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High density lipoprotein efficiently accepts surface but not internal oxidised lipids from oxidised low density lipoprotein

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ABSTRACT

Objective: Oxidised low density lipoprotein (oxLDL) contributes to atherosclerosis, whereas high density lipoprotein (HDL) is known to be atheroprotective due, at least in part, to its ability to remove oxidised lipids from oxLDL. The molecular details of the lipid transfer process are not fully understood. We aimed to identify major oxidised lipid species of oxLDL and investigate their transfer upon co-incubation with HDL with varying levels of oxidation.
Approach and results: A total of 14 major species of oxidised phosphatidylcholine and oxidised cholesteryl ester from oxLDL were identified using an untargeted mass spectrometry approach. HDL obtained from pooled plasma of normolipidemic subjects (N = 5) was oxidised under mild and heavy oxidative conditions. Non-oxidised (native) HDL and oxidised HDL were co-incubated with oxLDL, re-isolated and lipidomic analysis was performed. Lipoprotein surface lipids, oxidised phosphatidylcholines and oxidised cholesterols (7-ketocholesterol and 7β-hydroxycholesterol), but not internal oxidised cholesteryl esters, were effectively transferred to native HDL. Saturated and monounsaturated lyso-phosphatidylcholines were also transferred from the oxLDL to native HDL. These processes were attenuated when HDL was oxidised under mild and heavy oxidative conditions. The impaired capacities were accompanied by an increase in a ratio of sphingomyelin to phosphatidylcholine and a reduction in phosphatidylserine content in oxidised HDL, both of which are potentially important regulators of the oxidised lipid transfer capacity of HDL.
Conclusions: Our study has revealed the differential transfer efficiency of surface and internal oxidised lipids from oxLDL and their acceptance onto HDL. These capacities were modulated when HDL was itself oxidised.
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1. Introduction

Oxidised low density lipoprotein (oxLDL) contributes to atherosclerosis [1–3]. Lipid oxidation products including oxidised phosphatidylcholine (oxPC), oxidised cholesteryl ester (oxCE) and lyso-phosphatidylcholine (LPC) which is produced by the action of phospholipases on oxPC, have been detected and characterised in oxLDL [4], plasma [5] and atherosclerotic lesions [6]. These oxidation products may represent bioactive lipids with potential pro-inflammatory capacity that affect plaque progression and stability [4].

High density lipoprotein (HDL) is known to possess atheroprotective capacities including anti-oxidative properties [7,8]. Studies have characterised HDL anti-oxidative properties by its ability to delay LDL oxidation, typically monitored by the production of conjugated diene. There is also a growing body of evidence highlighting the defective anti-oxidative capacity of HDL in atherosclerosis. Decreased protection of LDL against oxidation [9,10], and triglyceride and serum amyloid A enrichment of HDL [11] have all been associated with its impaired anti-oxidative capacity. Earlier studies on HDL subpopulations from normolipidemic individuals showed that small dense HDL3c exhibited the greatest potency in inhibiting LDL oxidation. Analysis of the lipid composition demonstrated that the small dense HDL3c was preferentially enriched in sphingosine-1-phosphate, phosphatidylserine (PS), and phosphatidic acid, and was depleted in sphingomyelin (SM) [12,13].

HDL has also been reported to remove and inactivate lipid hydroperoxides from oxLDL upon co-incubation [14,15]. These processes are governed by the redox status of HDL-associated apolipoprotein AI (apoAI), and the surface rigidity of the phospholipid monolayer of the

Abbreviations: CE, cholesteryl ester; LC-MS, liquid chromatography coupled with mass spectrometry; LPC, lyso-phosphatidylcholine; MUFA, monounsaturated fatty acids; OxCE, oxidised cholesteryl ester; OxHDL, oxidised high density lipoprotein; OxLDL, oxidised low density lipoprotein; OxPC, oxidised phosphatidylcholine; PC, phosphatidylcholine; PS, phosphatidylserine; SFA, saturated fatty acids; SM, sphingomyelin.

