H]cholesterol, and treated with peroxynitrite (0–5 mM), before the addition of human recombinant LCAT [38–40,46] (Fig. 2D). Recombinant LCAT activity was decreased by about 40% in plasma treated with peroxynitrite (1 mM), suggesting modification of the endogenous LCAT substrate contributes to LCAT inhibition or inactivation.

3.3. Cholesteryl ester transfer in human plasma

The fractional conversion of cell-derived [^{3}H]cholesterol into cholesteryl ester is followed by the subsequent transfer of [^{3}H]cholesteryl esters from HDL to LDL and VLDL, via the action of CETP. As expected, [^{3}H]cholesteryl esters transferred to LDL/VLDL decreased substantially following peroxynitrite treatment, when expressed as a percentage of the total cholesterol efflux (Fig. 3A), reflecting the marked inhib-

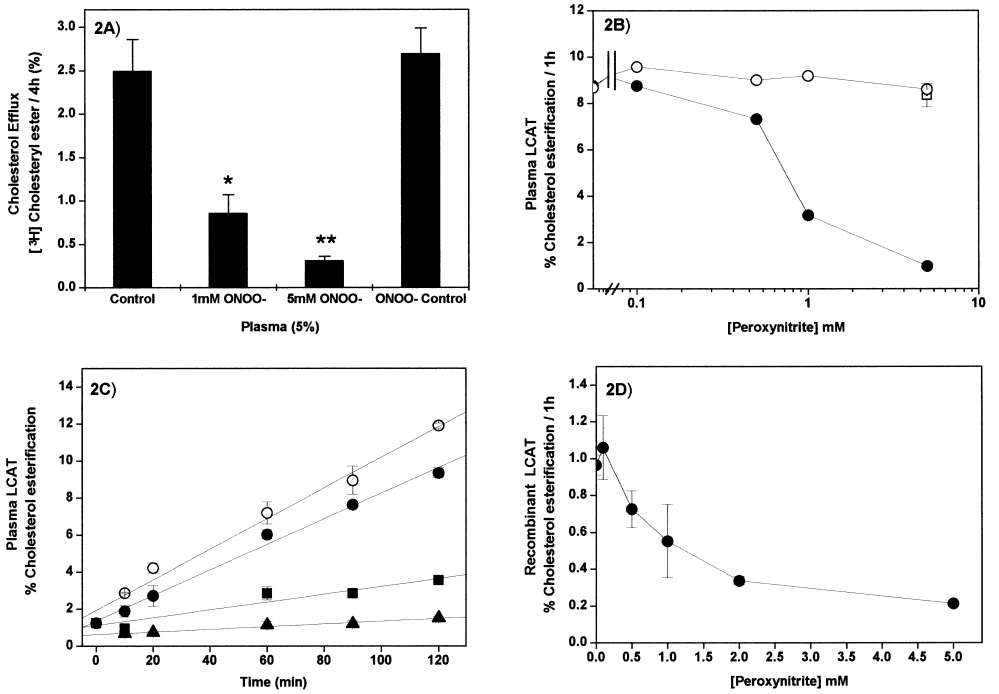

Fig. 2. A: Esterification of effluxed cholesterol. Esterification of effluxed cholesterol in plasma exposed to peroxynitrite ($ONOO^-$) or decayed peroxynitrite ($ONOO^\bullet$ control). Values are means ± S.D. of six plasma samples; * indicates values significantly different ($P < 0.05$) from control plasma; ** indicates values significantly different ($P < 0.05$) from both the control, and from plasma treated with 1 mM peroxynitrite. Results were confirmed in a further independent experiment. B: Plasma LCAT activity. Esterification of cholesterol in plasma exposed to varying concentrations of peroxynitrite (●), its vehicle (○) or decayed peroxynitrite (□), measured using an exogenous proteoliposome substrate (Section 2). Values are the means ± S.D. of triplicate measurements. C: Plasma LCAT activity. Esterification of cholesterol in plasma, in the absence (○) or presence of 0.5 mM (●), 1 mM (■) or 5 mM (▲) peroxynitrite, measured using an exogenous proteoliposome substrate (Section 2). Values are means ± S.D. of incubations performed in triplicate. D: Human recombinant LCAT activity in peroxynitrite-modified plasma. Esterification of cholesterol, by human recombinant LCAT, in heat-inactivated, [^{3}H]cholesterol-labelled plasma treated with varying concentrations of peroxynitrite (Section 2). Values are means ± ranges of duplicate incubations.

