Potential Role for Ceramide in Mitogen-activated Protein Kinase Activation and Proliferation of Vascular Smooth Muscle Cells Induced by Oxidized Low Density Lipoprotein

Proliferation of vascular smooth muscle cells (SMC) is a hallmark in the pathogenesis of atherosclerotic lesions. Mildly oxidized low density lipoproteins (UV-oxLDL), which are mitogenic to cultured AG-08133A SMC, activate the sphingomyelin (SM)-ceramide pathway. We report here the following. (i) UV-oxLDL elicited a biphasic and sustained activation of MBP kinase activity, phosphorylation and nuclear translocation of p44/42 mitogen-activated protein kinase (MAPK), and [3H]thymidine incorporation, which were inhibited by PD-098059, a MAPK kinase inhibitor. (ii) The use of preconditioned media (from SMC pre-activated by UV-oxLDL) transferred to native SMC and blocking antibodies against growth factors suggest that UV-oxLDL-induced activation of MAPK and [3H]thymidine incorporation seem to be independent of any autocrine secretion of growth factors. (iii) UV-oxLDL-induced activation of a neutral sphingomyelinase, SM hydrolysis, ceramide production, and [3H]thymidine incorporation were inhibited by two serine-protease inhibitors (serpins), suggesting that a serpin-sensitive proteolytic pathway is involved in the activation of the SM-ceramide signaling pathway. (iv) UV-oxLDL-induced MAPK activation and [3H]thymidine incorporation were mimicked by ceramide generated in the plasma membrane by bacterial sphingomyelinase treatment or by addition of the permeant C2-ceramide. Serpins did not inhibit the MAPK activation and [3H]thymidine incorporation induced by C2-ceramide, indicating that activation of the MAPK and [3H]thymidine incorporation is subsequent to the stimulation of the SM-ceramide pathway. Taken together, these data suggest that mitogenic concentrations of UV-oxLDL are able to stimulate the SM-ceramide pathway through a protease-dependent mechanism and activate p44/42 MAPK, leading to proliferation of vascular SMC.

Atherosclerosis, and its complications, namely myocardial infarction, stroke, and peripheral vascular diseases, is one of the most prevalent cause of morbidity and mortality in Western countries. During atherogenesis, focal lesions spread out progressively and lead to the formation of fibro-atheroma plaques, in which smooth muscle cell (SMC) proliferation plays a critical role (1, 2). Among the risk factors identified, low density lipoprotein (LDL) cholesterol level is strongly predictive of coronary heart disease. LDL are believed to have an important role in atherogenesis (3), following oxidative modifications (4–6), because oxidized LDL are present in atherosclerotic lesions (7) and possess a wide range of biological properties potentially occurring during atherogenesis in vivo (8). Oxidized LDL have recently been shown to be mitogenic to vascular SMC (9–11). These studies suggest that oxidized LDL may be considered as an additional mitogenic factor, alongside the classical growth factors implicated in SMC proliferation during atherogenesis (6). To date, the mechanism of the oxidized LDL proliferative effect is poorly elucidated and may result from the triggering of a mitogenic intracellular signal either directly by oxidized LDL or indirectly through an autocrine effect involving growth factor secretion and/or growth factor receptor over-expression.

Recently, sphingolipids have emerged as key signaling molecules involved in the regulation of cell growth and differentiation (for reviews, see Refs. 12–15). In particular, the sphingomyelin (SM; ceramide phosphocholine)-ceramide pathway appears as a prototypic sphingolipid signaling pathway implicated in the positive or negative regulation of cell growth. Activation of this pathway leads to SM hydrolysis and subsequent generation of ceramide, the backbone of all sphingolipids, which serves as an intracellular second messenger. To date, several agents have been described to stimulate the SM-ceramide pathway (reviewed in Refs. 12 and 14–17), including cytokines such as TNF-α, interleukin-1β, interferon-γ, nerve growth factor, anti-CD28, anti-Fas antibodies, anticancer drugs, and ionizing radiations (18–21). Cell-permeant ceram-
ides, or ceramide produced by treatment of intact cells with exogenous sphingomyelinase, can mimic the effects of various inducers of the SM-ceramide pathway. Thus, various cellular responses including cell proliferation (18), differentiation (22), or apoptosis (19, 20, 23) seem to be transduced by SM hydrolysis through ceramide generation. We have recently reported that the SM-ceramide pathway is involved in the mitogenic signaling triggered by mildly oxidized LDL (UV-ox-LDL) in SMC (11).

