Bis(Monoacylglycero)Phosphate Accumulation in Macrophages Induces Intracellular Cholesterol Redistribution, Attenuates Liver-X Receptor/ATP-Binding Cassette Transporter A1/ATP-Binding Cassette Transporter G1 Pathway, and Impairs Cholesterol Efflux

Céline Luquin-Costaz, Etienne Lefai, Maud Arnal-Levron, Daria Markina, Shota Sakai, Vanessa Euthine, Asami Makino, Michel Guichardant, Shizuya Yamashita, Toshihide Kobayashi, Michel Lagarde, Philippe Moulin, Isabelle Delton-Vandenbroucke

Objective.—Endosomal signature phospholipid bis(monoacylglycero)phosphate (BMP) has been involved in the regulation of cellular cholesterol homeostasis. Accumulation of BMP is a hallmark of lipid storage disorders and was recently reported as a noticeable feature of oxidized low-density lipoprotein–laden macrophages. This study was designed to delineate the consequences of macrophage BMP accumulation on intracellular cholesterol distribution, metabolism, and efflux and to unravel the underlying molecular mechanisms.

Approach and Results.—We have developed an experimental design to specifically increase BMP content in RAW 264.7 macrophages. After BMP accumulation, cell cholesterol distribution was markedly altered, despite no change in low-density lipoprotein uptake and hydrolysis, cholesterol esterification, or total cell cholesterol content. The expression of cholesterol-regulated genes sterol regulatory element–binding protein 2 and hydroxymethylglutaryl-coenzyme A reductase was decreased by 40%, indicative of an increase of endoplasmic reticulum–associated cholesterol. Cholesterol delivery to plasma membrane was reduced as evidenced by the 20% decrease of efflux by cyclodextrin. Functionally, BMP accumulation reduced cholesterol efflux to both apolipoprotein A1 and high-density lipoprotein by 40% and correlated with a 40% decrease in mRNA contents of ATP-binding cassette transporter A1, ATP-binding cassette transporter G1, and liver-X receptor α and β. Foam cell formation induced by oxidized low-density lipoprotein exposure was exacerbated in BMP-enriched cells.

Conclusions.—The present work shows for the first time a strong functional link between BMP and cholesterol-regulating genes involved in both intracellular metabolism and efflux. We propose that accumulation of cellular BMP might contribute to the deregulation of cholesterol homeostasis in atheromatous macrophages. (Arterioscler Thromb Vasc Biol. 2013;33:1803-1811.)

Key Words: ATP-binding cassette transporters ▪ atherosclerosis ▪ endosomes ▪ lipoproteins

Cholesterol cell homeostasis is regulated by a complex set of mechanisms that include cholesterol synthesis, uptake of low-density lipoproteins (LDL), cholesterol esterification, and cholesterol efflux. The bulk of cholesterol in macrophages originates from receptor-mediated endocytosis of LDL. Macrophages can export excess cholesterol to high-density lipoproteins (HDL) and apolipoprotein A1 (apoA1) by efflux processes that require the activity of ATP-binding cassette transporter A1 (ABCA1) and ATP-binding cassette transporter G1 (ABCG1). During atherogenesis, macrophages take up modified LDL in an unregulated manner via scavenger receptors, ultimately resulting in the deposition of large stores of cholesteryl esters and free cholesterol that characterize foam cell phenotypes. The formation of cholesterol-laden macrophages is a prominent feature of atherogenesis.

Late endosomes are an obligatory station for LDL-derived cholesterol that is next transported from this organelle to plasma membrane (PM) and endoplasmic reticulum (ER). Bis(monoacylglycero)phosphate (BMP), sometimes called lysobisphosphatidic acid, is a phospholipid highly abundant in the internal membranes of multivesicular late endosomes, in which it forms specialized lipid domains. Several reports...
have demonstrated the involvement of BMP in both structure and function of late endosomes/lysosomes.4 We and others have underlined the role of BMP in cellular cholesterol transport and distribution. It was first reported that treatment of cultured fibroblasts with anti-BMP antibody that accumulates in late endosomes resulted in a massive accumulation of cholesterol in this compartment.3,5 The modulation of BMP domains by the glycolipid inhibitor n-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol altered cellular cholesterol homeostasis in these cells.6 More recently, it was demonstrated that cholesterol exit and retention in late endosomes depend on BMP content in this compartment, through a mechanism involving the endosomal protein Alix.7 Our studies in both murine and human macrophages showed that endosomal accumulation of anti-BMP antibody altered cholesterol distribution in relation to reduced cholesterol efflux to HDL.8