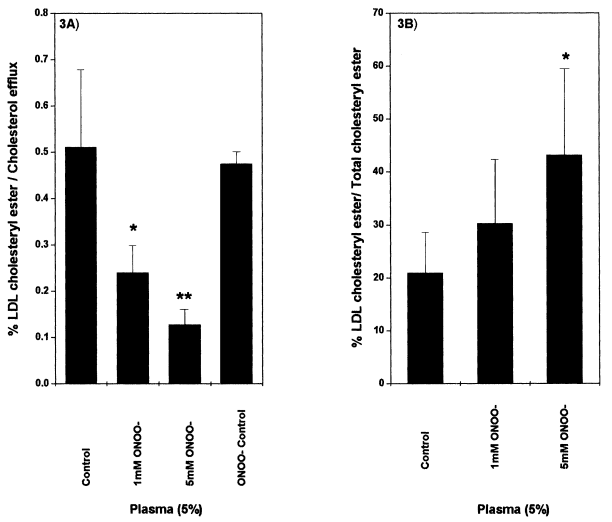

Fig. 3. Transfer of cholesteryl ester to LDL/VLDL. Transfer of effluxed, and esterified, cholesterol to LDL/VLDL, expressed as a percentage of total cholesterol efflux (A) or as a percentage of esterified cholesterol (B). Values are means ± S.D. of six plasma samples; * indicates values significantly different ($P < 0.05$) from control plasma; ** indicates values significantly different ($P < 0.05$) from both control plasma, and from plasma treated with 1 mM peroxynitrite. Results were confirmed in a further independent experiment.

ition of LCAT activity in these samples (Fig. 2A). However, when LDL/VLDL-[^{3}H]cholesteryl ester is expressed as a percentage of total [^{3}H]cholesteryl ester, it is evident a greater percentage of esterified cholesterol is entering the LDL/VLDL pool (Fig. 3B). This suggests CETP activity is not inhibited by peroxynitrite treatment of plasma; indeed, at higher concentrations a greater overall proportion of cholesteryl ester is transferred to LDL/VLDL.

4. Discussion

Reverse cholesterol transport, mediated by the interactions of HDL, LCAT and CETP, is a major way to eliminate cholesterol from peripheral tissues. Our study suggests that peroxynitrite, a reactive nitrogen species implicated in atherogenesis [34,35], has the potential to modulate this process. The efflux of cholesterol to plasma acceptors is only slightly reduced at high concentrations of peroxynitrite; this appears due to the protective effect of plasma constituents, as HDL is not inherently resistant to peroxynitrite modification. In contrast, plasma esterification of cholesterol, via LCAT, is markedly inhibited at much lower concentrations of peroxynitrite; inhibition appears to be exerted by both direct, and indirect, effects on the enzyme activity. As established elsewhere [48], endogenous LCAT activity does not determine the rate of cellular cholesterol efflux; rather, the conversion of cholesterol to cholesteryl ester by LCAT is thought to

reduce the 'back-transfer' of cholesterol from HDL to the macrophages. The subsequent transfer of cholesteryl ester to the LDL/VLDL plasma fraction is also decreased, but as a result of LCAT inhibition and not, apparently, to inhibition of CETP. Overall, therefore, generation of peroxynitrite would be predicted to decrease the efficiency of the plasma reverse cholesterol transport pathway, predominantly by inhibition of LCAT activity.