Cell proliferation promoted by extracellular growth stimuli involves various intracellular signaling pathways leading to activation of gene transcription, DNA synthesis, and cell division (24, 25). Protein phosphorylation, mediated by a complex regulatory network of protein kinases, plays an essential role in the signal transduction between cell surface and nucleus (26). Despite the broad diversity of mitogens and receptors, these signaling pathways often converge toward the mitogen-activated protein (MAP) kinases, a group of serine/threonine protein kinases also referred to as extracellular-regulated kinases (ERKs; p44/ERK1 and p42/ERK2, p44/42 MAPK) in mammalian cells (26). MAPKs are rapidly activated in response to stimulation of receptors for growth factors, hormones, or cytokines, G protein-coupled receptors, or in response to stress (25, 27–29). They are activated by phosphorylation on both Tyr and Thr residues (30) by the dual specificity MAPK kinases, which are activated by Raf (29). The activation of MAPKs, associated with their nuclear translocation, is essential to trigger entry into the S phase of cell cycle (31, 32).

The data reported in this study suggest that mitogenic concentrations of UV-ox-LDL elicit the activation of the SM-ceramide pathway through a protease-dependent process. Subsequently, it induced a sustained activation and nuclear translocation of p44/42 MAPK and [3H]thymidine incorporation in cultured vascular SMC.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—[3H]Thymidine ([3H]Tdr) and horseradish peroxidase-conjugated sheep anti-mouse Ig were obtained from Amersham (Les Ulis, France), [3H]ATP (5 Ci/mmol) and [3H]ATP (5 Ci/mmol) from Isotopichem (Genagone, France), [methyl-3H]choline chloride (86 Ci/mmol), [choline-methyl-14C]SM (54.5 Ci/mmol), and [9,10-3H]palmitic acid (52 Ci/mmol) from DuPont NEN (Les Ulis, France). Human recombinant growth factors (PDGF, bFGF, and EGF) were purchased from PeproTech-Tebu (Le Perray, France), and DiIC18 (1,1'-diiodotetramethylindocarbocyanine perchlorate) was from Molecular Probes. 14C-choline-methyl was from DuPont NEN (Les Ulis, France), [1,2]-3H]-ATP (100 Ci/mmol), and [1,2-3H]-ATP (7000 Ci/mmol) from Orsay (Orsay, France), and acid SMase activity, cells were homogenized by sonication in 0.1% Triton X-100 for the alkaline activity. For determining lipase activity, cells were homogenized by sonication (2 runs of 10 s as above indicated) in 0.1% Triton X-100, 20 mM HEPES (pH 7.4, containing 50 mM NaCl, 50 mM NaF, 20 mM sodium pyrophosphate, 1 mM Na3VO4, 100 mM 2-mercaptoethanol, and 10 mM MgCl2) and incubated with 100 mM of substrate [choline-methyl-14C]SM (120,000 dpm/assay) in 0.1% Triton X-100, 20 mM HEPES buffer, pH 7.4, containing 1 mM MgCl2 (and 10 mM EDTA when indicated) for the neutral activity, or in 250 mM Tris-HCl buffer, pH 8.5, containing 0.1% Triton X-100 for the alkaline activity. For determining acid SMase activity, cells were homogenized by sonication in 0.1% Triton X-100. Assays contained 100 mM of cell homogenate (100 μg of protein) and 100 μl of substrate [choline-methyl-14C]SM (120,000 dpm/assay) in 0.1% Triton X-100, 20 mM HEPES buffer, pH 7.4, containing 1 mM MgCl2 (and 10 mM EDTA when indicated) for the neutral activity, or in 250 mM Tris-HCl buffer, pH 8.5, containing 0.1% Triton X-100 for the alkaline activity.