Most human and animal cells or tissues contain relatively low amounts of BMP, not exceeding 1% to 2% of total phospholipids. However, the level of BMP was shown to dramatically increase in certain inherited lysosomal storage disorders, a group of diseases characterized by endolysosomal lipidosis, such as Niemann–Pick (NP) and Gaucher diseases.4 Gaucher disease, that displays lipid-engorged cells of the monocyte-macrophage lineage, has been associated with low HDL cholesterol concentration, evocating a deregulation of cholesterol homeostasis.9 Furthermore, BMP accumulation has been recently reported as a noticeable feature of oxidized LDL (oxLDL)–laden macrophages.10 To date, literature dealing with the understanding of cholesterol-related functions of BMP is rather scarce. We have previously reported that cellular BMP content was significantly increased after incubation of RAW 264.7 macrophages with the precursor dioleoyl-phosphatidylglycerol (18:1/18:1-PG).11 This approach was considered as a valuable model of BMP accumulation regarding that BMP produced from exogenous PG by RAW macrophages has been shown to keep the unique sn1:sn1 stereoconfiguration of natural BMP.12 In addition, we here report that 18:1/18:1-BMP was primarily increased after supplementation with 18:1/18:1-PG, thus mimicking the conditions found in lysosomal storage disorder for both cellular and plasma BMP accumulation.13 Our results show for the first time that endosomal BMP accumulation impacts on cholesterol distribution and efflux in association with changes in cholesterol-regulating genes. Molecular mechanisms involving liver-X receptor (LXR)/ABCA1/ABCG1 are proposed to contribute to the effects of BMP on cholesterol homeostasis and, especially, efflux in macrophages.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Supplementation With 18:1/18:1-PG Induced the Specific Accumulation of BMP in Late Endosomes

We previously reported that supplementation of cultured RAW macrophages with 30 µmol/L of 18:1/18:1-PG induced cellular accumulation of BMP with no change in other phospholipase, namely phosphatidylcholine and phosphatidylethanolamine.11 Here, we further show that PG content did not change in this condition, whereas BMP content was increased 2-fold (Figure 1A). The conversion of PG to BMP in RAW macrophages requires a PG-selective phospholipase A2, whose activity toward 16:0/16:0-PG is low compared with oleate-containing PG.14 Consistently, we found that BMP content was unchanged in cells supplemented with 30 µmol/L of 16:0/16:0-PG, whereas PG content was significantly increased. Total PG plus BMP reached similar amounts in both 16:0/16:0-PG– and 18:1/18:1-PG–supplemented cells, about twice that in controls (7333, 7889, and 3799 pmol/mg protein for the 2 added PG and control samples, respectively), indicating that both PG were taken by the cells at equal rate, with no conversion of 16:0/16:0-PG and total conversion of 18:1/18:1-PG to BMP. As shown in Table 1, 18:1/18:1-BMP was the major BMP molecular species in 18:1/18:1-PG–supplemented cells. Overall, molecular species composition of BMP in these cells was similar to control cells. Supplementation with 16:0/16:0-PG resulted in a large increase in 16:0/16:0-PG at the expense of other PG molecular species (Table 2), which confirms previous reports indicating that 16:0/16:0-PG was not metabolized in RAW macrophages.

Figure 1. Quantification of bis(monoacylglycero)phosphate (BMP) and cellular localization. A, BMP and phosphatidylglycerol (PG) from control cells and 18:1/18:1-PG– and 16:0/16:0-PG–supplemented cells were quantified by liquid chromatography–mass spectrometry as described in Materials and Methods in the online-only Data Supplement. Data are the mean±SD of 3 independent determinations. B, Control and BMP–enriched cells were fixed and stained with Alexa 546–conjugated anti-BMP antibody (6C4) and with Alexa 488–conjugated anti-CD63 antibody. Scale bar, 10 µm.
Table 1. Molecular Species of BMP

<table>
<thead>
<tr>
<th>BMP Molecular Species</th>
<th>Control 18:1/18:1 PG mol%</th>
<th>16:0/16:0 PG mol%</th>
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RAW macrophages were cultured in the absence or presence of 30 μmol/L 18:1/18:1-PG or 16:0/16:0-PG for 24 h. After total lipid extraction, analysis of BMP molecular species was performed by electrospray mass spectrometry as described in Materials and Methods in the online-only Data Supplement. Data are expressed as mole percent and are the average of 3 independent determinations.

