Role of Sphingosine 1-Phosphate in the Mitogenesis Induced by Oxidized Low Density Lipoprotein in Smooth Muscle Cells via Activation of Sphingomyelinase, Ceramidase, and Sphingosine Kinase*

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Oxidized LDL (oxLDL) have been implicated in diverse biological events leading to the development of atherosclerotic lesions. We previously demonstrated that the proliferation of cultured vascular smooth muscle cells (SMC) induced by oxLDL is preceded by an increase in neutral sphingomyelinase activity, sphingomyelin turnover to ceramide, and stimulation of mitogen-activated protein kinases (Augé, N., Escargueil-Blanc, I., Lajoie-Mazenc, I., Suc, I., Andrieu-Abadie, N., Pieraggi, M. T., Chatelut, M., Thiers, J. C., Jaffrézou, J. P., Laurent, G., Levade, T., Nègre-Salvayre, A., and Salvayre, R. (1998) J. Biol. Chem. 273, 12893–12900). Since ceramide can be converted to other bioactive metabolites, such as the well established mitogen sphingosine 1-phosphate (SIP), we investigated whether additional ceramide metabolites are involved in the oxLDL-induced SMC proliferation. We report here that incubation of SMC with oxLDL increased the activities of both acidic and alkaline ceramidases as well as sphingosine kinase, and elevated cellular sphingosine and SIP. Furthermore, the mitogenic effect of oxLDL was inhibited by D-erythro-2-(N-myristoylamino)-1-phenyl-1-propanol and N,N-dimethylsphingosine which are inhibitors of ceramide and sphingosine kinase, respectively. These findings suggest that SIP is a key mediator of the mitogenic effect of oxLDL. In agreement with this conclusion, exogenous addition of sphingosine stimulated the proliferation of cultured SMC, and this effect was abrogated by dimethylsphingosine but not by fumonisin B1, an inhibitor of the acylation of sphingosine to ceramide. Exogenous SIP also promoted SMC proliferation. Altogether, these results strongly suggest that the mitogenic effect of oxLDL in SMC involves the combined activation of sphingomyelinase(s), ceramidase(s), and sphingosine kinase, resulting in the turnover of sphingomyelin to a number of sphingolipid metabolites, of which at least SIP is critical for mitogenesis.

Oxidized low density lipoproteins (LDL)§ are believed to play a critical role in atherosclerosis (1, 2). Oxidized LDL exert diverse biological effects on the different cell types, including smooth muscle cells (SMC), which are present in the atherosclerotic lesions (3). The responses of cultured SMC depend on the degree of oxidation and on the extracellular concentration of oxidized LDL, and include production of growth factors (3, 4), chemotaxis (5), and cell proliferation (6–8) as well as induction of cytotoxicity (7, 9), all of which are considered to be key events in the development of atherosclerosis (3, 10).

Oxidized LDL (oxLDL) induce the proliferation of cultured SMC (11, 12), which has recently been shown to be accompanied by the activation of a neutral, magnesium-independent sphingomyelinase that induces sphingomyelin (SM) hydrolysis and ceramide generation (13). Ceramide (N-acethylsphingosine) belongs to the family of sphingolipids (14), and has recently emerged as an important signaling molecule that is involved in the regulation of cell growth, differentiation, or most notably apoptotic cell death (15–20).

In previous studies, the induction of SM turnover was shown to be mitogenic for SMC through the activation of the mitogen-activated protein kinases p42 and p44 (13); however, the exact nature of the SM metabolite(s) that were responsible for this response was not elucidated. The ceramide formed from SM turnover might be hydrolyzed by ceramidases to liberate the sphingoid base backbone (sphingosine), which can be re-acylated to ceramide or phosphorylated to sphingosine 1-phosphate (SIP) by sphingosine kinase (14, 20) (see Fig. 1). While ceramide and sphingosine have most frequently been described as potent inhibitors of cell growth or as cytotoxic agents (20), SIP has been found to be growth stimulatory for fibroblasts (21) and SMC (22, 23). The mitogenic effects of SIP have been attributed to calcium mobilization, activation of phospholipase D, and generation of the second messenger phosphatidic acid, engagement of the MAPK pathway, and activation of the transcription factor AP-1 (24); furthermore, SIP can inhibit apoptosis (25).

