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Human native, enzymatically modified and oxidized low density lipoproteins show different lipidomic pattern

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ABSTRACT

In the present paper we have performed comparative lipidomic analysis of two prototypic atherogenic LDL modifications, oxidized LDL and enzymatically modified LDL. Oxidization of LDL was carried out with different chemical modifications starting from the same native LDL preparations: (i) by copper oxidation leading to terminally oxidized LDL (oxLDL), (ii) by moderate oxidization with HOCl (HOCl LDL), (iii) by long term storage of LDL at 4 °C to produce minimally modified LDL (mmLDL), or (iv) by 15-lipoxygenase, produced by a transfected fibroblast cell line (LipoxLDL). The enzymatic modification of LDL was performed by treatment of native LDL with trypsin and cholesteryl esterase (eLDL).

Free cholesterol (FC) and cholesteryl esters (CE) represent the predominant lipid classes in all LDL preparations. In contrast to native LDL, which contains about two-thirds of total cholesterol as CE, enzymatic modification of LDL decreased the proportion of CE to about one-third. Free cholesterol and CE in oxLDL are reduced by their conversion to oxysterols. Oxidization of LDL preferentially influences the content of polyunsaturated phosphatidylcholine (PC) and polyunsaturated plasmalogen species, by reducing the total PC fraction in oxLDL. Concomitantly, a strong rise of the lysophosphatidylcholine (LPC) fraction can be found in oxLDL as compared to native LDL. This effect is less pronounced in eLDL. The mild oxidation of LDL with hypochlorite and/or lipoxygenase does not alter the content of the analyzed lipid classes and species in a significant manner.

The lipidomic characterization of modified LDLs contributes to the better understanding their diverse cellular effects.

1. Introduction

Atherosclerosis is a chronic vascular disease characterized by lipid retention and inflammation in the vessel wall. In response to modified low-density lipoproteins (LDL), endothelial cells and vascular smooth muscle cells release diverse cytokines and chemoattractants, leading to monocyte activation, migration and subendothelial macrophage differentiation. Activated macrophages accumulate modified LDL such as enzymatically degraded LDL (eLDL) and oxidatively modified LDL (oxLDL) [1].

Oxidatively modified LDL (oxLDL) has been commonly suggested as atherogenic lipoprotein [2]. Upon oxidative modification of LDL, several types of modified lipids are formed, including oxidized fatty acids, lysophosphatidylcholines (LPCs), lipid ester-bound aldehydes, sterols, and oxysterols. Fatty acid oxidation occurs via radical fragmentation of double bonds in cholesteryl esters, non-esterified fatty acids, phospholipids, sphingolipids, plasmalogens, free sterols, tri-, di-, and monoacylglycerides, whereas phospholipids are much more susceptible to oxidation than sterols. The formation of these modified lipids is responsible for a variety of distinct atherogenic properties to oxLDL such as mitogenic activity, cytotoxicity, and lipoapoptosis [3,4]. Representatives of such lipids are oxysterols, which are oxygenated derivatives of cholesterol. The cytotoxic effect of oxLDL is directly connected to oxysterols including 7-hydroxycholesterol and 7-ketocholesterol. Therefore, the determination of oxysterols in oxLDL is an important issue to explore the mechanisms for oxLDL-induced atherosclerosis, or in recently implicated neurodegenerative processes [5].

Whereas terminally oxidized LDL such as copper oxidized LDL is modified at both its lipid and protein part, minimally modified LDL (mmLDL) is mainly oxidized at its lipid part, and thus exhibits unique properties which are different from those of oxLDL. Long term storage mmLDL exposure of endothelial cells enhances monocyte endothelial interactions and induces resistance to the toxic effects of mmLDL [6]. OxLDL are recognized by charge-/motif-receptors e.g., CD36, or other scavenger receptors [7]. Furthermore, it was demonstrated that lipoxygenase oxidized LDL (LipoxLDL) selectively activates macrophages via toll-like receptor-4 (TLR4) [8]. Downregulation of the sterol biosynthetic pathway by LipoxLDL was indicative of LDL uptake and intracellular cholesterol accumulation [9]. Furthermore, hypochlorite-oxidized LDL (HOCl LDL) has been shown to be recognized by macrophages and

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evidence was provided that HOCl LDL is internalized via class B scavenger receptors [10].

The recognition of enzymatically modified LDL (eLDL) is based on the observation that unesterified cholesterol and fatty acids, generated by intimal lipid hydrolases, lead to the formation of eLDL which express conformational epitopes of apo B that are recognized by eLDL specific antibodies in atherogenic lesions [11–13].

Unmodified LDL, which is entrapped in the intima, is susceptible to enzymatic proteolysis and lipid modification. There are enzymes found in atherosclerotic lesions which mediate the modification of LDL, including lysosomal lipid hydrolases, lipoxygenases and proteases. These enzymes are secretory products of immune cells, platelets, subendothelial fibroblasts, and smooth muscle cells in the local microenvironment [12]. Finally, free/unesterified cholesterol and free fatty acids are enriched in eLDL particles as compared to native LDL [14]. The free fatty acids and lysoPC (LPC) species in eLDL promote cell activation and cytokine secretion at lower concentrations [15] and become cytotoxic at higher levels resulting in the atherogenic and apoptotic potential of eLDL [16]. Upon cellular uptake of these particles by macrophages the free cholesterol content of eLDL is rapidly esterified, leading to formation of lipid droplets and foam cells [17].

ELDL particles for in vitro experiments are commonly generated by enzymatic modifications of native LDL with cholesterol esterase and mild trypsin or plasmin exposure, and these particles are internalized by diverse opsonin receptors of macrophages.

The exact lipid composition of modified LDLs as compared to native LDL is still insufficiently characterized.

