The Sphingomyelin-Ceramide Signaling Pathway Is Involved in Oxidized Low Density Lipoprotein-induced Cell Proliferation*

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Nathalie Augeé, Nathalie Andrieut, Anne Nègre-Salvayre, Jean-Claude Thiers, Thierry Levade, and Robert Salvayre§

From the Laboratory of Biochemistry, Metabolic Disease Section (INSERM C) F-9206, Rangueil Faculty of Medicine, Paul Sabatier University, 31054 Toulouse Cedex, France

Development of atherosclerosis is believed to involve proliferation of smooth muscle cells (SMC). Our laboratory previously demonstrated that the growth of bovine aortic SMC was stimulated by mildly oxidized low density lipoproteins (oxLDL) and that the mitogenic effect of oxLDL was greater than that induced by native LDL (Augeé, N., Pieraggi, M. T., Thiers, J. C., Nègre-Salvayre, A., and Salvayre R. (1995) Biochim. J. 309, 1015–1020). Since the lipid mediator ceramide has been described to be growth positive, the present work aimed at studying the potential involvement of the so-called sphingomyelinceramide pathway in the signal transduction cascade induced by oxLDL. Incubation of SMC with UV-oxidized LDL induced sphingomyelin hydrolysis (32%), which peaked at 60 min and was accompanied by a concomitant increase of intracellular ceramide level. The effect of oxidized LDL on sphingomyelin turnover exhibited the same LDL dose dependence as their mitogenic effect. Exogenous bacterial sphingomyelinase induced sphingomyelin hydrolysis and ceramide generation and also stimulated cell growth, in contrast to exogenous phospholipases A2, C, or D. This mitogenic effect was reproduced by incubating the cells with the cell-permeant ceramides, N-acetyl- and N-hexanoylsphingosines. Altogether, these data strongly suggest for the first time that activation of the sphingomyelin-ceramide pathway may play a pivotal role in the oxLDL-induced SMC proliferation and atherogenesis.

Recently, sphingolipids have emerged as key signaling molecules involved in the regulation of cell growth and differentiation (1–5). In particular, the so-called sphingomyelin (ceramide phosphocholine; SPM)1 cycle appears as a prototypic sphingolipid signaling pathway implicated in the positive or negative regulation of cell growth (6). Activation of this pathway leads to SPM hydrolysis and subsequent generation of ceramide, the backbone of all sphingolipids, which serves as an intracellular second messenger (7, 8). To date, several agents have been described to stimulate the SPM-ceramide pathway: vitamin D3 (9), cytokines such as TNF-α (10–14), interleukin-1β (15, 16), interferon-γ (10), nerve growth factor (17), as well as anti-CDC8 (18, 19) and anti-Fas (20, 21) antibodies. Thus, various cellular responses including cell proliferation (18, 19), differentiation (9, 10), or apoptosis (8, 20–22) appear to be transduced by SPM hydrolysis through ceramide generation. This conclusion has further been supported by the observation that cell-permeant ceramides or ceramide produced by treatment of cells with exogenous sphingomyelinase can mimic the effects of various inducers of the SPM cycle (9, 10, 15–17, 19, 21–23).

Atherosclerosis and its complications, namely heart attack, stroke, and peripheral vascular disease, are the most prevalent causes of human death in Western countries. During atherogenesis, focal lesions spread out progressively and lead to the formation of fibroatheroma plaques in which accumulation of macrophagic foam cells and proliferation of smooth muscle cells (SMC) play a crucial role (24, 25). Oxidatively modified low density lipoproteins (LDL) are present in atheromatous areas (26, 27) and exhibit a variety of biological properties potentially involved in atherogenesis (reviewed in Ref. 28). LDL oxidation is a progressive process leading at first to the formation of mildly oxidized LDL (oxLDL), which are defined by a low content of lipid peroxidation derivatives and slight apolipoprotein B modifications, and later to extensively oxidized LDL which contain high levels of lipid peroxidation products and severe apolipoprotein B alterations (29).