**Measurement of MAP Kinase Activity**—MAP kinase activity was determined on immunoprecipitates according to a procedure described previously (39) but in the presence or absence of EDTA under the previously used conditions (21). Briefly, cells were homogenized by sonication (2 runs of 10 s as above indicated) in 0.1% Triton X-100, 10 mM MgCl2, 5 mM dithiothreitol, 0.1 mM Na3VO4, 10 mM β-glycerophosphate, 750 mM ATP, 1 mM PMSF, 2 mM EDTA, 10 mM leupeptin, and 10 mM pepstatin. SMase assays contained 100 μl of cell homogenate (100 μg of protein) and 100 μl of substrate [choline-methyl-14C]SM (120,000 dpm/assay) in 0.1% Triton X-100, 20 mM HEPES buffer, pH 7.4, containing 1 mM MgCl2, and 10 mM EDTA when indicated for the neutral activity, or in 250 mM Tris-HCl buffer, pH 8.5, containing 0.1% Triton X-100 for the alkaline activity. For determining acid SMase activity, cells were homogenized by sonication in 0.1% Triton X-100. Assays contained 100 μl of cell homogenate (100 μg of protein) and 100 μl of substrate [choline-methyl-14C]SM (120,000 dpm/assay) in 250 mM acetic acid buffer, pH 5.0, containing 0.1% Triton X-100. After 2 h of incubation at 37 °C, the liberated [methyl-14C]choline was partitioned by the Folch procedure (38) under the previously used conditions (21) and quantitated by liquid scintillation counting.
Tris-HCl, pH 7.4, containing 10 g/liter Triton X-100, 1 g/liter SDS, 50 mM NaCl, 50 mM NaF, 5 mM EDTA, 20 mM sodium pyrophosphate, 1 mM Na3VO4, 100 μg/ml PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). Samples were centrifuged (12,500 × g for 10 min, 4 °C). A 5-μl aliquot of the supernatant was used for protein determination (37). The supernatant was precleared for 1 h at 4 °C with 5 mg of protein A-Sepharose. After centrifugation (12,500 × g for 5 min), the supernatant was incubated for 4 h at 4 °C with 5 μl of anti-ERK1 antibody (100 μg/ml), and 5 mg of protein A-Sepharose was added for 1 h at 4 °C. After centrifugation (12,500 × g for 5 min), the pellet was washed twice with buffer A, solubilized in 200 μl of extraction buffer (20 mM-Tris-HCl, pH 7.4, 60 mM glycerophosphate, 10 mM EDTA, 10 mM MgCl2, 0.1 mM NaF, 2 mM dithiothreitol, 1 mM Na3VO4, 20 mg/ml leupeptin, and 1 mM PMSF) and used for determining the MAP kinase activity by phosphorylation of MBP in the presence of [γ-32P]ATP (300,000 dpm assay) as described (40, 41). The reaction mixtures were spotted on phosphocellulose discs, which were washed with 10% cold trichloroacetic acid containing 10 mM sodium pyrophosphate. After drying, the discs were counted by liquid scintillation. Alternatively, proteins were separated by electrophoresis in a 10% polyacrylamide gel, and the radioactivity was visualized by autoradiography (Biomax-MR, Eastman Kodak).

