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Inserm, U1060, CarMeN Laboratory, Villeurbanne, F-69621, France; INSA-Lyon, IMBL, Villeurbanne, F-69621, France; Université Lyon 1, Villeurbanne, F-69622, France.

Corresponding author: Isabelle Delton-Vandenbroucke, UMR 1060 INSERM, INSA-Lyon, IMBL Building, 20 Ave A. Einstein, 69621 Villeurbanne, France. Tel.: 33-4-72-43-72-36; Fax:33-4-72-43-85-24. E-mail: isabelle.vandenbroucke@insa-lyon.fr.

ABSTRACT

Atherosclerosis is a major cardiovascular complication of diseases associated with increased oxidative stress that favours 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 towards 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.

Keywords : Low density liprotein- Oxysterol-Cholesterol-Macrophages-Apoptosis

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

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 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 towards 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 24h after seeding by pre-incubation without (control) or with 30 µM PG liposomes (BMP-enriched) [15] for 24h. 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 24h 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 (5mg/ml) (80°C, 1h), 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 (60m x 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.

3. Results

3.1. OxLDL exposure enhances the production of oxysterols in RAW macrophages

We first aimed to evaluate the capacity of native and oxidatively modified LDL to induce the production of oxysterols in cultured RAW macrophages. The content of oxysterols in native LDL (nLDL) and oxidized LDL (oxLDL) used for cell incubations is shown in Table 1. No oxysterols could be detected in nLDL. By contrast, as expected from exposure to oxidative conditions ($CuSO_4$ 10 µM), oxLDL contained important amounts of oxysterols. The main oxysterol species were 7-ketocholesterol (58.6%) and 7β-hydroxycholesterol (35.1%), and to a lesser extent 7α-hydroxycholesterol.
The two types of LDLs were then incubated on macrophages and oxysterol production was assayed using two experimental approaches. In a first series of experiments, cells were labelled with [^{3}H] cholesterol during exposure with nLDL or oxLDL (100 or 200 µg/ml) and cholesterol oxidation was determined as the percentage of radioactivity recovered in free and esterified oxysterols. As shown in Figure 1, the proportion of [^{3}H]oxysterols is very low (1.9%) in control cells and increased only slighlty (3%) after incubation with both concentrations of nLDLs. By contrast, it was significantly augmented, by nearly 5-fold, in cells incubated with 100 µg/ml or 200µg/ml of oxLDL. Cell free incubation indicate only moderate oxidation of [^{3}H]cholesterol in the medium during the 24h incubation with the highest concentration of oxLDL (less than 3% compared to nearly 13% in cells), which supports the conclusion that [^{3}H]oxysterols recovered in oxLDL-incubated cells derive from cellular activity. Although only slight and not significant difference was observed between the two concentrations of LDLs, the highest concentration of oxLDL was used in the next experiments to maximize oxysterol production while the lowest concentration of nLDL was preferred as a reference condition with minimal oxysterol production.

In a second set of experiments, cells were incubated in absence or in presence of different LDLs and individual oxysterols were quantified by GC-MS/MS. The quantities of each oxysterol species generated by cells were calculated as the sums of intracellular oxysterols and oxysterols released into the extracellular medium after subtraction, for the oxLDL condition, of the quantity of oxysterols provided by loaded oxLDL (Table 2). In unloading conditions, the level of total oxysterols was very low (450 ng/mg protein), with a majority of 7β-hydroxycholesterol (54%), 7-ketocholesterol (26%), and 7α-hydroxycholesterol (10%). Similar low amount of total oxysterols was recovered upon incubation with 100 µg/ml of nLDL, with almost the same proportion of each oxysterol species, at the exception of a trend to decrease 25 and 27-hydroxycholesterols ($p=0.13$ and $p=0.18$, respectively). Incubation with 200 µg/ml of oxLDL induced a huge increase by 270-fold in total oxysterol production, with an outstanding increase of the amount of 7β-hydroxycholesterol (333-fold), 7-ketocholesterol (132-fold) and 7α-hydroxycholesterol (358-fold). Interestingly, 25- and 27-hydroxycholesterol were significantly increased, while only traces were detected in basal condition and in nLDL- incubated cells.
Taken together, these results show the cell-mediated formation of oxysterols in macrophages exposed to oxLDL.