In atherosclerotic areas, SMC proliferation may be mediated through a complex network of growth factors (30). We recently demonstrated that UV-treated LDL, that is mildly oxidized LDL, are mitogenic to cultured bovine aortic SMC (31), an effect also observed with copper-oxidized LDL (32). We report here that the mitogenic effect of oxLDL is transduced through the SPM-ceramide pathway in cultured SMC as shown by the induction of SPM hydrolysis and as mimicked by exogenous sphingomyelinase or short-chain synthetic C2-n-C6-ceramides.

EXPERIMENTAL PROCEDURES

Chemicals—RPMI 1640 containing Glutamax®, penicillin, streptomycin, trypsin-EDTA, and fetal calf serum (FCS) were from Gibco BRL Life Technologies (Cergy-Pontoise, France). Anti-BrdUrd monoclonal antibody, 5-fluorodeoxyuridine, and Bacillus cereus phospholipase C were from Boehringer Mannheim (Meylan, France). Anti-mouse biotinylated sheep Ig, [3H]thyminde (5 Ci/mmol), and [9,10-3H]palmitic acid (53 Ci/mmol) were obtained from Amersham (Les Ulis, France) and [methyl-3H]choline chloride (86 Ci/mmol) from DuPont NEN (Courta-
Oxidized LDL Activate the Sphingomyelin Pathway

19252

boeuf, France). Fluorescein isothiocyanate-streptavidin was from Dako (Trappes, France). N-Acetyl- and N-hexanoylsphingosines (C<sub>2</sub> and C<sub>5</sub>-ceramides), B. cereus sphingomyelinase, Naja naja phospholipase A2, calf lung phospholipase D, and BHT were obtained from Sigma. All solvents and other reagents obtained from Merck (Darmstadt, Germany) were of analytical grade.

Cell Culture—Bovine aortic SMC (AG-08133A cell line, from the NIA Aging Cell Repository, Camden, NJ) and human endothelial cells (CRL-1998 cell line, from the ATCC) were routinely grown in RPMI 1640 Glutamax® medium supplemented with 10% FCS, penicillin (100 units/ml), and streptomycin (100 μg/ml), at 37 °C in humidified CO₂ (5%) atmosphere.

Lipoprotein Isolation and Oxidation by UV-C or Human Endothelial Cells—LDL (d 1.019–1.063) and lipoprotein-depleted serum were isolated from human pooled fresh sera by sequential ultracentrifugation (33), dialyzed, sterilized by filtration (0.2-μm Millipore membrane), and stored at −20 °C under nitrogen until use (up to 2 weeks), as described previously (31). The electrophoretic mobility was monitored by electrophoresis on Hydragel®. Cholesterol and apolipoprotein B concentrations stored at 4°C under nitrogen until use (up to 2 weeks), as described

OxLDL were obtained by UV-oxidation. LDL in 0.15 n NaCl containing 0.3 mM EDTA were irradiated by UV-C (0.5 milliwatts/cm² for 1 h) under the previously used conditions (31). Alternatively, cells were obtained by cell-mediated oxidation. Endothelial cells (100,000 cells/ml) were seeded in RPMI containing 10% FCS, and further incubated with native LDL (50, 100, or 200 μg of apoB/ml) for 16 h in medium containing 1% FCS. Then, the culture medium was collected, filtered, and added to SMC. The procedures were set up to obtain the formation of mildly oxidized LDL, i.e. LDL characterized by a moderate amount of lipoperoxidation products (34) (2.5 ± 0.3 and 3.6 ± 0.4 nM of TBARS/mg of apolipoprotein B, for cell- and UV-oxidized oxLDL, respectively), by a minor loss of trinitrobenzene sulfonic acid-reactive amino groups (35), and by a cellular uptake at a rate similar to that of nonoxidized LDL (31).