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acceptor HDL (as determined by the ratio of sphingomyelin/phosphatidylcholine) [14]. This ability to transfer oxidised lipids from oxLDL to HDL may play an important part of the overall HDL anti-oxidative capacity. However, the molecular details of the oxidised lipid transfer capacity of HDL are not yet fully elucidated. Our knowledge on the identities of the oxidised lipids that are actually transferred by HDL is limited partly because studies have relied heavily on conjugated diene measurement and HPLC chemiluminescence coupled with UV detection measurement of lipid hydroperoxides. Here we aimed to oxidise lipoproteins in an artificial system using copper chloride and to identify major oxidised lipid species of oxLDL including oxPC, oxCE, and oxidised cholesterols and investigate their transfer upon co-incubation with HDL with varying levels of oxidation.

2. Materials and methods

2.1. Isolation of human LDL and HDL

Five healthy non-diabetic normolipidemic male and female volunteers aged between 25 to 65 years were recruited for the study. All subjects provided informed written consent. They fasted overnight (≥12 h) prior to the blood collection. Venous blood was collected from the antecubital vein into sterile EDTA tubes. Plasma was isolated, pooled, and sucrose was added (final concentration of 0.6%) as a cryoprotectant for lipoproteins [16,17] prior to storage at -80 °C. Each aliquot of plasma was thawed only once before the isolation of lipoproteins.

LDL and HDL were isolated by sequential ultracentrifugation [18]. EDTA was added to plasma to give a final concentration of 2 mmol/l. The samples were centrifuged in an Optima MAX-TL ultracentrifuge (Beckman Coulter, New South Wales, Australia) using TLA 120.2 rotor at 435,680 xg (100,000 rpm), 16 °C for 3 h for the isolation of each lipoprotein. LDL (density of 1.019 g/ml - 1.063 g/ml) and HDL (density of 1.063 g/ml to 1.21 g/ml) were dialysed against phosphate-buffered saline, pH 7.4 containing 5 μmol/l of EDTA with three buffer changes (225 × sample volume) over 24 h. Sucrose was added to aliquots of lipoprotein fractions to give a final concentration of 10% (*w*/*v*); this is to preserve the lipoprotein function [19] prior to storage at -80 °C.

2.2. Lipid extraction

Aliquots of isolated lipoproteins (100 μl of approximately 0.1 mg protein/ml of LDL and 0.3 mg protein/ml of HDL) were lyophilised and subsequently reconstituted in 10 μl of deionised water for lipid extraction. Lipoprotein lipids were extracted as previously described [20]. Briefly, lipoprotein samples (10 μl) were combined with 15 μl of internal standards mix (Supplemental Table I) containing 10,000 pmol cholesterol (d7), 1000 pmol cholesteryl ester 18:0 (d6), 100 pmol 7-ketocholesterol (d7), 100 pmol lysophosphatidylcholine 13:0, 100 pmol phosphatidylcholine 13:0/13:0, 100 pmol phosphatidylserine 17:0/17:0, and 200 pmol sphingomyelin C12:0, per sample. The lipids were extracted using 200 μl chloroform:methanol (2:1). The mixtures were briefly vortexed, mixed for 10 min (on a rotary mixer), sonicated for 30 min and then allowed to stand at room temperature for 20 min before they were centrifuged at 16,000 × g, room temperature for 10 min. The supernatant containing extracted lipids were dried under a stream of nitrogen at 40 °C and subsequently reconstituted in 100 μl of a mixture of water saturated butanol and methanol (1:1) containing 5 mmol/l of ammonium formate.

2.3. Identification of oxPC and oxCE by untargeted lipidomics

To identify the major oxidised lipids in oxLDL, we performed a time-course oxidation of LDL (1 mg protein/ml) with 8 μmol/l copper sulphate for 1, 2, 4, 8, and 24 h at 37 °C. The lipids were subsequently extracted and analysed using an Agilent 1200 liquid chromatography system with an Agilent 6520 quadrupole-time of flight mass spectrometer (LC-MS). Lipid extracts (5 μl) were injected on a 2.1 × 100 mm C18 Zorbax-Eclipse column (Agilent, USA) at 250 μl/min. The following gradient conditions were used: 0% B to 40% B over 4 min, 40% B to 63.6% B over 13 min, 63.6% B to 100% B over 12 min, 100% B over 3 min, and a return to 0% B over 1 min, followed by 0% B over 7 min. Solvents A and B consisted of water:terahydrofuran:methanol in the ratio of 60:20:20 and 5:75:20 respectively, both containing 10 mmol/l ammonium formate. Analysis was conducted using MZmine 2.10, developed by Orešič et al. [21,22] and Pluskal et al. [23].