3.2. BMP regulates the oxLDL-induced production of oxysterols in RAW macrophages

We then aimed to verify whether BMP could modulate cholesterol oxidation as observed in acellular system [15]. To this purpose, cell content of BMP was specifically increased by

supplementation with the precursor dioleylphosphatidylglycerol (PG 30 µM, hereinafter called BMP-enriched cells) as previously described [15]. The consequences of BMP accumulation was evaluated on cellular content of oxysterols as assayed by radiochemical and mass analyses.
As reported above the formation of [^{3}H]oxysterols was significantly increased upon cell incubation with 200 µg/ml of oxLDL compared to 100 µg/ml of nLDL and unloading conditions (Figure 2A). Noteworthy, oxLDL-induced formation of [^{3}H]oxysterols was significantly decreased, by about 30%, in BMP-enriched cells ($p=0.017$). By contrast, BMP accumulation had no effect on [^{3}H]oxysterol formation in unloaded cells or cells incubated with nLDL. We previously reported that PG supplementation increased cellular BMP content in a dose-dependent manner, from nearly 2-fold at 15 µM PG up to 4-fold at 30µM and 60 µM PG [15]. Of interest, the production of intracellular oxysterols was significantly and similarly reduced after supplementation with 30 or 60 µM PG, whereas no change was observed with 15µM PG (figure 2B). This result supports the correlation between BMP content and oxysterol formation.

The distribution of oxysterol species recovered in control and BMP-enriched cells incubated with 200 µg/ml of oxLDL is shown in Figure 2C. The cellular amounts of 7α-hydroxycholesterol and 7β-hydroxycholesterol were unchanged in BMP-enriched cells compared to controls. By contrast, that of 7-ketocholesterol was significantly decreased by about 40% ($p=0.04$). Noteworthy, oxLDL-induced accumulation of 25- and 27-hydroxycholesterol was totally suppressed in BMP-enriched cells. It should be noted that the uptake of both nLDL and oxLDL was unchanged in BMP-enriched cells compared to control cells (data not shown), indicating that changes in cellular amount of oxysterols are not attributable to a different supply of LDL-associated oxysterols.
As shown in Figure 2D, incubation with 200 µg/ml of oxLDL elicited free cholesterol accumulation compared to unloading conditions or incubation with 100 µg/ml of nLDL. Concomitant with the decreased formation of oxysterols, oxLDL-induced cholesterol accumulation was specifically increased, by about 30% ($p=0.049$), in BMP-enriched cells compared to controls.
Altogether, these results showed that increased amount of cellular BMP reduced oxysterol formation/accumulation in oxLDL-loaded macrophages.

3.3. Protective action of BMP against cytotoxic effect induced by oxLDL in RAW macrophages

We then examined whether the reduced accumulation of oxysterols in BMP-enriched cells was correlated with a lower sensitivity to oxLDL-induced cytotoxicity. Cell viability was first assessed by colorimetric MTT assay and was expressed as a percentage of maximum cell viability determined in control unloaded cells (Figure 3A). BMP accumulation alone did not significantly change cell viability. Loading with 100 µg/ml of nLDL induced a moderate cell toxicity from about 20%, and no significant effect of BMP accumulation was observed. After loading with 200 µg/ml of oxLDL, cell viability was greatly decreased, reaching up to 50% in control cells, thus highlighting the toxicity of oxidized LDL. Most importantly, oxLDL-induced toxicity was significantly reduced in BMP-enriched cells. To further investigate the toxic effect of oxLDL, apoptosis was measured by Tunnel assay (Figure 3B). A negligible percentage of apoptotic cells, below 1% of total cells, was detected in both unloaded cells and cells incubated with 100 µg/ml of nLDL. BMP accumulation had no effect under these conditions. By contrast, when cells were incubated with 200 µg/ml of oxLDL, the percentage

of apoptotic cells reached 50% in control cells, thus emphasizing the pro-apoptotic effect of oxLDL as already described [21]. Noteworthy, oxLDL-induced apoptosis was significantly less pronounced in BMP-enriched cells. Since the uptake of both nLDL and oxLDL was unchanged in BMP-enriched cells compared to control cells (data not shown), these changes in cell viability/apoptotic cells are not attributable to a different supply of apoptotic compounds carried by oxLDL.