Sphingosine kinase is activated by PDGF, phorbol esters,
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Metabolic Labeling of Cellular Lipids—For SM determination, SMC were metabolically labeled to apparent equilibrium with [methyl-3H]choline (0.5 μCi/ml) in MEM medium containing 1% FCS for 48 h. The cells were washed once with PBS and incubated for 2 h in fresh medium containing 1% FCS (to allow them to adjust to the medium change) and then incubated with 0.1 mCi/ml Ox-LDL at 37 °C for 24 h. At the indicated times, cells were washed with ice-cold PBS, harvested, and sedimented by centrifugation (300 × g for 5 min). Cell pellets were immediately frozen at −20 °C. Alternatively, SM and ceramide contents were determined by [9,10-3H]palmitic acid labeling using the previously reported experimental conditions (12). For the estimation of cellular ceramide concentrations, subconfluent cells were incubated for 24 h with phosphatase-free DMEM medium containing [32P]phosphoric acid (40 μCi/ml). After 1 h of chase in phosphate-free medium, cells were treated as indicated above.

Lipid Extraction and Analyses—Cell pellets were suspended in 0.6 ml of distilled water and homogenized by sonication (2 × 10 s, using a MSE probe sonicator). An aliquot was saved for protein determination (29). Lipids from 0.5 ml of the cell lysate were extracted by 2.5 ml of chloroform/methanol (30). The lipid phase was evaporated under nitrogen. [3H]Choline-labeled SM was quantified as described (31). The [9,10-3H]palmitic acid-labeled lipids were separated by TLC on Silica Gel G-60 analytical plates, using 3 successive runs, one with chloroform/methanol/water (100:42.6, by volume) up to 14 cm, a second run with chloroform/methanol (90:10, by volume) up to 18 cm, and a last run with petroleum ether/diethyl ether (80:20, by volume) up to 19.5 cm. Radioactive lipids were localized using a Berthold radiocromatoscan and after exposure to iodine vapors. For S1P quantification, lipids were extracted exactly as described for the experiments with radioactive choline, that is after mild alkaline hydrolysis. [3P]-Labeled phospholipids were separated by TLC using chloroform/methanol/water (60:35:8, by volume) as developing solvent. Radioactive S1P was localized by autoradiography (Kodak BioMax film) and by iodine vapors. All radioactive spots were scraped off and counted by liquid scintillation. Sphingosine mass was determined as described previously (27).

In Vitro Ceramidase Assay—After stimulation with or without ox-LDL, SMC were washed with ice-cold PBS, scraped from two 60-mm dishes, pooled and sedimented, and ceramidase assays were performed as described previously (27). After incubation for 1 h at 37 °C in 10 ml Tris, pH 7.2 (for neutral ceramidase assay), 0.5 ml acetate buffer, pH 5 (for acidic ceramidase), or 10 ml Hepes-Tris, pH 8 (for alkaline ceramidase), the reaction was stopped and the products were analyzed by HPLC as described below. Alternatively, the enzymatic reaction (after 2 h incubation at 37 °C) was terminated by adding chloroform/methanol, and the lipids were extracted, separated on aluminum TLC plates using chloroform/methanol (95:5, by volume), and then petroleum ether/diethyl ether (80:20, by volume). The fluorescent bands were cut, eluted in chloroform/methanol (2.1, by volume), and quantified using a Jobin-Yvon 3D spectrofluorometer (at 466 and 536 nm, for the excitation and emission wavelengths, respectively).

In Vivo Ceramidase Assay—SMC were incubated with C3-NBD-ceramide (6 μM) for 6 h before addition of ox-LDL. After varying incubation times, the cells were washed with ice-cold PBS and 1.5 ml of methanol/water/phosphoric acid (850:150:1.5, by volume) was added, and the suspension was incubated for 1 h at 37 °C with gentle shaking. The insoluble material was removed by centrifugation and the supernatant was analyzed by HPLC as described in the following section.