Table 2. Molecular Species of PG

<table>
<thead>
<tr>
<th>PG Molecular Species</th>
<th>Control 18:1/18:1 PG mol%</th>
<th>16:0/16:0 PG mol%</th>
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<td>16:0/16:0</td>
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<td>22:6/22:6</td>
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<td>0.02</td>
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</table>

RAW macrophages were cultured in the absence or presence of 30 μmol/L 18:1/18:1-PG or 16:0/16:0-PG for 24 h. After total lipid extraction, analysis of PG molecular species was performed by electrospray mass spectrometry as described in Materials and Methods in the online-only Data Supplement. Data are expressed as mole percent and are the average of 3 independent determinations. PG indicates phosphatidylglycerol.

BMP Accumulation Does Not Modify LDL-Derived Cholesterol Delivery to Macrophages

Free cholesterol (FC) delivery to macrophages after LDL endocytosis depends on LDL uptake, hydrolysis of LDL-associated cholesteryl esters (CE) in the late endosomal/lysosomal compartment, and cholesterol re-esterification in the ER by acetyl-coenzyme A acetyltransferase. No difference in LDL uptake, as assessed using [3H]cholesterol-oleate-LDL, was observed between control and BMP-enriched cells (Figure 2A). Hydrolysis of LDL-associated CE was measured in the presence of 2 μg/mL acetyl-coenzyme A acetyltransferase inhibitor F1394 to avoid cholesterol re-esterification. At this concentration, F1394 led to complete inhibition of cholesterol esterification as assayed by [3H]oleate incorporation test (not shown). Figure 2B indicates similar rate of CE hydrolysis in both control and BMP-enriched cells. Cholesterol esterification was measured as the proportion of radioactivity recovered in CE after incubation with [3H]cholesterol-LDL or [3H]cholesterol. Under all tested conditions, no difference was observed between control and BMP-enriched cells (Figure 2C). These data suggest no change in the esterification process after BMP accumulation.

BMP Accumulation Is Associated With Redistribution of LDL-Derived Cholesterol

The consequences of BMP accumulation on LDL-derived cholesterol transport to PM and ER were examined. We previously showed that cholesterol efflux to methyl-β-cyclodextrin is indicative of the proportion of LDL-derived cholesterol transported to the PM in RAW macrophages. As shown in Figure 3A, cholesterol efflux to methyl-β-cyclodextrin was significantly reduced in BMP-enriched cells compared with controls (≈−20%; P≤0.05), suggesting a decreased transport of cholesterol to PM.

The content of cholesterol in ER is known to regulate the expression of sterol regulatory element–binding protein (SREBP) 2 and SREBP2 target genes, such as hydroxymethylglutaric-ylcoenzyme A reductase. As expected, cell loading with LDL, which is assumed to increase ER cholesterol, significantly decreased SREBP2 mRNA level (≈−40% versus controls; P=0.0016; Figure 3B). Treatment with U18666A (1 μg/mL), which has been reported to inhibit cholesterol transport to ER in many cells, including RAW macrophages, consistently reversed LDL-induced suppression of SREBP2.
and increased SREBP2 expression above control values (1.4-fold versus controls; \( P = 0.024 \)). Conversely, LDL-induced suppression of SREBP2 was exacerbated in BMP-enriched cells (\( \approx -80\% \) versus control; \( P < 0.0001 \) and \( \approx -60\% \) versus LDL; \( P = 0.0014 \)). Similar variations were observed for hydroxymethylglutaryl-coenzyme A reductase mRNA (Figure 3C). These results suggest that BMP accumulation is associated with an increase in ER cholesterol.