Although various analytical methods have been previously reported, isotope dilution GC-MS has been the standard method for the determination of oxysterols [18–20]. GC-MS was successfully applied to the determination of oxysterols in oxLDL. As an alternative approach, HPLC has also been successfully introduced to the determination of oxysterols in oxLDL [21]. Both GC and HPLC are reliable methods for the accurate determination of oxysterols, but they often require derivatization and chromatographic separation, which make these methods time-consuming.

For the detection and identification of phospho- and sphingolipids in biological samples, mass spectrometry (MS) is a sensitive and useful technique. Recently, we have developed a fast and reliable liquid chromatography tandem mass spectrometry (LC-MS/MS) method for sphingolipid profiling based on hydrophilic interaction chromatography (HILIC) which allows the simultaneous quantification of sphingoid bases and their methylated products, and established a detailed sphingolipid profile in lipoprotein fractions [22,23].

In the present paper we demonstrate a comparative lipidomic profiling of native and modified LDLs.

2. Experimental

2.1. Lipoprotein preparation

For preparation of lipoproteins normolipidemic healthy volunteers (regular blood donors) with apoE3/E3 genotype were selected. Written informed consent and approval of the local Ethical Committee were obtained. After 12 hour fasting a standard plasmapheresis (on a Trima Accel® automated blood collection system, Terumo BCT or on an Amicus Fenwal® blood separator, Fresenius-Kabi) was performed according to transfusion medicine's procedure. The plasmapheresis was carried out by using sterile ACD-A (Fenwal) as anticoagulant and stabilizator (22.0 g sodium citrate, 8.0 g citrate monohydrate, 24.5 g glucose monohydrate ad 1000 mL distilled water). The plasma was recalcified by adding of calcium chloride dehydrate (final concentration: 0.0002 mM) for 10 min at room temperature. The coagulation was carried out in 50 mL aliquots for 30 min at 37 °C water bath. The clotted plasma was centrifuged for 30 min with 4000 rpm at room temperature. The clot was carefully removed and the serum fraction was subjected to density gradient ultracentrifugation by using potassium bromide as described by Chapman et al. [24]. Part of LDL was enzymatically modified as described below. A commercial trypsin-EDTA solution (Sigma), recommended for cell culture, was applied for the enzymatic modification of LDL. The product consists of 0.5 g/L porcine trypsin and 0.2 g/L EDTA in Hank's balanced salt solution with phenol red. The enzyme activity of the porcine trypsin was determined photometrically at 253 nm by the manufacturer, by using BAEE (benzoyl L-arginine ethyl ester) as substrate: 0.003 μmol/min.

A commercial cholesterol esterase (Seigaku) was also used for the enzymatic modification of LDL. The cholesterol esterase was assayed by the manufacturer spectrophotometrically in a 3 mL reaction mixture containing 0.143 U cholesterol esterase, 1.4 mM cholesteryl oleate, 0.14% (*w*/*v*) NaCl, 0.25% (*w*/*v*) taurocholic acid, and 287 mM potassium phosphate at pH 7.0. The specific activity was found: >200 U/gram protein (biuret). In all of our experiments the same charge of cholesterol esterase was used.

Oxidation of LDL (oxLDL) was performed according to published protocols [25]. Because of the significant cellular ceramide elevation upon copper oxidized LDL and due to simplicity and reproducibility we finally decided to use the classical copper oxidation method for oxLDL preparation. Briefly, LDL was diluted to 1 mg/ml protein in phosphate-buffered saline and dialyzed against 5 μM Cu^{2+} (42 h, 4 °C).

Hypochlorite-oxidized lipoproteins (HOCl-LDL) were prepared by adding 3 mmol/l NaOCl to lipoprotein solutions. Oxidation of LDL was carried out in an ice bath for 30 min [26].

In order to obtain LipoxLDL 50 μg/ml of LDL was incubated in serum-free DMEM for 18 h with murine fibroblast cells overexpressing human 15-lipoxygenase [27–29].

We obtained mmLDL by storage of LDL at 4 °C for 3 months in evacuated tubes [30].

Native LDL from 4 donors was used to preparations of the full data set (LDL, eLDL, oxLDL, HOCl LDL, Lipox LDL, and mmLDL). 11 further LDL preparations (11 donors) were performed just for LDL, eLDL, oxLDL. The modified lipoproteins were stored at 4 °C and used for further cell uptake experiments within a week.

For lipid analysis all LDL samples were frozen at −80 °C and measured in batch mode.

2.2. Protein determination

Protein concentrations were measured using bicinchoninic acid as described [31].

2.3. Lipid extraction

Lipids were extracted according to the procedure described by Bligh and Dyer [32] in the presence of not naturally occurring lipid species as internal standards. The chloroform phase was dried in a vacuum centrifuge and dissolved as described below for quantitative lipid analysis. For oxysterol analysis the extraction was performed according to a modified protocol [33]. The lipids were dried under vacuum without nitrogen or inert gas. A slight increase of oxysterols which are generated by autoxidation can't be excluded during the whole work up procedure. However, the most sensitive step is the alkaline hydrolysis to cleave ester bonds. This step is done under inert gas atmosphere.

2.4. Mass spectrometry

Lipids were quantified by direct flow injection electrospray ionization tandem mass spectrometry (ESI-MS/MS) in positive ion mode using the analytical setup and strategy described previously [34,35]. A precursor ion of *m*/*z* 184 was used for phosphatidylcholine (PC), sphingomyelin (SM) [34] and lysophosphatidylcholine (LPC) [36]. Neutral loss fragments were used for the following lipid classes: Phosphatidylethanolamine (PE) 141, phosphatidylserine (PS) 185,