HPLC Analysis of NBD Lipids—NBD lipids were injected into a reverse-phase column (Nova-Pak, C18, Bio-Rad) and eluted with methanol/water/phosphoric acid (850:150:1.5, by volume) as described (27). Under these conditions, NBD fatty acids appear as a single peak at 1.5 min and C3-NBD-ceramide at 10.3 min. The NBD fluorescence was monitored with excitation at 455 nm and emission at 530 nm.

Sphingosine 1-Kinase Assay—Sphingosine kinase activity was determined as described previously (32) with minor modifications. After incubation with or without ox-LDL, cells were washed with ice-cold PBS, harvested, and sedimented by centrifugation. Cell pellets were immediately solubilized in the kinase buffer (see Ref. 32) and frozen in liquid nitrogen. The kinase assay was performed by mixing protein samples (50 μg with 10 μl of 1 ml sphingosine (dissolved in 5% Triton X-100) and [32P]ATP (1 μCi, 20 μCi/mol) with 5 ml of the kinase buffer for 5 min). After incubation for 30 min at 37 °C, the reaction was stopped by addition of 20 μl of 1 N HCl, and the [32P]ATP-labeled S1P was extracted, isolated by TLC, and quantified as described (32). Alternatively, a nonradioactive method was used. Cells were extracted in the kinase buffer as described above and the kinase reaction was performed by incubating 50 μg of protein sample with 50 μM octyl-β-D-glucopyranoside, 20 μM sphingosine...
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TABLE I

| Inhibitor | [3H]Thymidine incorporation (% of control) |
|-----------|------------------------------------------|
|           | Time | OxLDL | SMase | C2- ceramide | Sphingosine | SIP     |
|-----------|------|-------|-------|-------------|-------------|---------|
| None      | 24   | 134 ± 12 | 211 ± 13 | 129 ± 6 | 145 ± 16 | 145 ± 7 |
| D-MAPP (20) | 24 | 152 ± 19 | 143 ± 12 | 134 ± 12 | 126 ± 6 | 151 ± 16 |
| DMS (1)   | 48   | 88 ± 14 | 58 ± 6 | 81 ± 20 | 137 ± 12 | 138 ± 10 |
|           | 48   | 88 ± 16 | 60 ± 17 | 86 ± 11 | 120 ± 7 | 138 ± 10 |

*S, p < 0.001; according to Student t test.

RESULTS

Previous studies have shown that oxLDL induce SMC proliferation as reflected in small, but significant, increases in [3H]thymidine incorporation (6, 8, 11, 13), increases in cell number (6, 11), and 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assays,3 and this growth stimulation by oxLDL is thought to be significant as a contributor to atherogenesis (3, 10). We have shown in bovine aortic SMC that oxLDL induced SM hydrolysis to ceramide, and that this was strongly associated with the mitogenesis induced by oxLDL (12, 13). Therefore, this study investigated the role of sphingolipid metabolites in greater detail using the related model, rabbit femoral SMC, which also display oxLDL-induced mitogenesis (see Table I).

OxLDL Activate SM Turnover in Rabbit SMC—As shown in Fig. 2, oxLDL at a concentration that is mitogenic for SMC (10 μg of apoB/ml; see Table I and Fig. 3) induced a time-dependent decrease in SM. Maximal hydrolysis of [3H]choline-labeled SM (38 ± 11.5%) was observed within 40–70 min after oxLDL addition, then [3H]SM returned progressively toward the original level (Fig. 2A). Similar results were obtained when using cells labeled with [3H]palmitic acid (Fig. 2B). OxLDL also transiently increased the amounts of labeled ceramide (Fig. 2B), but the amount of radiolabel in ceramide was about 60% compared with the loss in SM, supporting the conclusions that not only oxLDL stimulate a sphingomyelinase activity but also that some of the ceramide is being hydrolyzed to other products.