**Figure 2.** Intact cholesterol delivery from internalized low-density lipoprotein (LDL) in bis(monoacylglycerol) phosphate (BMP)–enriched cells; control and BMP-enriched cells were incubated with 50 \( \mu \)g/mL [\( ^{3}H \)]cholesteryl oleate-LDL. A, LDL uptake is expressed as nCi/mg cell protein. B, Cholesterol ester (CE) hydrolysis is expressed as the percentage of [\( ^{3}H \)]cholesteryl oleate converted to [\( ^{3}H \)]cholesterol in the absence or presence of the acetyl-coenzyme A acetyltransferase inhibitor F1394. C, Control and BMP-enriched cells were incubated with [\( ^{3}H \)]cholesterol-LDL or [\( ^{3}H \)]cholesterol in the absence or presence of 100 \( \mu \)g/mL LDL for 12 hours. Cholesterol esterification was measured as the proportion of radioactivity recovered in CE. D, Control and BMP-enriched cells were incubated in the absence (unloaded) or presence (loaded) of 100 \( \mu \)g/mL LDL for 12 hours. Sterol contents are expressed as nmol/mg cell protein. Data are the means±SD of 4 wells and representative of 2 independent experiments.

**Figure 3.** Low-density lipoprotein (LDL)–derived cholesterol redistribution in bis(monoacylglycerol) phosphate (BMP)–enriched cells. A, Control and BMP-enriched cells were incubated with 50 \( \mu \)g/mL of [\( ^{3}H \)]cholesteryl oleate-LDL for 12 hours. Cholesterol efflux was then stimulated by incubation with 10 mmol/L methyl-\( \beta \)-cyclodextrin for indicated times. Control, BMP-enriched cells, or U18666A-treated cells were incubated in the absence or presence of 100 \( \mu \)g/mL LDL for 12 hours. Cells were analyzed for sterol regulatory element–binding protein (SREBP) 2 (B) and hydroxymethylglutaryl-coenzyme A reductase (HMGCoAR; C) mRNA content as described in Materials and Methods in the online-only Data Supplement. Data are the means±SD of 4 wells and representative of 3 independent experiments. A–C, Significantly different groups (multiple means comparisons by ANOVA and Tukey–Kramer method with \( \alpha = 0.05 \)). *\( P < 0.05 \) compared with control by \( t \) test.

**BMP Accumulation Decreased HDL- and ApoA1-Stimulated Cholesterol Efflux**

Macrophages were then challenged for their capacity to release cholesterol in response to HDL and apoA1 after cell loading with LDL. As shown in Figure 4A, both HDL- and apoA1-stimulated efflux were reduced by \( \approx 40\% \) in BMP-enriched cells compared with controls (\( P < 0.0005 \) and \( P < 0.001 \), respectively). Significant diminution of HDL-stimulated cholesterol efflux could be observed throughout time-course experiments (Figure 4B). As control for BMP specificity, cholesterol efflux to HDL was measured in PG-enriched cells (supplemented with 16:0/16:0-PG), and no difference was observed with controls (20.7±2.3 versus 22.3±2.2%, respectively; mean±SD of 3 determinations).

Data from methyl-\( \beta \)-cyclodextrin–mediated cholesterol efflux (Figure 3A) suggest that the transport of LDL-derived cholesterol to PM is decreased in BMP-enriched cells, which could explain the lower cholesterol efflux in LDL-loaded cells. In another series of experiments, cells were incubated with free [\( ^{3}H \)]cholesterol that can exchange with PM and label cellular pools independent of LDL loading. A decrease in cholesterol efflux to HDL and apoA1 was then observed in BMP-enriched cells compared with controls (HDL: \(-52\%;\ P < 0.0005\); apoA1: \(-46\%;\ P < 0.05\); Figure 4C). Altogether,
these results suggest that the decrease in LDL-derived cholesterol efflux in BMP-enriched cells did not result from lower cholesterol availability in PM.

