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Bis(monoacylglycero)phosphate reduces oxysterol formation and apoptosis in macrophages exposed to oxidized LDL

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ABSTRACT

Atherosclerosis is a major cardiovascular complication of diseases associated with increased oxidative stress that favors oxidation of circulating low density lipoproteins (LDLs). Oxidized LDL (oxLDL) is considered as highly atherogenic as it induces a strong accumulation of cholesterol in subendothelial macrophages leading to the formation of foam cells and emergence of atherosclerotic plaque. OxLDL is enriched in oxidation products of cholesterol called oxysterols, some of which have been involved in the ability of oxLDL to induce cellular oxidative stress and cytotoxicity, mainly by apoptosis.

Little is known about the possible contribution of cell-generated oxysterols toward LDL-associated oxysterols in cellular accumulation of oxysterols and related apoptosis. Using both radiochemical and mass analyzes, we showed that oxLDL greatly enhanced oxysterol production by RAW macrophages in comparison with unloaded cells or cells loaded with native LDL. Most oxysterols were produced by non-enzymatic routes (7-ketocholesterol and 7α/β-hydroyxycholesterol) but enzymatically formed 7α-, 25- and 27-hydroxycholesterol were also quantified. Bis(monoacylglycero)phosphate (BMP) is a unique phospholipid preferentially found in late endosomes. We and others have highlighted the role of BMP in the regulation of intracellular cholesterol metabolism/traffic in macrophages. We here report that cellular BMP accumulation was associated with a significantly lower production of oxysterols upon oxLDL exposure. Of note, potent pro-apoptotic 7-ketocholesterol was the most markedly decreased. OxLDL-induced cell cytotoxicity and apoptosis were consistently attenuated in BMP-enriched cells.

Taken together, our data suggest that BMP exerts a protective action against the pro-apoptotic effect of oxLDL via a reduced production of intracellular pro-apoptotic oxysterols.

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1. Introduction

Atherosclerosis is a major cardiovascular complication of diseases associated with increased oxidative stress such as diabetes and chronic inflammation. It is characterized by dysregulation of cholesterol homeostasis in the blood plasma and vascular cells, especially resident macrophages in the subendothelial space in relation with oxidative modification of circulating low density lipoproteins (LDLs) [1]. The so-called oxidized LDL (oxLDL) is considered as highly atherogenic as it induces a strong accumulation of cholesterol in subendothelial macrophages due to unregulated uptake through scavenger receptors, ultimately leading to formation of foam cells and emergence of atherosclerotic plaque [2]. This event mainly depends from the oxidative alteration of apolipoprotein A1 that is no more recognized by the regulated LDL receptor. In addition, oxLDL contain increased levels of oxidation products of cholesterol called oxysterols.

This large family of compounds includes those oxygenated on the sterol ring, mainly at the 7-position (e.g., 7-ketocholesterol and 7α/β-hydroxycholesterol) and those oxygenated on the side-chain (e.g., 24S-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol). Generally, ring-oxygenated sterols tend to be formed non-enzymatically, whereas side-chain oxygenated sterols usually derived from specific enzymes belonging to the cytochrome P450 family. However, 25-hydroxycholesterol and

Abbreviations: BMP, bis (monacylglycero)phosphate; LDL, low density lipoprotein; LE, late endosomes; LXR, liver-X receptor; PG, phosphatidylglycerol.

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7α-hydroxycholesterol can be produced by both enzymatic and non-enzymatic routes [3]. OxLDL has been shown to essentially contain 7-derivatives such as 7β-hydroxycholesterol and 7-ketocholesterol. These compounds have been involved in the ability of oxLDL to induce cellular oxidative stress and cytotoxicity, mainly by apoptosis [4]. In macrophages, 7-ketocholesterol, 7β-hydroxycholesterol, 5,6-epoxycholesterol and 25-hydroxycholesterol are implicated in the activation of apoptosis [5] and are believed to contribute to plaque formation. Atherosclerotic lesions are indeed enriched in 7β-hydroxycholesterol and 7-ketocholesterol, but also some products of enzymatic cholesterol oxidation such as 27-hydroxycholesterol, and 7α- and 25-hydroxycholesterol in lower concentrations and cholesterol 5,6-epoxides [6–9]. 27-hydroxylation of cholesterol is an important pathway for nuclear receptors LXR (Liver X Receptors) activation in response to cholesterol overload [10].

Oxysterol levels in cultured macrophages are very low under normal conditions, but can increase dramatically in response to various perturbations such as excessive cholesterol loading using modified acetylated LDL or upon LPS exposure [10,11]. However, the contribution of cell-mediated generation of oxysterols toward LDL-associated oxysterols is poorly known. To our knowledge, only one study clearly demonstrated the intracellular formation of oxysterols in macrophages upon exposure to aggregated LDL. Similarly, the respective contribution of cellularly generated oxysterols vs LDL-associated oxysterols in inducing apoptosis has been little or not studied.

Bis(monoacylglycero)phosphate (BMP), a unique phospholipid preferentially found in late endosomal membranes, participates in the intracellular cholesterol metabolism/traffic in macrophages and regulates cholesterol efflux via HDL [12–14]. Importantly, using acellular models, we previously reported that BMP could exert protective action against cholesterol oxidation [15]. Of note, it was shown that late endosomes where BMP is concentrated exhibit high oxidant status and could be a site of cholesterol oxidation [16,17]. Very interestingly, it was recently shown that oxLDL induced an increase in cellular content of BMP [18], supporting a link between cellular oxidative stress and BMP.