Western Blotting—Phosphorysosine proteins were detected in SMC by Western blotting (42). Cells (2 × 106/assay) were solubilized in 100 μl in lysis buffer B for 30 min at 4 °C, centrifuged (12,500 × g for 10 min at 4 °C). One tenth of protein of the supernatant was used to SDS-polyacrylamide gel electrophoresis (100 V, 9 mA for 16 h). Proteins were transferred to nitrocellulose membranes (300 V, 200 mA for 5 h), blocked with 5% nonfat milk in Tris-buffered saline-Tween 20 (0.1%) at 4 °C for 2 h and incubated overnight at 4 °C with anti-phosphotyrosine 4G10 monoclonal antibody (dilution 1:500). The membranes were washed four times in Tris-buffered saline-Tween 20, and antibody reactions were detected using horseradish peroxidase-conjugated sheep anti-mouse IgG (dilution 1:3000) and the ECL chemiluminescent detection reagents (Amersham) according to the manufacturer's instructions. Alternatively, the cell lysate was precleared on protein A-Sepharose and immunoprecipitated with an anti-phosphotyrosine (PY20) monoclonal antibody exactly under the conditions described above for immunoprecipitating MAPKs with anti-ERK. Then, tyrosine-phosphorylated proteins, heated (90 °C for 10 min) and dissolved in the electrophoresis buffer, were resolved by SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and probed with anti-ERK1 or anti-ERK2 antibody, as indicated above.

Indirect Immunofluorescence—SMC, grown on uncoated glass coverslips, were fixed in PBS and 3% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100, and incubated with anti-ERK1 antibody (1:401 for 30 min. After washing three times with PBS, incubation with a secondary fluorescein isothiocyanate-conjugated anti-rabbit IgG antibody (dilution 1:3000) and the ECL chemiluminescent detection reagents (Amersham) according to the manufacturer's instructions. Alternatively, the cell lysate was precleared on protein A-Sepharose and immunoprecipitated with an anti-phosphotyrosine protein antibody, resolved on SDS-polyacrylamide gel electrophoresis, and revealed with anti-ERK1/ERK2 antibody.

RESULTS

MAPK Activation in AG-08133A SMC Treated by UV-oxLDL—The UV-oxLDL used here were defined by moderate lipid peroxidation (4 ± 1 nmol of thio-barbituric acid-reactive substances/mg of apoB) and only minor modifications of apoB (as shown by minor alterations of the electrophoretic mobility, no significant loss of surface amino groups and cellular uptake through the apoB/E receptor) (34, 43). We utilized here a concentration of UV-oxLDL (100 μg of apoB/ml) that gave maximal mitogenic effect to cultured AG-08133A SMC (as shown by [3H]thymidine incorporation) and only a low level of cytotoxicity, as assessed by LDH release (10, 11).

Mitogenic concentrations of UV-oxLDL induced a biphasic increase of MBP kinase activity in SMC (Fig. 1, A and B). A first, transient peak of MAPK activity, maximal at 1 h, followed by a second rise, sustained for at least 3 h. Native LDL induced a weak transient peak, both of the second sustained peak of MBP kinase (Fig. 1, A and B). Similar results were obtained when using whole cell extracts (Fig. 1A) and anti-ERK immunoprecipitates (data not shown). To provide more evidence for the MAPK activation in SMC stimulated by mitogenic doses of UV-oxLDL, tyrosine phosphorylation of MAPK was examined by immunoblotting. As reported in Fig. 1C, UV-oxLDL induced an increase of tyrosine phosphorylation of p44 and p42 that was evident after 1 h of incubation and plateaued at 3–5 h. As shown in Table I, both the MAPK activation (peaks at 1 and 3 h) and the proliferative effect induced by UV-oxLDL were inhibited by 10 μM PD-098059, a MAPK kinase inhibitor (44). This suggests the involvement of the MAPK cascade in the mitogenic signaling triggered by UV-oxLDL.

Subsequently to mitogenic stimulation, activated MAPKs have been shown to be translocated into the nucleus (31, 45, 46). Therefore, we investigated, by immunofluorescence microscopy, the subcellular localization of MAPKs in SMC incubated with mitogenic doses of UV-oxLDL. As illustrated in Fig. 2, MAPKs underwent a nuclear translocation, which was maximal between 3 and 4 h after the beginning of the incubation of SMC with UV-oxLDL.

MAPK Activation and Proliferation Triggered by UV-oxLDL in AG-08133A SMC Do Not Result from an Autocrine Secretion of Growth Factors—As oxidized LDL have been shown to enhance the expression of various growth factors (6), we investigated whether an autocrine mechanism (secretion of growth
Proliferation of vascular SMC induced by mito-
secreted (autocrine) mediators, such as growth factors.