The biological significance of our findings may not be immediately apparent, given the high concentrations of peroxynitrite employed. However, the short half-life (<1 s) of this oxidant at physiological pH means that a bolus dose of 1 mM peroxynitrite is equivalent to an exposure of 26 μM min^{-1} [30,49]. Thus, it is not the absolute concentration, but rather the effective exposure (concentration × exposure time) that determines the effect of peroxynitrite treatment [30,49]. Furthermore, formation of peroxynitrite at rates of 0.1 nmol/10^6 cells/min is reported for activated murine macrophages [50] and human neutrophils [51], leading to suggestions that local concentrations of peroxynitrite may rise as high as 0.5–1.0 mM min^{-1} under certain pathological conditions [50]. The inhibition of LCAT activity by peroxynitrite could potentially, therefore, be important during inflammation in vivo. Indeed, LCAT activity and/or expression, are decreased during the acute phase response in animal models [52], following cytokine-induced hyperlipidaemia [53], and in human patients infected with *Schistosomiasis mansoni* [54]; decreased LCAT activity has also been noted in patients with diabetes mellitus [55] and coronary artery disease [56].

The precise mechanism by which peroxynitrite inhibits LCAT activity has not been the focus of this study; nevertheless, our results indicate that LCAT activity can be inhibited directly by this reactive species. In particular, both plasma LCAT activity and human recombinant LCAT is inhibited by peroxynitrite, when assessed using an intact proteoliposome substrate. Similar results have been described for the effect of aldehydes isolated from the gas phase of cigarette smoke [10,13–15]. Inhibition of LCAT by aldehydes is independent of lipid peroxidation, protected by *N*-acetyl cysteine and reversible by disulphide-reducing agents [13–15]; these results indicate that covalent modification of free thiol groups Cys-31 and Cys-184, near the active site of the enzyme, inhibits LCAT activity [13–15]. Free thiol groups are an obvious target for peroxynitrite attack [29,49]; alternatively, however, LCAT contains a number of tyrosine residues, which may undergo nitration and possibly affect LCAT activity [28]. Indeed, a naturally occurring LCAT mutation, Tyr156Asn, decreases the activity of LCAT by around 70%, possibly via an disturbed enzyme-lipid interaction [57].

However, plasma LCAT may also be inhibited indirectly by peroxynitrite treatment. Plasma treated with peroxynitrite appears to be a less effective substrate for human recombinant LCAT; this could be, of course, due either to modification of the LCAT substrate, or to the formation of an inhibitory species within the plasma. LCAT can be inhibited by mildly [11] and heavily oxidized LDL [12], and by lipid peroxides [13]; oxidation of plasma, by copper or peroxyl radicals, also leads to apoAI and apoAII crosslinking, which can abrogate LCAT activation [13,58]. Our evidence suggests that plasma HDL is relatively resistant to functional modification by peroxynitrite; equally, only trace amounts of cholesteryl ester hydroperoxides have been detected following peroxynitrite treatment of plasma [30]. The latter suggests lipid peroxidation is not the major route by which peroxynitrite modifies the ability of plasma to support LCAT activity; our preliminary evidence also indicates that treatment of peroxynitrite-modified plasma with Ebselen to reduce lipid hydroperoxides does not restore LCAT activity (data not shown). However, plasma LDL does exhibit increased levels of nitrotyrosine following peroxynitrite treatment [28]. Thus, multiple mechanisms may exist by which peroxynitrite modifies LCAT activity, which require further study.

This study indicates that peroxynitrite can inhibit the activity of a key component of the reverse cholesterol pathway. However, the implications of LCAT inhibition for atherogenesis are not, as yet, entirely clear, mainly because the protective role of LCAT in atherosclerotic vascular disease is not established. Studies in transgenic animal models have indicated that over-expression of human LCAT induces hyperalphaproteinaemia, and a less atherogenic lipoprotein profile, in both mice [59] and rabbits [60]; paradoxically, however, LCAT over-expression can either exacerbate [61], or protect against [62] development of atherosclerosis in these animal models. In man, LCAT deficiency causes HDL deficiency, elevated triacylglycerol levels, and clinical symptoms of corneal opacification, anaemia, proteinuria and renal disease, but not overt premature atherosclerosis [63]. An unequivocal demonstration of the protective role of LCAT in atherogenesis therefore remains elusive.

In summary, therefore, we have demonstrated that peroxynitrite can modify components of the plasma reverse cholesterol transport pathway, and that LCAT activity is particularly susceptible to attack by this reactive species. The precise mechanism, and physiological significance, of LCAT inhibition by peroxynitrite will be the subject of future studies.

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