**Decreased Expression of ABCA1 and ABCG1 Transporters and LXR Receptors Is Involved in Decreased Cholesterol Efflux in BMP-Enriched Cells**

HDL and apoA1-stimulated efflux of cholesterol in macrophages have been shown to essentially depend on the expression/activity of ABC transporters G1 and A1, respectively. As shown in Figure 5A and 5B, loading with LDL increased ABCA1 and ABCG1 mRNA levels, by 4- and 2-fold, respectively, versus controls (P<0.0001). These results are pertinent because previous studies have only reported a decrease in ABCA1 expression/activity of ABC transporters G1 and A1, respectively.17,18

As previously reported, LXRβ is more highly expressed than LXRα in RAW macrophages. Loading with LDL increased both LXRα (1.3-fold; P=0.0012) and LXRβ (1.4-fold; P=0.016) mRNA levels. LXRα expression was significantly reduced in BMP-enriched cells on LDL loading (LDL+18:1/18:1-PG versus LDL, −50%; P<0.0001) and under basal conditions, although to a lower extent (18:1/18:1-PG versus controls, −22%; P=0.0014; Figure 5D). Similar effects were obtained for LXRβ (LDL+18:1/18:1-PG versus LDL, −65%; P<0.0001 and 18:1/18:1-PG versus controls, −29%; P=0.045; Figure 5E). These effects paralleled those observed for ABCA1 and ABCG1, suggesting that a downregulation of LXRs could participate in downregulating these ABC transporters. Again, no difference was observed in PG-enriched cells (supplemented with 16:0/16:0-PG) versus controls (LXRα, 42.2±4.3 versus 40.7±2.2, and LXRβ, 247±39 versus 203±8, relative values, mean±SD of 3 determinations), which is consistent with unchanged expression of ABCA1 and G1 transporters in these cells.

**oxLDL–Induced Foam Cell Formation Is Exaggerated in BMP-Enriched Cells**

Another functional consequence of BMP accumulation was evaluated by foam cell formation assay. As expected, Nile red–positive cells were significantly increased after exposure to oxLDL compared with controls (oxLDL versus controls, +30%; P<0.0001; Figure 6A and 6B). Most importantly, Nile red staining was further enhanced in BMP-enriched cells (oxLDL+18:1/18:1-PG versus controls, +48%; oxLDL+18:1/18:1-PG versus oxLDL; P=0.007), indicating that BMP accumulation contributes to foam cell formation. As control for BMP specificity, Nile red staining was measured in PG-enriched cells (supplemented with 16:0/16:0-PG), and no difference was observed with cells exposed to oxLDL only.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Decreased cholesterol efflux by high-density lipoprotein (HDL) and apolipoprotein A1 (apoA1) in bis(monoacylglycerol)phosphate (BMP)–enriched cells. Control and BMP-enriched cells were incubated with (A and B) 50 µg/mL of [3H]cholesterol for 12 hours. Cholesterol efflux was then stimulated by incubation with (A and C) 100 µg/mL HDL for 8 hours or 10 µg/mL apoA1/0.5 mmol/L 8brcAMP for 24 hours; (B) 100 µg/mL HDL for the indicated times. Data are the mean±SD of 4 wells and representative of 3 independent experiments. *P<0.05, **P<0.001 compared with control by t test.
Discussion

Our current study reveals not only the suppressive effects of BMP accumulation on cholesterol efflux in macrophages but also the underlying molecular mechanisms. Indeed, we demonstrated that BMP accumulation reduces the expression of key cholesterol regulatory genes, including ABCA1-, ABCG1-, SREBP2-, and SREBP2-dependent genes. We further connect the regulation of ABC transporters to the LXR pathway, with the putative involvement of LXR ligand oxy-sterols. Furthermore, we draw a possible link between BMP accumulation and atherogenesis through the exacerbated foam cell formation in BMP-enriched cells.

Our conclusions are established from studies based on the supplementation with 18:1/18:1-PG to induce BMP accumulation in RAW macrophages. Interesting studies from the Gruenberg team have been previously performed in BHK cells and fibroblasts using exogenous BMP provided by liposomes. By this approach, the authors were able to compare different BMP molecular species and BMP with different stereo-configurations and demonstrated that both of these structural characteristics were relevant for BMP functions.7 In our study, the aim was to evaluate the consequences of increased BMP closest to natural BMP. According to previous data, PG was converted into BMP with the natural sn-1:sn-1′ stereoconfiguration in RAW macrophages,12 and we here show that molecular species composition of BMP was not significantly changed after supplementation with 18:1/18:1-PG. Furthermore, accumulated BMP was recovered in late endosomes. Importantly, we can reasonably conclude that BMP accumulation is responsible for the reported effects because key effects, such as cholesterol efflux and ABCG1 expression, were not reproduced under supplementation with 16:0/16:0-PG that accumulated in cells but was not converted to BMP.