Cell Proliferation and Cytotoxicity Measurements—After trypsinization, bovine aortic SMC were seeded at a density of 50,000 cells/well in 6-well Nunc culture plates, in medium containing 10% FCS for 24 h. Then the medium was removed, and cells were washed once with RPMI and grown for 24 h in RPMI medium containing 1% FCS (without any loss of cell viability). Cells were then incubated with the indicated concentration of mitogenic agent (native LDL, oxLDL, sphingomyelinate, short-chain ceramides, or phophatidylserines) for 24, 48, or 72 h and labeled for the last 12 h of the experiment with [3H]thymidine (0.5 μCi/ml). Cells were washed 3 times with PBS, harvested, precipitated by 3% perchloric acid, and centrifuged at 1000 × g of apoB/ml) or bacterial sphingomyelinase (100 milli-

RESULTS AND DISCUSSION

Exposure of LDL to cultured endothelial cells or to UV-C irradiation results in mild lipoprotein oxidation (29, 34, 41, 42). In contrast to extensively oxidized LDL, mildly oxidized LDL used here are taken up by cultured cells (42), including SMC (data not shown), through the apoB/E receptor pathway. As used here are taken up by cultured cells (42), including SMC (data not shown), through the apoB/E receptor pathway. As used here are taken up by cultured cells (42), including SMC (data not shown), through the apoB/E receptor pathway.

FIG. 1. Mitogenic effect of cell- or UV-mediated mildly oxLDL on cultured vascular SMC. Bovine aortic SMC were incubated with endothelial cell-oxidized (A) or UV-oxidized (B) LDL (50, 100, or 200 μg of apoB/ml, filled triangles, circles, and squares, respectively) or with native LDL (100 μg/ml, empty circles). [3H]Thymidine incorporation was evaluated during the last 12 h of incubation. At each time, the values are expressed as percent of the radioactivity measured in cells grown in medium containing 1% FCS (means ± S.E. of at least 4 separate experiments). B, inset, [3H]thymidine incorporation in SMC grown for 60 h under the above described conditions, in the presence (+) or absence (−) of 10 μM BHT and 100 μg/ml apoB of native (nat) or UV-oxidized (ox) LDL. C and D, microphotographs of cells grown for 48 h in RPMI containing 1% FCS in the absence (C) or presence (D) of 100 μg of apoB/ml of oxLDL and labeled for the last 16 h with BrdUrd in the presence of 10 μM 5-fluorodeoxyuridine. Cells that have incorporated BrdUrd were immunologically detected as described under “Experimental Procedures.”

[3H]Choline-labeled SPM was quantified as described previously (40). The [9,10-3H]palmitic acid-labeled lipids were separated by TLC on Silica Gel G60 analytical plates, using 4 successive runs, 1 run with chloroform/methanol/water (100:42:6, by volume) up to 14 cm, and 3 successive runs with hexane/diethylether/formic acid (55:45:1, by volume) up to 19 cm. Radioactive lipids were localized using a Berthold radiodichromatoscop, SPM and ceramide spots were scraped off and counted by liquid scintillation.

Metabolic Labeling of Cellular Choline Phospholipids—For SPM determination, SMC were metabolically labeled to equilibrate with [methyl-3H]choline (0.5 μCi/ml) in RPMI medium containing 1% FCS. After 48 h of incubation, cells were washed once with PBS and chased for 2 h in fresh RPMI containing 1% FCS. Then, the medium was replaced by fresh 1% FCS-containing medium with or without UV-oxLDL (100 μg of apoB/ml) or bacterial sphingomyelinase (100 milli-