Both [3H]palmitate-labeled SM and ceramide levels recovered to baseline within 3 h. These results are similar to the responses seen previously with UV-oxidized LDL (12), and provide further evidence that oxidized LDL stimulates transient ceramide release from SM via sphingomyelinase activation.

OxLDL-induced SMC Proliferation is Abrogated by Inhibitors of Ceramidase and Sphingosine Kinase—The increase in ceramide might mean that this metabolite is responsible for the mitogenic response of SMC to oxLDL, however, it is also possible that subsequent metabolite(s) are involved. To explore this possibility, the cells were treated with oxLDL in the presence of D-MAPP, a ceramidase inhibitor (33) or DMS, an inhibitor of sphingosine kinase (34, 35). The proliferative effect of oxLDL was completely abolished by co-incubation with 20 μM

Fig. 2. Activation of SM turnover in cultured SMC by oxLDL.

A, SMC were metabolically labeled with [methyl-3H]choline and incubated for 12 h with the inhibitor. [3H]Thymidine incorporation was evaluated after 24 h with (solid bars) or without (open bars) oxLDL (10 μg of apoB/ml) at the indicated times, incubations were stopped and SM levels determined as described under “Experimental Procedures.” Results are expressed as % of the SM amounts measured in untreated cells (about 400,000 dpm/mg of cell protein) at the corresponding time. B, cells metabolically labeled for 48 h with [9,10-3H]palmitic acid (1 μCi/ml) were treated as above. At the indicated times, lipids were extracted and separated by TLC; SM and ceramide (Cer) spots were scraped off and counted by liquid scintillation. Radioactive lipids (mean ± S.E. of three independent experiments) are expressed as % of the amounts measured at time 0 (about 200,000 and 80,000 dpm/mg of cell protein for SM and ceramide, respectively).

Fig. 3. OxLDL-induced SMC proliferation is inhibited by both ceramidase and sphingosine kinase inhibitors. Rabbit SMC were incubated for 24 h with (solid bars) or without (empty bars) oxLDL (10 μg of apoB/ml) in the presence or absence of the indicated concentration of D-MAPP, DMS, or FB1. In the case of D-MAPP, cells were pretreated for 12 h with the inhibitor. [3H]Thymidine incorporation was evaluated during the last 12 h of incubation. The values are expressed as % of the radioactivity measured in cells grown in medium containing 1% FCS (mean ± S.E., n = three separate experiments, each being performed in triplicate).
D-MAPP or 1–2 μM DMS (Fig. 3). In contrast, the mitogenic effect was not blocked by an inhibitor of ceramide synthase, i.e. fumonisin B1 (except at the concentration of 50 μM which also affected SMC proliferation in the absence of oxLDL). These data suggested the involvement of both ceramidase and sphingosine kinase in the mitogenic effect induced by oxLDL, therefore, these activities were next assayed.

**OxLDL Activate in Situ and in Vitro Ceramidase Activities**—To test whether oxLDL stimulate ceramide turnover, cells were incubated with the fluorescent ceramide analog C₆-NBD-ceramide and then exposed to oxLDL for varying times. The ratio of fluorescent fatty acid to ceramide, which is a reflection of in situ ceramidase activity (27), was higher at all time points for the cells treated with oxLDL (Fig. 4). Consistent with this increased turnover of ceramide, there was an increase in the amount of sphingosine in the cells treated with oxLDL versus the untreated control (Fig. 4, inset). The effect of oxLDL was also determined by measuring in vitro ceramidase activities under acidic (pH 5) and alkaline (pH 8) conditions (Fig. 5). Under basal conditions, the specific activity of ceramidase at pH 5 (6.0 ± 0.3 nmol/mg of protein/h) was somewhat higher than the activity at pH 8 (3.8 ± 0.4 nmol/mg of protein/h) (assays were also conducted at pH 7.2, but no activity was detected at neutral pH, data not shown). Figure 5 shows that incubation of SMC with oxLDL resulted in enhanced ceramidase activity at both acidic and alkaline pH. This increase was detected by 60 min and appeared to be maximal between 120 and 150 min.