Previous works including from our group have highlighted the involvement of BMP in the regulation of cholesterol transport, especially with respect to exit/retention in the endosome–lyosome compartment.5–8 Our present study examined the cholesterol distribution in downstream compartments, including PM and ER. We thereby showed that BMP accumulation compromised LDL-derived cholesterol transport to PM. Based on the repression of SREBP2 and HMG-coenzyme A reductase in BMP-enriched cells, we also concluded that BMP accumulation is associated with an increase in ER cholesterol content. In apparent contradiction, LDL uptake was not modified in BMP-enriched cells, whereas the expression of LDL receptor is known to be regulated by the SREBP2 pathway. Recent studies have reported that LDL receptor could be degraded by inducible degrader of the LDL receptor (an E3 ubiquitin ligase), whose expression is controlled by LXR.21 Because LXR’s are repressed in BMP-enriched cells, one possible mechanism is a less inducible degrader of the LDL receptor–dependent degradation of LDL receptor, which could compensate for its putative suppression by the SREBP2 pathway. Also surprising with respect to increased ER cholesterol is the fact that cholesterol esterification was not increased in BMP-enriched cells, suggesting that ER cholesterol accumulation would then specifically impact on SREBP2- and SREBP2-dependent genes. It could be that these genes respond to smaller variation of ER cholesterol content compared with acetyl-coenzyme A acetyltransferase activity. Alternatively,
it has been suggested that specific FC pools in ER could be involved for acetyl-coenzyme A acetyltransferase activation, and this pool may not be modified in BMP-enriched cells.

Mechanisms responsible for altered distribution of cholesterol to ER and PM are quite difficult to investigate because pathways of intracellular cholesterol trafficking at the molecular level are poorly understood. It is, however, assumed that the NP type C proteins NP type C1 and NP type C2 are involved in the normal egress of cholesterol out of late endosomes, and several studies have suggested functional interaction between BMP and NP type C proteins. Although it is generally accepted that ABCA1 functions in cholesterol efflux at the PM, it has been suggested that localization and trafficking of ABCA1 into intracellular compartments, including late endosomes, may play an important role in modulating ABCA1 transporter activity. The possibility that BMP accumulation in late endosomes affects ABCA1 trafficking through this organelle is thus to investigate. Previously, we reported that anti-BMP antibody altered LDL-derived cholesterol distribution in RAW macrophages. Further experiments have revealed cholesterol redistribution among PM-specific cholesterol pools (unpublished observations). Together with our present data, these observations support the hypothesis that endosomal BMP is important for normal targeting of cholesterol to functional pools.

One major functional consequence of BMP accumulation is the suppressive effects on both HDL and apoA1-stimulated cholesterol efflux, which correlated with the repression of ABCG1 and ABCA1 transporters. The contribution of oleoyl moieties in the downexpression of ABC transporters in BMP-enriched cells (ie, supplemented with 18:1/18:1-PG) should be considered regarding that unsaturated fatty acids, including oleic acid, have been shown to repress these transporters or to be inactive. When exerting suppressive effects, oleic acid was supplied as a nonesterified fatty acid at relatively high concentrations (from 0.1 to 1 mmol/L), which is several orders of magnitude higher than in our experimental conditions (esterified oleate, 30 μmol/L PG). LXR data are also informative in this respect because BMP accumulation attenuated the expression of LXR{s}, whereas oleic acid was reported to have no effect. All these observations weaken the possibility that oleic acid alone mediates the effects of 18:1/18:1-PG.

In contrast to BMP accumulation, antibody blocking BMP did not change the expression of cholesterol-regulating genes ABCA1, ABCG1, SREBP, and LXRs (not shown). Reduced cholesterol efflux by anti-BMP antibody may then result, as mentioned above, from cholesterol redistribution among PM-specific cholesterol pools not available to HDL.