Taken together, these results suggest a protective role of BMP against the toxic/apoptotic effect of oxLDL in RAW macrophages.

4. Discussion

Apoptosis has been involved in the evolution of atherosclerotic plaques, as supported by the presence of apoptotic macrophages in areas of plaque rupture [22]. Macrophage apoptosis can be induced either by excessive loading with free cholesterol or by uptake of oxLDL [22-24]. We here demonstrate that oxLDL elicits apoptosis in macrophage with concomitant increase of oxysterol production. In addition, we report for the first time the protective effect of BMP against oxLDL-induced apoptosis, most likely through a reduced formation of pro-apoptotic oxysterols.

Oxidation of LDL is an intricate process during which lipids and proteins of the particles undergo oxidative changes and form complex products [25]. LDL oxidation is promoted by metal ions (e.g. copper, iron), enzymes (e.g. lipoxygenase, or superoxide-generating enzymes), or reactive oxygene species, [25, 26]. The composition of LDL is greatly altered during its oxidation catalyzed by transition metals, including loss of cholesterol and cholesteryl esters, and simultaneous formation of free and esterified oxysterols. We therefore characterized oxysterol compositions in native and oxidized LDL used in our studies. Whereas no oxysterol were detected in nLDL, copper-oxidation at 37°C induced the production of about 70 mg of oxysterols/mg LDL protein, which is close to the quantity reported in same oxidative condition by Gerry et al [27]. Of interest, these authors showed that this method of oxidizing LDL specifically yields to oxysterol-rich form, thus providing a useful method to study the effects of oxysterols carried by LDL on cell function [27]. In agreement with the literature [27-29], the most abundant oxysterols found in oxLDL are 7-ketocholesterol and 7β-hydroxycholesterol, derivatives of non-enzymatic pathway whereas enzymatically produced oxysterols such as 25- or 27-hydroxycholesterol were not detected. Using both radiochemical and mass assays, we show that formation of oxysterols was promoted upon cell exposure with oxLDL while it remained very low in basal conditions or during incubation with nLDL. This result is important because it reinforces the idea that oxidized LDL induces cellular accumulation of oxysterols, deriving not only from oxysterols it contains, but also from enhanced intracellular production of these compounds [17]. Another possibility to consider is that oxysterols would not be generated intracellularly but would rather derive from an enhanced oxidation of oxLDL in the medium. In such scenario, oxLDL would initiate cellular oxidative stress [4] that would in turn promote oxidation of LDL present in the incubation medium with subsequent cellular uptake. Although we cannot settle this issue, we may extrapolate from observations collected from relative studies using THP1 macrophages. In these cells indeed, we could also measure [^{3}H]oxysterols in pulse label conditions, *i.e.* [^{3}H]cholesterol labeling followed by [^{3}H]cholesterol removal and subsequent incubation with oxLDL, supporting the conclusion that oxLDL favours intracellular cholesterol oxidation. The formation of enzymatically produced 27-hydroxycholesterol in RAW macrophages also corroborated this conclusion. Enhanced formation of non-enzymatically produced oxysterols may then result from cellular cholesterol auto-oxidation, as elicited by increased oxidant stress in oxLDL-loaded macrophages. Sterol 27-hydroxylase (CYP27A1), the enzyme responsible for the formation of 27-hydroxycholesterol, is highly expressed in macrophages [30-32]. To our knowledge, the formation of 27-hydroxycholesterol upon oxLDL exposure has not been reported before. Increased formation of 27-hydroxycholesterol has been previously reported in human macrophages following excessive cholesterol loading, *i.e.* high concentration of acetylated LDL for 72h [10]. 25- and 27-hydroxycholesterol have been detected in RAW macrophages exposed to Kdo-lipid A, an active component of lipopolysacccharide functioning as Toll-like receptor 4 [11].