**OxLDL Induce Sphingosine Kinase Activation and S1P Production in SMC**—The possibility that sphingosine kinase is activated in response to oxLDL was examined using the in vitro assay described by Spiegel et al. (32) (Fig. 6A) as well as by an assay that analyzes the product mass by HPLC (Fig. 6B). OxLDL induced a 40% increase in sphingosine kinase activity when cell homogenates were assayed 90–120 min after addition of the oxLDL (Fig. 6). As shown in Fig. 7A, cells treated with oxLDL also exhibited higher amounts of [³²P]-labeled S1P, with an apparent maximum (about 2-fold over the control) at 120 min. This increase in S1P in response to oxLDL was abrogated by the serpin TPCK (Fig. 7B), which was previously shown to inhibit SM hydrolysis (13, 36, 37).

The amount of labeled S1P was also elevated when cells were treated for 1 h (data not shown) or 2 h with exogenous bacterial sphingomyelinase, C₆-ceramide, or sphingosine (Fig. 7C). The increases with exogenous sphingomyelinase and C₂-ceramide demonstrate, again, that SMC can metabolize endogenously generated and exogenously added ceramide(s) to S1P. In all cases, including in the presence of oxLDL, the production of S1P was considerably inhibited by co-administration of the sphingosine kinase inhibitor DMS or the ceramidase inhibitor D-MAPP (Fig. 7C) at concentrations that abrogated SM conversion (see Fig. 3). Note also that D-MAPP did not inhibit sphingosine induction of proliferation (see Table I). Thus, these results strongly implicate the involvement of ceramidase and sphingosine kinase, as well as sphingomyelinase, in the response of SMC to oxLDL, thereby resulting in the formation of S1P.

**SMC Proliferation Is Induced by Exogenous Sphingomyelini- nase, C₂-ceramide, Sphingosine, and S1P**—To further define the role of downstream metabolites of ceramide in the mito-

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*It should be noted that a very low concentration of DMS was employed because this compound exhibited a strong cytotoxicity for SMC above 5 μM.*
Cells were grown for 24 h in a phosphate-free medium containing [32P]phosphate. After a 1-h chase, cells were incubated with oxLDL (10 μg/ml) for various times (A), or for 2 h with oxLDL (10 μg/ml) in the presence or absence of 5 μM TPCCK (B). In C, cells were incubated for 2 h with oxLDL (10 μg/ml), exogenous sphingomyelinase (SMase; 0.1 units/ml), C2-ceramide (C2-cer; 5 μM), or sphingosine (Sph; 5 μM), in the presence or absence of d-MAPP or DMS. d-MAPP was incubated with the cells for 12 h before other additions, while TPCCK and DMS were added 1 h before. Cellular S1P was quantified as described under “Experimental Procedures.” Each experiment was performed at least 3 times (except for C where the data are from two separate experiments with a variation averaging 10%).

**DISCUSSION**

The backbones of sphingolipids are now recognized as important players in the regulation of both normal and abnormal cell function (16, 20, 38). Among these molecules, not only ceramide (15, 16, 18), but also ceramide 1-phosphate, sphingosine, sphingosine 1-phosphate, and sphingosylphosphocholine, are highly bioactive in a growing number of species (20, 24, 38). In general, the biological effects of ceramides range from induction of cell differentiation to apoptosis, with ceramide acting as a mediator of stress (16). In contrast, S1P is usually a positive modulator of cell growth stimulation (24, 39–41), and can protect cells from apoptosis (25, 42, 43).

In previous studies on SMC, oxLDL induced sphingomyelinase activation, SM hydrolysis, and ceramide generation, which was followed by MAPK stimulation and mitogenesis (12, 13), suggesting that ceramide might mediate the oxLDL-induced SMC proliferation. However, since S1P is a more likely candidate in mitogenic signaling (24), we investigated whether this sphingolipid could be responsible for the cell proliferation triggered by oxLDL. Regarding SMC, this hypothesis was supported by previous observations showing a mitogenic role for S1P in guinea pig airway SMC (23) and human arterial SMC (22).