ABCA1 and ABCG1 transporter genes are well-known targets of LXRs, whose regulation depends on several parameters, including protein synthesis and activation by ligands. Very little is known about the regulation of LXR expression. Our data indicate that both LXRα and LXRβ genes are induced after loading with native LDL. Because LDL-induced LXR expression was reduced by BMP-enriched cells, we propose that downregulation of LXRs contributes to the downregulation of ABCA1 and ABCG1 genes. This hypothesis is supported by the fact that overexpression of LXRα or to be inactive. All these observations weaken the possibility that oleic acid alone mediates the effects of 18:1/18:1-PG.
Further research in this area is certainly warranted and should provide better understanding of the role of endogenous oxysterols in the LXR/ABCA1-ABCG1 pathway in macrophages.

In conclusion, our findings shed some light on the still enigmatic late endosomal phospholipid BMP. We propose a functional link between endosomal BMP and the LXR/ABCA1/ABCG1 pathway underlying the effects of BMP on the regulation of cholesterol homeostasis in macrophages (Figure 7). Of pathophysiological relevance, we show that foam cell formation induced by oxLDL was exaggerated in BMP-enriched cells, supporting a role of BMP in atherogenesis.

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Disclosures

None.

References

This study deals with the cellular role of bis(monoacylglycero)phosphate (BMP), a specific and natural phospholipid from late endosomes. Previous reports have underlined the involvement of BMP in the regulation of cholesterol homeostasis in various cell types, including macrophages. We developed an experimental approach to specifically accumulate BMP in late endosomes, mimicking BMP accumulation in lysosomes. We, thus, propose a scheme illustrating how different cellular events related to cholesterol metabolism and trafficking that are modified after exposure to oxidized low-density lipoprotein. These findings will arouse new interests to investigate the potential of BMP as a possible target in atherosclerosis.
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Materials and methods

Materials
Tissue culture media were purchased from Eurobio (Les Ulis, France). [1,2,3H]cholesteryle oleate and [1,2,3H]cholesterol (50 Ci/mmol) were from Perkin Elmer Life Science (Paris, France). Alexa 488 and Alexa 546 were from Molecular Probes (Eugene, OR). Monoclonal antibody against BMP (anti-LBPA antibody, 6C4) was obtained as described. Antibody to the tetraspanin CD63/LAMP3 (clone M13) was from Santa Cruz Biotechnology (CA, USA).

Lipoprotein preparation
Human low-density lipoproteins (LDL) and high-density lipoproteins (HDL) were isolated from plasma by sequential ultracentrifugation. LDL were labeled with [3H]cholesteryl oleate or [3H]cholesterol (40 µCi per 1mg LDL protein) as previously described.

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 and passaged by trypsination. Experiments were started by incubation without (control) or with 30 µM 18:1/18:1-PG liposomes for 24h. Supplementation with 18:1/18:1-PG up to 72h did not affect cell viability (96.2 % ± 3.8 of controls according to colorimetric MTT assay). The incubation with 18:1/18:1-PG was maintained during subsequent incubations with lipoproteins. Incubations with lipoproteins were done in 5% lipoprotein deficient serum (LPDS)-containing medium. Other details of incubation conditions are given below and/or in figure legends.

LDL uptake and LDL-associated CE hydrolysis
Cells were incubated with 50 µg/ml of [3H]cholesteryl oleate-LDL. Aliquot of cell lysates (in 0.1% Triton) was counted by liquid scintillation and LDL uptake was calculated as nCi/mg cell protein. Total lipids were extracted from cell lysates by the method of Bligh and Dyer. [3H]FC (free cholesterol) and [3H]CE (cholesterol esters) were separated by TLC (hexane/diethyl ether/acetic acid, 80/20/1, v/v) and detected with a radioactivity analyzer (Raytest, France). LDL-associated CE hydrolysis was assayed as the percentage of total radioactivity recovered as [3H]FC.

Cholesterol Esterification
Cells were incubated with 50 µg/ml of [3H]cholesterol-LDL. The distribution of [3H]FC and [3H]CE was analyzed as detailed above. Cholesterol esterification was expressed as the percentage of total radioactivity recovered as [3H]CE. Cholesterol esterification was also evaluated by the conversion of [3H]FC to [3H]CE in cells labeled with [3H]FC (1 µCi/ml).