The putative regulatory role of BMP on oxysterol production was then challenged using a model of cell enrichment in BMP. The results indicate that oxLDL-induced accumulation of

oxysterols, especially 7-ketocholesterol and 27-hydroxycholesterol, was significantly decreased in BMP-enriched cells. The correlation between BMP content and reduced oxysterol production was further supported by concentration-dependent effect of PG. Of interest, the differences were mainly accounted for oxysterols recovered in cells as similar amounts of oxysterols were measured in media (data not shown). This suggests that BMP may control cellular formation of oxysterols and/or their degradation but would not modify oxysterol efflux. It is important to note that the decreased production of 7-ketocholesterol and 27-hydroxycholesterol in BMP-enriched cells is correlated to a 30% increase of free cholesterol level, which may be related to a reduced oxidation of cholesterol and/or an inhibition of cholesterol efflux via the decrease of the LXR agonist, 27-hydroxycholesterol.
Mechanisms underlying the reduction of cellular cholesterol oxidation in BMP-enriched cells remain to be determined. Regarding that cholesterol oxidation has been reported to occur in late endosomes [16, 17], the specific localization of BMP in this compartment may be an important factor. We previously showed in acellular system that the 22:6/22:6-BMP species protects against non-enzymatic cholesterol oxidation, related to the high sensitivity of this species to oxidative stress which would confer anti-oxidant activity towards neighboring lipids. This putative mechanism is unlikely to be involved in BMP-enriched cells as the protocol used to accumulate BMP was shown to primarily induce the synthesis of 18:1/18:1-BMP [15]. The loss of 27-hydroxycholesterol formation in BMP-enriched cells after oxLDL exposure suggest that BMP may regulate CYP27A1 activity/and or expression. 27-hydroxycholesterol appears as a potent LXR activator in macrophages [33]. Noteworthy, some of our relative studies indicate that expression of several genes involved in cholesterol metabolism/transport including some LXR dependent genes are modified in BMP-enriched cells (unpublished data). Besides regulating oxysterol production, BMP could promote redistribution of oxysterols leading to their degradation, through interaction with the cytoplasmic oxysterol binding proteins, involved in various cellular processes such as vesicular transport, cholesterol metabolism and cell signaling [34]. A possible interaction of BMP with ORP could induce a redistribution of oxysterols leading to their degradation thereby preventing apoptosis. Most studies have concluded that apoptotic effect of oxLDLs was at least partly mediated by their associated-oxysterols. Generally, 7-ketocholesterol, 7ß-hydroxycholesterol, and to a lesser extend 27-hydroxycholesterol induce apoptosis on various cell types, whereas 25-hydroxycholesterol has a more or less pronounced cytotoxicity depending on the cell considered [4].

The last part of this study aimed to evaluate the apoptotic effect of oxLDL and the consequences of BMP accumulation in relation with oxysterol production. Our data indicate that oxLDL induced apoptosis with a concomitant formation of pro-apoptotic 7-ketocholesterol and 7ß-hydroxycholesterol. By contrast, no apoptosis was detected in cells incubated with nLDL, in which no increase of oxysterol production was measured. Noteworthy, we showed that oxLDL-induced apoptosis could be partially prevented in BMP-enriched cells in correlation with a lower formation of apoptotic 7-ketocholesterol. These observations strongly support the role of cellularly generated oxysterols in oxLDL-induced apoptosis, in addition to direct effect of oxLDL-associated oxysterols. Also of interest is that BMP accumulation did not reverse nLDL-induced decrease of cell viability that is most likely independent of oxysterols. The findings that BMP may regulate oxysterol formation/accumulation is of particular relevance regarding that BMP accumulation has been reported as a noticeable feature of oxLDL laden macrophages [18]. In this context, the decreased formation of 27-hydroxycholesterol would be rather detrimental since this compound is believed to play an essential role in counteracting cellular cholesterol overload [35]. It is the most abundant oxysterol in the human artery wall [3, 36-38] and is not only