Lipid Extraction and Analyses—Cell pellets were suspended in 0.6 mL of distilled water and homogenized by sonication (2 × 10 s, using a MGE probe sonicator). An aliquot was saved for protein determination (38). Lipids from 0.5 ml of the cell lysate were extracted by 2.5 ml of chloroform/methanol (39). The lipid phase was evaporated under nitro-
ceramide signaling pathway, SMC phospholipids were metaboli-
cally labeled to equilibrium either with [methyl-\(^{3}H\)]choline or [9,10-\(^{3}H\)]palmitic acid. As shown in Fig. 2, A and B, effective
mitogenic doses of UV-oxidized LDL (100 \(\mu g\) of apoB/ml) in-
duced a time-dependent degradation of SPM. Maximal hydro-
dysis of [\(^{3}H\)]choline-labeled SPM (32 ± 9\%) was observed within
50–70 min after addition of oxLDL. Then, SPM levels returned
progressively toward baseline (Fig. 2A). Similar results of SPM
hydrolysis were obtained when using cells labeled with
[\(^{3}H\)]palmitic acid (Fig. 2B). Activation of SPM hydrolysis was
accompanied by a concomitant increase of cellular ceramide
levels (Fig. 2B), supporting the conclusion that oxLDL stimu-
late a sphingomyelinase activity. Both SPM and ceramide lev-
els recovered to baseline within 2 h, similarly to responses seen
with various activators of the SPM cycle, such as vitamin D3
(9), TNF-\(\alpha\) (10, 43), interferon-\(\gamma\) (10), nerve growth factor (17),
or anti-Fas (20).

To examine further the connection between oxLDL-induced
cell proliferation and activation of the SPM pathway, the dose
dependence of the effects of oxLDL on SPM degradation was
investigated. While 100 \(\mu g\) of apoB/ml of oxLDL consistently
promoted about 30\% SPM hydrolysis, a concentration of 50 \(\mu g/ml\)
causd only a 15\% reduction (which was also shorter in dura-
tion), and, quite unexpectedly, no significant SPM degradation
was detected after incubation (for 15 to 120 min) with 200
\(\mu g/ml\) oxLDL (Fig. 2C). Furthermore, as observed with thymi-
dine incorporation assays (see Fig. 1B, inset), addition of BHT
did not significantly inhibit the oxLDL-induced activation of
SPM breakdown (Fig. 2C). Thus, the effects of oxLDL on SPM
turnover closely paralleled their effects on mitogenesis (see Fig.
1, A and B).

Since native LDL were also found to stimulate SMC growth
(Fig. 1 and Ref. 31), the effects on increasing concentrations of
native LDL on SPM turnover were examined. Interestingly,
only elevated concentrations of native LDL promoted SPM
hydrolysis (Fig. 2D). It is noteworthy that the extent of SPM
degradation induced by 100 \(\mu g\) of apoB/ml of native LDL ap-
proximated that induced by 50 \(\mu g\) of apoB/ml of oxLDL, thereby
 correlating with the effects on cell growth (see Fig. 1, A and B).
Finally, the effects of native LDL on SPM degradation were
severely inhibited by BHT, again in accordance with the inhi-
bition by BHT of the mitogenic effect of native LDL. Based on
these observations, it is tempting to speculate that native LDL
may stimulate SPM hydrolysis and subsequent cell prolifera-
tion because they get oxidized when added to the cell culture.
FIG. 4. Mitogenic effect of synthetic short-chain ceramides, N-acethylspingosine (A) or N-hexanoylsphingosine (B). Cell-permeant ceramides (squares, 1 μM; circles, 5 μM; triangles, 10 μM) were introduced into the culture medium at time 0, and [3H]thymidine incorporation was determined at the indicated times, exactly under the conditions described in Fig. 1. Inset, cytotoxicity evaluated by LDH activity (IU/liter) released into the culture medium (means ± S.E. of 3 separate experiments).