The effect of RNA and protein synthesis inhibitors on MAPK activation elicited by UV-oxLDL was investigated by preincubating the cells for 2 h with 50 nM actinomycin D or 10 μM cycloheximide just before addition of 100 μg of apoB/ml of UV-oxLDL. This treatment did not block the MAPK activation (neither the first transient nor the sustained peaks) induced by UV-oxLDL (Table I). This suggests that the MAPK activation (both peaks) triggered by UV-oxLDL does not require RNA and protein synthesis (e.g. synthesis of growth factors).

In an attempt to understand whether the activation of the SM-ceramide and MAPK pathways triggered by UV-oxLDL were independent or causally related, two sets of experiments were performed using SM hydrolysis inhibitors and exogenous ceramide.

TABLE I

Effect of PD-098059, a MAPK kinase inhibitor, and RNA and protein synthesis inhibitors on MAPK activity and proliferation of AG-08133A SMC induced by UV-oxLDL

Mean ± S.E.M. of three to four experiments.

| Inhibitor          | UV-oxLDL | MAPK activity | [3H]Thymidine incorporation |
|--------------------|----------|---------------|-----------------------------|
|                    | μM       | μg apoB/ml    | 1 h | 3 h | a   | b |
| None (control 1)   | 0        | 0             | 104 ± 13 | 112 ± 18 | 100 ± 10 |
| None (control 2)   | 0        | 100           | 885 ± 122 | 992 ± 115 | 167 ± 12 |
| PD-098059          | 10       | 0             | 57 ± 12 | 64 ± 11 | 101 ± 10 |
| PD-098059          | 10       | 100           | 175 ± 15 | 214 ± 28 | 108 ± 12 |
| Cycloheximide      | 10       | 0             | 101 ± 10 | 108 ± 15 | ND |
| Cycloheximide      | 10       | 100           | 855 ± 112 | 1042 ± 137 | ND |
| Actinomycin D'     | 0.05     | 0             | 111 ± 12 | 106 ± 16 | ND |
| Actinomycin D''    | 0.05     | 100           | 867 ± 117 | 975 ± 118 | ND |

a MAPK activity was expressed as pmol/min/mg cell protein.
b [3H]Thymidine incorporation was evaluated under standard conditions (at time 24 h) and expressed as percent of control 1, in which [3H]thymidine incorporated into DNA ranged between 330,000 and 448,000 dpm/mg cell protein. ND, not determined.
c Before UV-oxLDL addition, cells were preincubated for 10 min, 2 h, or 2 h with PD-098059, cycloheximide, or actinomycin D, respectively. d p < 0.01 % (comparison with control 2).

Serine protease inhibitors (serpins) have recently been shown to be able to block the activation of the SM-ceramide pathway involved in daunorubicin-induced apoptosis (47). In our experimental model system, effective concentrations of the two serpins used here, DCIC and TPCK, inhibited concomi-
tantly SMase stimulation (Fig. 3F). In longer time-course experiments (24 h), DCIC and TPCK were only slightly toxic, as evaluated by trypan blue staining. Therefore, the inhibitory effect of TPCK on [3H]thymidine incorporation may result from a broad toxic effect. Over the time of experiment (5 h), we did not detect any significant toxicity of the two serpins (at the used concentrations, 10 μM) (Fig. 3F). In longer time-course experiments (24 or 48 h), DCIC was toxic to SMC, and TPCK was only slightly toxic, as evaluated by trypan blue staining. Therefore, the inhibitory effect of TPCK on [3H]thymidine incorporation in SMC treated by UV-oxLDL (Table IV) does not seem to result from a toxic effect but rather from an inhibition of [3H]thymidine

reaction buffer (data not shown), thus suggesting that this neutral SMase is magnesium-independent.