The present study demonstrates that oxLDL stimulate an enzymatic cascade leading to the production of S1P from SM. The oxLDL-stimulated enzymes include neutral sphingomyelinase (13), ceramidase(s), and sphingosine kinase, as shown in Fig. 1. This conclusion is supported by our observations that not only are each of these activities increased in a relevant time scale after exposure of SMC to oxLDL, but also, by the expected variations in the intracellular amounts of the corresponding products. Furthermore, the notion that S1P is a critical mediator was strengthened by the inhibition of S1P formation, and mitogenesis, by d-MAPP and DMS.

This metabolic scheme has been corroborated by previous reports that have, typically, focused on one or two of the enzymatic steps of the cascade. Spiegel and associates (21) demonstrated that PDGF induces formation of sphingosine, activation of sphingosine kinase, and the subsequent production of S1P in Swiss 3T3 cells. A PDGF-stimulated increase in S1P levels has been reproduced by others on airway SMC (23). In embryonic rat thoracic aorta SMC, PDGF was shown to produce an increase in sphingosine levels (44), and later studies using primary rat mesangial cells reported the activation of a neutral sphingomyelinase and an alkaline ceramidase in response to PDGF (26). Besides PDGF and other mitogens, nerve growth factor, a neurotrophin described to trigger SM breakdown and ceramide generation (45), has also been reported to activate sphingosine kinase (42). The same is true for vitamin D3 (43). In addition, recent studies of the regulation of CYP2C11 in rat hepatocytes have shown that interleukin-1β activates SM hydrolysis (46, 47), ceramide breakdown (via an increase in ceramidase activity), and S1P formation, although the latter was based only on the use of sphingosine kinase inhibitors (27). Finally, the tumor necrosis factor-induced expression of adhesion molecules by endothelial cells has recently been reported to be preceded by sphingomyelinase and sphingosine kinase activation (48). Thus, as far as we are aware, the current study of the mitogenic effects of oxLDL on SMC provides one of the only analyses of all three metabolites and the activities of enzymes that form them. The subcellular location of the metabolic cascade initiated by oxLDL that leads to S1P still remains to be elucidated.

The mechanism(s) by which the oxLDL-stimulated S1P triggers SMC proliferation(s) are not yet clarified. However, several lines of evidence indicate that the MAPK pathway is activated downstream of S1P. First, it should be borne in mind that S1P has both extracellular and intracellular receptors, which complicates interpretation of its site(s) of action (49). Nonetheless, it is clear that formation of S1P intracellularly plays a role in the mitogenicity of oxLDL because inhibition of...
sphingosine kinase by DMS blocked the growth stimulation by oxLDL. Second, several reports suggest that the Raf/MEK/MAPK pathway is stimulated by SIP (24, 50). We (13) and others (51) have previously shown that mildly oxidized LDL are able to stimulate MAPK in cultured SMC, and that the oxLDL-induced DNA synthesis is blocked by a MAPK kinase inhibitor. In addition, incubation of SMC with the serpin TPCK which blocked SM hydrolysis was found to abolish the activation of MAPK and mitogenesis (13). Finally, SIP is known to enhance the DNA binding activity of the transcription factor AP-1 (52), a molecular event believed to take place downstream of MAPK.

In conclusion, this study has emphasized the concept that a given agonist can activate an entire cascade of sphingolipid metabolism, as exemplified by the triggering of SM hydrolysis in SMC by oxLDL leading to the formation of SIP and mitogenesis. However, this entire pathway might not be activated in other cell types in response to the same stimulus. As a possible example, oxLDL have recently been reported to induce apoptotic cell death in endothelial cells, a phenomenon that was preceded by the generation of ceramide (37, 54). It is, therefore, tempting to speculate that oxLDL may exemplify the ceramide/SIP rheostat that was proposed by Spiegel and coworkers (24) for the modulation of cell growth and survival, with the outcome depending on the regulation of several key sphingolipid metabolizing enzymes.

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