As the SPM-ceramide pathway has been reported to be involved in cell growth regulation (5, 8, 44), the above data suggested that SPM hydrolysis might be involved in the intracellular signaling triggered by oxLDL in vascular SMC. To further define the potential role of SPM degradation and subsequent ceramide generation, we investigated the ability of membrane SPM hydrolysates and of cell-permeant ceramide analogs to mimic the mitogenic effect of oxLDL. Treatment of SMC by bacterial sphingomyelinase, under nontoxic conditions, induced an intense incorporation of [3H]thymidine (205 ± 20% of the control after a 48-h incubation; Fig. 3A), that was associated with an extensive hydrolysis of radiolabeled SPM (72 ± 9% degradation of cellular radiolabeled SPM within 15 min) and a concomitant production of ceramide (Fig. 3B). In contrast, under nontoxic conditions, addition of exogenous phospholipases A2, C, and D led to the hydrolysis of 35–40% of cellular radiolabeled phospholipids, in particular phosphatidylcholine, but induced no significant [3H]thymidine incorporation (Fig. 3C and D). This strongly suggests that, under the used conditions, hydrolysis of the SPM present in the plasma membrane triggers a mitogenic signal in vascular SMC, whereas, under similar conditions, phosphatidylcholine hydrolysis by various phospholipases is unable to produce any mitogenic effect.

Among the various metabolites derived from SPM hydrolysis, ceramide has been shown to be mitogenic per se in Swiss 3T3 fibroblasts (45, 46). This led us to test whether addition of cell-permeable, short-chain ceramide analogs, such as C2-ceramide (N-acetylspingosine) and C6-ceramide (N-hexanoylsphingosine) were mitogenic to the vascular SMC used here. Fig. 4 shows that treatment of SMC with 1 μM C2-Ceramide or 5 μM C6-Ceramide (i.e. under nontoxic conditions), resulted in a significant [3H]thymidine incorporation, thereby mimicking the effect of endogenously produced ceramide.

Taken together, the present findings demonstrate that mildly oxidized LDL activate the SPM-ceramide pathway in SMC with the same dose dependence as their effect on cell growth. In addition, the mitogenic effect of oxLDL can be recapitulated by production or addition of ceramides. Such a proliferative effect has previously been documented on Swiss 3T3 cells directly treated with synthetic ceramides or exogenous sphingomyelinase (45, 46). On the same cells, ceramide has also been shown to potentiate the proliferation induced by platelet-derived growth factor (47). While in those studies the effect of exogenously added ceramides or of ceramides produced by bacterial sphingomyelinase was investigated, the question still remained whether the mitogenic response could be elicited by the natural ceramide generated in situ through the action of an extracellular agent. The present study strongly supports this idea, corroborating previous reports which indicated that triggering of the SPM-ceramide pathway is involved in cell proliferation. For instance, the SPM-ceramide pathway has been shown to be activated in the TNFα or interleukin-1β-induced proliferation of human skin fibroblasts (40), as well as in the T cell proliferation triggered by anti-CD28 (19).

To our knowledge, the present study is the first report on the initiation of the SPM-ceramide turnover in SMC and on the overlap of SPM and oxLDL signaling pathways. Since ceramide can induce the phosphorylation and activation of mitogen-activated protein kinases (MAPK) (47, 48), which are classically associated with cell proliferation signaling, it is tempting to speculate that the mitogenic effect of oxLDL is mediated by the stimulation of ceramide-activated MAPK. Indeed, preliminary results from our laboratory show that oxLDL stimulate MAPK (2). The proliferative effect of ceramide might also be due to another metabolite of SPM hydrolysis (5). However, it has been demonstrated that exogenous ceramides most probably exert their effect without any metabolic conversion (13, 45), suggesting that ceramide actually represents the effective mediator.

Oxidized LDL exhibit a variety of biological properties, being able to modulate gene expression of growth factors, adhesion molecules, and tissue factor, altering the motility of monocytes/macrophages and the vasomotor properties of arteries, being cytotoxic (reviewed in Ref. 28) and mitogenic to vascular SMC (31, 32). The signaling pathways involved in the cellular effects triggered by oxidized LDL are only poorly known, and no coherent picture has emerged to account for all of the data. Although the participation of other signaling pathways cannot be excluded (49), our data provide strong evidence for the involvement of the SPM-ceramide signal transduction pathway in the mitogenic effect of mildly oxidized LDL on vascular SMC.

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Oxidized LDL Activate the Sphingomyelin Pathway

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