The aim of the present study was to assess the capacity of RAW macrophages to generate oxysterols and to evaluate the putative role of BMP in this process, in relation with the oxysterol-induced apoptosis.

2. Materials and methods

2.1. Reagents

Cells culture products were from Life Technologies (Saint Aubin, France). [1,2,-^{3}H]cholesterol (50 Ci/mmol) was from Perkin Elmer Life Science (Paris, France). 1,2-dioleoyl-sn-glycero-3-phospho-rac-1-glycerol (18:1/18:1-PG) and stigmasterol, bis(trimethylsilyl)trifluoroacetamide (BSTFA), bis(trimethylsilyl)trifluoroacetamide-trimethylchlorosilane (TMCS-BSTFA) and hydrochloride methoxylamine pyridine were from Sigma (Saint Quentin-Fallavier, France). All solvents were analytical grade from SDS (Peypin, France). Silica gel 60 plates were supplied by Merck (Fontenay Sous Bois, France). Lipids standards were from Avanti Polar lipids (Alabaster, AL). Bio-Rad Protein Assay was from Bio-Rad (Marnes-la-Coquette, France), Cell proliferation Kit I and In Situ Cell Death Detection Kit, Fluorescein were from Roche (Meylan, France).

2.2. Cell culture and treatments

Murine macrophage-like RAW 264.7 were obtained from RIKEN Bioresource Center (Tsukuba, Japan). Cells were cultured in MEM supplemented with non-essential amino acids, 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. They were routinely grown in 100 mm dishes at 37 °C in an atmosphere of 5% CO_2 and subcultured by trypsination at a 1:10 ratio. Experiments were started 24 h after seeding by pre-incubation without (control) or with 30 μM PG liposomes (BMP-enriched) [15] for 24 h. The addition of PG was maintained through the whole experiments. Cells were then incubated in basal conditions (unloaded) or in presence of native or oxidized LDL (loaded) for 24 h at physiological concentrations (100–200 μg/ml). Incubations with LDL were done in 5% lipoprotein deficient serum (LPDS)-containing medium. Other details of incubation conditions are given below and/or in figure legends.

2.3. Lipoprotein preparation and oxidation

Human LDLs were isolated from plasma by sequential ultracentrifugation [19]. LDL oxidation was done by dialysis for 5 h at 37 °C against buffer 10 mM Tris, 150 mM NaCl, pH 7.4 supplemented with 10 μM of $CuSO_4$. A second dialysis was done overnight at 4 °C against buffer 10 mM Tris, 150 mM NaCl, pH 7.4 containing 2 mM EDTA to eliminate $CuSO_4$ and stop oxidation. LDL oxidation was evaluated by GC–MS/MS quantification of their associated oxysterols compared to native LDL.

2.4. Evaluation of cytotoxicity and apoptosis

After treatments, cells were washed with PBS and cell viability was assessed using a colorimetric MTT assay (Cell proliferation Kit I, Roche) according to the manufacturer's instructions. MTT cleavage was determined by reading the absorbance at 560 nm. Cell viability in control and BMP-enriched cells was expressed as the percentage of maximum cell viability as determined in control unloaded cells. Apoptosis was assayed by tunnel assay (In Situ Cell Death Detection Kit, Fluorescein, Roche) and expressed as % apoptotic cells as determined by fluorescent staining.

2.5. Radiochemical analysis

Cells were incubated with 2 μCi/mL [^{3}H]cholesterol in absence or in presence of LDL. Total lipids were extracted from cell lysates and media according to the method of Bligh and Dyer [20]. Labeled sterols, namely [^{3}H]cholesterol, [^{3}H]cholesterol esters and their oxidation products [^{3}H]oxysterols and [^{3}H]oxidized cholesterol esters were separated by TLC (hexane/diethyl ether/acetic acid/methanol, 50:50:1:5; v/v:) and quantified with a radioactivity analyzer (Raytest, France). Cholesterol oxidation was determined as the percentage of total radioactivity recovered as [^{3}H]oxysterols and [^{3}H]oxidized cholesterol esters.

2.6. GC-MS/MS quantification of oxysterols and cholesterol

Total lipids were extracted from cell lysates and media after addition of internal standard stigmasterol and sterols were separated by TLC as described above. Cholesterol and oxysterols were extracted from silica (hexane/diethyl ether, 1:1; v:v), and dried under nitrogen. Derivatization of cholesterol was done using bis(trimethylsilyl)trifluoroacetamide (BSTFA, room temperature, overnight) to yield trimethylsilyl ethers. Derivatization of oxysterols was done using hydrochloride methoxylamine pyridine (5 mg/ml) (80 °C, 1 h), followed by bis(trimethylsilyl)trifluoroacetamide-trimethylchlorosilane (TMCS-BSTFA, room temperature, overnight). Sterols were analyzed by gas chromatography using a Hewlett Packard (HP-6890) and a capillary column J & W 122-4762 (60 m × 0.25 mm). The eluted compounds were detected at the column outlet by a mass spectrometer (Hewlett Packard MS-5973) and quantified using internal standard stigmasterol.

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