2.4. Assessment of the transfer of oxidised lipids from oxLDL to HDL

The ability of HDL to act as an acceptor of oxidised lipids from oxLDL was assessed by incubating HDL with oxLDL at a protein ratio of 3:1 (HDL:LDL). This ratio provides approximately equal amounts of HDL and LDL lipids in the assay system. The lipoproteins were then re-isolated and the lipid composition was analysed by liquid chromatography electrospray ionisation tandem mass spectrometry.

Prior to co-incubation with HDL, LDL (0.6 mg protein/ml) was oxidised with 6 μmol/l copper chloride for 2 h. Oxidised LDL and HDL with final protein concentrations of 0.1 mg/ml and 0.3 mg/ml respectively were co-incubated in the presence of 120 μmol/l EDTA at 37 °C for 2 h. To assess the effect of oxidation on the ability of HDL as an acceptor of oxidised lipids, HDL (1.8 mg protein/ml) was oxidised with 6 μmol/l copper chloride for 60 and 200 min to obtain mildly and heavily oxidised forms of HDL, respectively. The mildly- and heavily-oxHDL were co-incubated with oxLDL as described above. Following co-incubation, the lipoproteins were re-isolated using a similar density ultracentrifugation procedure method as described above. Briefly, aliquots of 400 μl mixture of LDL and HDL were adjusted to a final density of 1.063 g/ml using NaBr solution and then overlayed with NaBr solution (density of 1.063 g/ml) to a total volume of 1.0 ml. The samples were centrifuged (435,680 × *g*, 16 °C, 3 h). The LDL (density of 1.019 g/ml–1.063 g/ml) was aspirated with the top 400 μl of the density gradient. The HDL (density of 1.063 g/ml to 1.21 g/ml) was isolated in the lower 400 μl of the density gradient. The intermediate layer (200 μl) in between the LDL and HDL was analysed and found to contain only 7–10% of lipids, demonstrating clear separation of the LDL and HDL fractions. Further, SDS-PAGE analysis of the re-isolated oxLDL and HDL showed no cross- contamination of apoAI and apoB (data not shown). The re-isolated lipoproteins were dialysed against phosphate-buffered saline (100 × total sample volume) containing 5 μM EDTA overnight. Aliquots of 100 μl of the samples were frozen at −80 °C and then lyophilised. They were reconstituted in 10 μl of deionised water prior to lipid extraction as described above.

2.5. Lipid analysis by liquid chromatography electrospray ionisation tandem mass spectrometry

Lipids were quantified using multiple reaction monitoring on an Agilent 1200 HPLC system coupled to a QTrap 4000 triple quadruople mass spectrometer (Applied Biosystems) using methodology similar to that described previously [20] (Supplemental Table I). Liquid chromatography separation was performed on a 2.1 × 100 mm C18 Poroshell column (Agilent, USA) at 300 μl/min. The following gradient conditions were used: 10% B to 100% B over 13 min, 100% B over 3 min, and a return 10% B over 1 min, followed by 10% B over 3 min. Solvents A and B consisted of water:tetrahydrofuran:methanol in the ratio of 60:20:20 and 5:75:20 respectively, both containing 10 mmol/l ammonium formate.

The oxPC and oxCE species identified using the untargeted LC-MS approach described above (Table 1) were added to the multiple reaction monitoring list for quantification using Q3 product ion values of 184.1 and 369.4 for oxPCs and oxCEs respectively; Declustering, entrance, and cell exit potential were set at 100, 10, and 11 V, respectively for

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