capable of inducing ABCA1, ABCG1, and apoE expression via LXR activation [39], its secretion may also be a significant form of HDL-independent cholesterol efflux [36]. On the contrary, the decrease of proapoptotic 7-ketocholesterol is a beneficial effect, especially as it seems to be responsible for the protective role of BMP against apoptosis induced by oxLDL. Our data suggest that BMP accumulation could constitute a defense against deleterious oxLDL and its associated oxysterols.
In conclusion, this study supports a role of BMP in modulating cellular disturbances induced by oxLDL in macrophages, especially oxysterol production and apoptosis, with potential relevance in pro-atherogenic conditions.

Aknowledgments

This work was supported by grants from INSA-Lyon, INSERM.

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Figure legends

Fig. 1. Production of oxysterols by RAW macrophages. Cells were incubated with 2µCi [^{3}H]cholesterol without (unloaded) or with nLDL or oxLDL at 100 or 200 µg/ml for 24h. The conversion of [^{3}H]cholesterol into [^{3}H]oxysterols was expressed as percentage of total cellular radioactivity. Data are the means ± SD of 3 wells and representative of two independent experiments. a, $p<0.05$ compared to unloaded; b, $p<0.05$ compared to the same concentration of nLDL.

Fig. 2. Protective action of BMP against oxLDL-induced production of intracellular oxysterols. (A) Control and BMP-enriched cells were incubated with 2µCi [^{3}H]cholesterol without (unloaded) or with 100 µg/ml of nLDL or 200 µg/ml of oxLDL for 24h. [^{3}H]oxysterol formation was determined as in figure 1. (B) Cells were supplemented with different concentrations of PG and incubated with 2µCi [^{3}H]cholesterol without (unloaded) or with 200 µg/ml of oxLDL for 24h. [^{3}H]oxysterol formation was determined as in figure 1. (C, D) Control and BMP-enriched cells were incubated without (unloaded) or with 100 µg/ml of nLDL or 200 µg/ml of oxLDL for 24h. Analyses of oxysterol species (C) or cholesterol (D) were performed by GC-MS/MS as described in material and methods. Data are the means ± SD of three wells and representative of at least two independent experiments. a, $p<0.05$ compared to unloaded; b, $p<0.05$ compared to nLDL; c, $p<0.05$ compared to oxLDL.

Fig. 3. Protective action of BMP against toxic and apoptotic effects induced by oxLDL. Control and BMP-enriched cells were incubated without (unloaded) or with 100 µg/ml of nLDL or 200 µg/ml of oxLDL for 24h. Cell viability (A) and apoptosis (B) were evaluated. Data are the means ± SD of three wells and representative of three independent experiments. a, $p<0.05$ compared to unloaded; b, $p<0.05$ compared to nLDL; c, $p<0.05$ compared to oxLDL.

Table 1
Oxysterol composition of native and oxidized LDL

	nLDL	oxLDL	
7α-hydroxycholesterol	n.d.	4.3	4.5
7β-hydroxycholesterol	n.d.	29.8	25.4
7-ketocholesterol	n.d.	28.9	42.4
25-hydroxycholesterol	n.d.	n.d.	n.d.
27-hydroxycholesterol	n.d.	n.d.	n.d.
Total oxysterols	n.d.	62.9	72.4

Data are expressed in μg/mg of LDL protein

Table 2
Quantification of oxysterol species produced by RAW macrophages after exposure to LDL.

	Unloaded	nLDL	oxLDL
7α-hydroxycholesterol	48.5 ± 11.9	53.3 ± 10.8	16164.7 ± 326.2 [a,b]
7β-hydroxycholesterol	246.9 ± 43.7	239 ± 29.8	88468.4 ± 1952.5 [a,b]
7-ketocholesterol	120.8 ± 30.2	101.3 ± 19.9	15915.3 ± 623.4 [a,b]
25-hydroxycholesterol	16.3 ± 4.2	n.d.	754.8 ± 13.6 [a,b]
27-hydroxycholesterol	17.5 ± 3.5	4.2 ± 3.8	82.6 ± 8.5 [a,b]
Total oxysterols	450.8 ± 83.8	397.8 ± 37.8	121353.5 ± 2272.6 [a,b]

Data are expressed in ng/mg of cell protein, are the mean ± SD of 3 wells and representative of two independent experiments. a, $p<0.05$ compared to unloaded; b, $p<0.05$ compared to nLDL.