Free cholesterol (FC) and cholesterol esters (CE) content
Cells were incubated in basal culture conditions or in presence of 100µg/ml LDL (loading conditions) for 12h. Total lipids were extracted from cell lysates, and sterols (FC, CE) were separated by TLC (hexane, diethyl ether, methanol, acetic acid, 50/50/5/1, v/v). FC was measured by GC-MS using stigmasterol as an internal standard. CE was measured by GC of fatty acid methyl esters using cholesteryl heptadecanoate as an internal standard.
Cholesterol efflux to HDL, apoA1 and mβCD
Cells were pre-incubated with 50 µg/ml of [3H]cholesteryl oleate-LDL or 1 µCi/ml [3H]cholesterol for 12 h. Cholesterol efflux was then stimulated by incubation with 10 mM MβCD, 100 µg/ml HDL or 10 µg/ml apoA1 + 0.5 mM 8 BrcAMP in 5% LPDS-containing medium. The radioactivity in cells and in media was determined by liquid scintillation counting. More than 95% of the radioactivity in media was recovered as FC. Cholesterol efflux was expressed as the percentage of radioactivity released into the medium relative to total radioactivity in cells plus media. The values correspond to the net efflux after subtraction of spontaneous efflux measured in 5% LPDS-medium.

Quantification of BMP and PG species by LC-MS
An Agilent 1100 series LC (Agilent Technologies, Santa Clara, CA) coupled to a 4000 QTRAP hybrid triple quadrupole mass spectrometer (AB SCIEX, Foster City, CA) was used to quantify the individual BMP and PG species. Extracted lipids (2 µl) were injected onto a reversed phase C18 column (CAPCELL PAK C18 MG III, 2.0 × 50 mm, Shiseido CO., LTD., Tokyo, Japan) and were eluted with an isocratic flow (100 µl/min) of methanol : acetonitrile (90:10) containing 5 mmol/L ammonium formate and 0.1% formic acid. MS analysis was run in the positive ion mode. Multiple-reaction monitoring (MRM) mode was used to measure the PG and BMP species, containing different acyl chains. BMP and PG contents in cells were calculated by relating the peak areas of each species to the peak area of the corresponding internal standard (C14:0/C14:0-BMP or C14:0/C14:0-PG) and the standard curve of each internal standard (0-20 pmol). Data acquisition and analysis were performed using Analyst Software version 1.4.1 (AB SCIEX).

Foam cell formation assay
Macrophages were plated on coverslips and incubated with 50 µg/mL oxidized LDL (10 µM CuSO$_4$, 37°C, 5h) for 24h, rinsed three times with PBS, and then fixed at room temperature for 20 min with 4% para-formaldehyde. Macrophages were then rinsed twice with PBS and stained 30 min at room temperature with 0.1µg/ml Nile Red. Cells were rinsed twice with PBS, mounted and observed under Olympus IX81 microscope. Fluorescence intensity was determined using cell^F software (Soft Imaging System).

BMP staining
Cells were fixed and stained with Alexa 488– or Alexa 546-conjugated primary antibodies (anti-BMP (6C4) and anti-CD63 antibodies) as described. The specimens were mounted with Mowiol and examined under Zeiss LSM 510 confocal microscope equipped with the C-Apochromat 63XW Korr (1.2 n.a.) objective.

Quantification of mRNAs by real-time RT-PCR
Cells were incubated in basal culture conditions or in presence of 100µg/ml LDL (loading conditions) for 12h. Total RNA was isolated using the Trizol reagent according to manufacturer's instructions. First-strand cDNAs were synthesized from 500 ng of total RNAs in the presence of 10 U of Superscript II and a mixture of random hexamers and oligo(dT) primers. Real-time PCR assays were performed with Rotor-GeneTM 6000 (Corbett Research, Mortlake, Australia). A list of the primers and real-time PCR assay conditions are available upon request (lefai@univ-lyon1.fr). The results were normalized using HPRT mRNA concentration, measured as a reference gene in each sample.

Statistical analysis
Data are presented as mean values ± SD. All statistical analyses were performed using the JMP 10.0.2 software (SAS Institute, Inc.). Multiple comparisons were performed by one-way ANOVA. Means comparisons of all pairs were performed by the Tukey-Kramer Honestly Significant Difference method based on ANOVA with $\alpha = 0.05$; significantly different pairs are indicated in the figures by different capital letters. Comparisons of experimental means from
two groups were performed by unpaired Student's t-test; significant differences at p≤0.05 and p≤0.001 are indicated in the figures by * and ***, respectively.