Figure 1

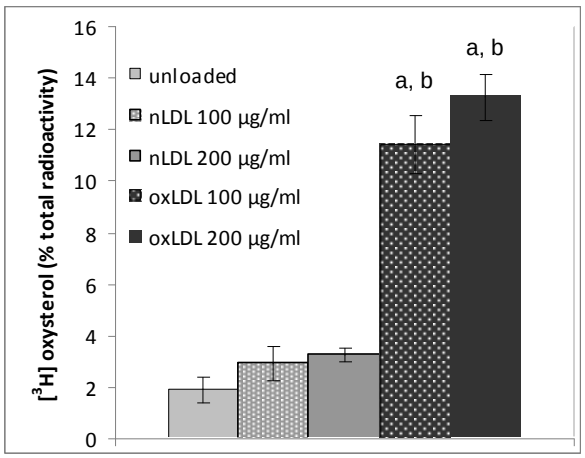

16
14
12
10
8
6
4
2
0
[3H] oxysterol (% total radioactivity)
unloaded
nLDL 100 µg/ml
nLDL 200 µg/ml
oxLDL 100 µg/ml
oxLDL 200 µg/ml
a, b
a, b

Figure 2

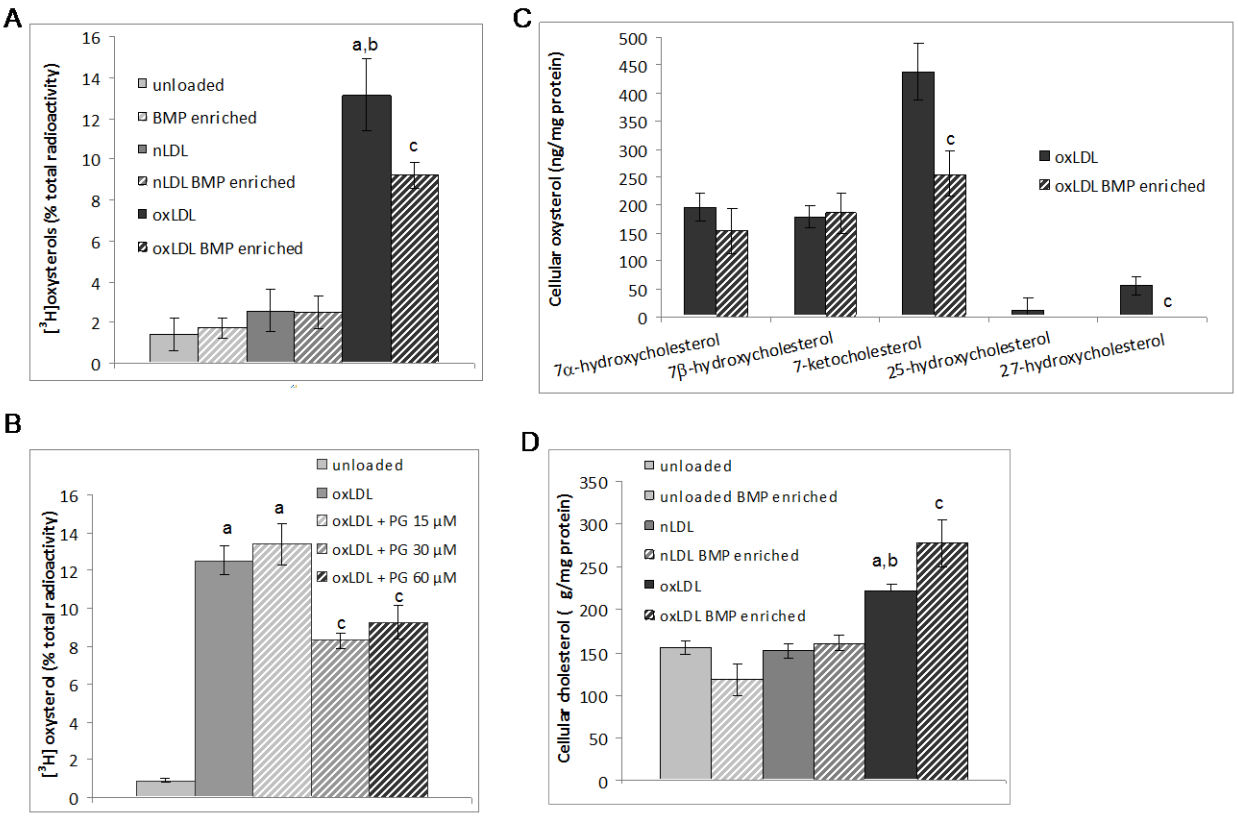
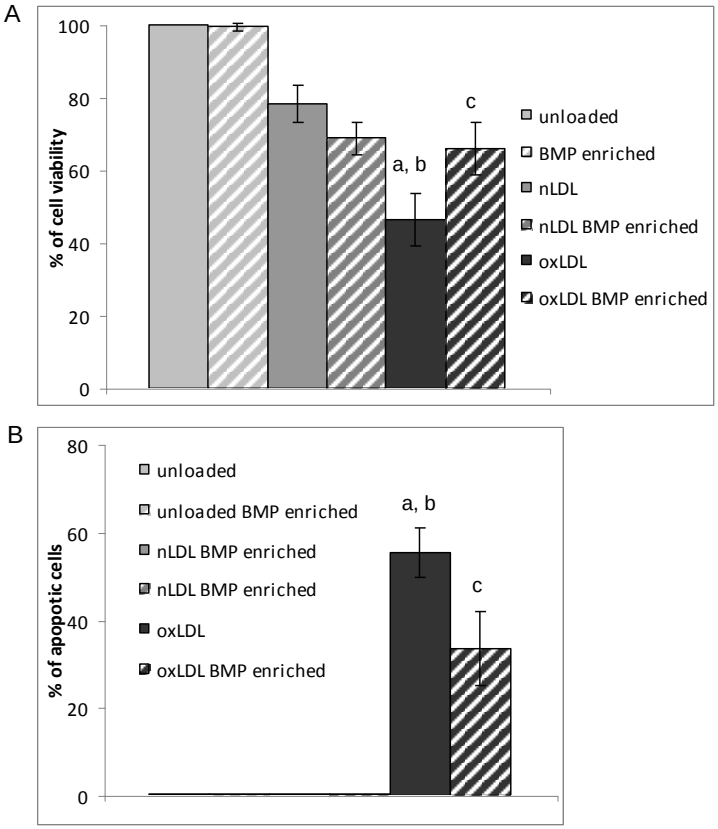

A
[3H]oxysterols (% total radioactivity)
unloaded
BMP enriched
nLDL
nLDL BMP enriched
oxLDL
oxLDL BMP enriched
a,b
c
C
Cellular oxysterol (ng/mg protein)
oxLDL
oxLDL BMP enriched
c
7α-hydroxycholesterol
7β-hydroxycholesterol
7-ketocholesterol
25-hydroxycholesterol
27-hydroxycholesterol
B
[3H] oxysterol (% total radioactivity)
unloaded
oxLDL
oxLDL + PG 15 µM
oxLDL + PG 30 µM
oxLDL + PG 60 µM
a
a
c
c
D
Cellular cholesterol (g/mg protein)
unloaded
unloaded BMP enriched
nLDL
nLDL BMP enriched
oxLDL
oxLDL BMP enriched
a,b
c

Figure 3


A
100
80
60
40
20
0
% of cell viability
a, b
c
unloaded
BMP enriched
nLDL
nLDL BMP enriched
oxLDL
oxLDL BMP enriched
B
80
60
40
20
0
% of apopotic cells
unloaded
unloaded BMP enriched
nLDL BMP enriched
nLDL BMP enriched
oxLDL
oxLDL BMP enriched
a, b
c