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Ceramide-dependent Activation of MAPK by Oxidized LDL

The present study shows that, in cultured vascular AG-08133A SMC, (i) mitogenic concentrations of UV-ox-LDL elicit the activation of the SM-ceramide pathway and the stimulation and nuclear translocation of MAPK and (ii) ceramides (natural ceramide generated in the plasma membrane by SMase treatment or short chain permeant C2-ceramide) are effective mitogenic mediators, which are also able to induce activation and nuclear translocation of MAPK and subsequent stimulation of [3H]thymidine incorporation.

Oxidized LDL have been shown to induce a significant increase of [3H]thymidine incorporation and proliferation of cultured aortic SMC (9–11). This mitogenic effect is stronger than that of native LDL (10, 11), which were only poorly mitogenic to SMC, despite their ability to induce some intracellular signaling (49).

Moderate concentrations of UV-ox-LDL (100 μg of apoB/ml) induced a significant mitogenic response in vascular SMC, in a medium supplemented with either human lipoprotein-depleted serum (10) or 1% FCS (11). In G0-arrested cells, initiation of DNA synthesis is a complex process that requires potent growth factors enabling cells to pass the G1 restriction point controlling S phase entry (50). We report here that, similarly to growth factors, the proliferative effect of UV-ox-LDL on SMC was associated with a biphasic, sustained stimulation of MBP kinase activity, tyrosine phosphorylation, and nuclear translocation of p44/p42 MAPKs. In contrast, native LDL induced only a transient activation of MAPK. The role of MAPK activation in the proliferation of SMC induced by UV-ox-LDL was supported by the inhibitory effect of PD-098059, a MAPK kinase inhibitor, which blocked both the MAPK activation (45) and the SMC proliferation.

An early and transient activation of MAPK has been reported to be induced by native LDL (44, 51, 52) or ox-LDL (44, 52–54), but, in these studies, no information was provided on the late events (over 5 h) and nuclear translocation of MAPK, which are thought to play a key role in the full mitogenic response (55). When monitoring the MAPK activation during a longer period of time, we observed an obvious difference between MAPK activation triggered by native LDL and UV-ox-LDL, as only UV-ox-LDL induced the biphasic and sustained activation of MAPK. This response is similar to that observed with classical mitogens that induce a full mitogenic response (30, 31, 55).

We therefore considered the possibility that oxidized LDL may promote indirectly cell growth via an autocrine paradoxical mechanism resulting from enhanced expression of growth factors. Several data obtained suggest that a role of autocrine growth factors is unlikely in our model system (cultured vascular AG-08133A SMC used under the described experimental conditions). (i) The interval of time between cell contact with and MAPK activation by UV-ox-LDL (the first peak occurred after a 1-h pulse with UV-ox-LDL) is relatively short for an autocrine signaling involving stimulation of growth factor synthesis by UV-ox-LDL. For instance, as ox-LDL-induced PDGF-A mRNA peaked after 2 h of incubation (49) and protein synthesis and secretion of PDGF need some additional time, PDGF secretion cannot explain the MAPK activation occurring at 1 h. (ii) Similarly, MAPK activation triggered by exogenous SMase (which mimics the mitogenic effect of UV-ox-LDL) occurs very rapidly (first peak at 5 min), thereby excluding a role of growth factors (gene induction, protein synthesis, and secretion of growth factors being very unlikely in this very short time). (iii) Inhibition of mRNA or protein synthesis by effective doses of actinomycin D or cycloheximide did not block the MAPK activation induced by UV-ox-LDL. (iv) MAPK activation by ceramide (ce-

### Table II

| Mitogen | Antibody | [3H]Thymidine incorporation
|---------|----------|-------------------------------|
|         |          | µg/ml | %                           |
| None (control) | None | 100 ± 9 |                            |
| Growth factor (ng/ml) |          |          |                            |
| PDGF, 10 | None | 126 ± 10 |                            |
| PDGF, 10 | Anti-PDGF, 25 | 97 ± 6 |                            |
| EGF, 60 | None | 102 ± 14 |                            |
| bFGF, 1 | None | 97 ± 10 |                            |
| UV-ox-LDL (µg apoB/ml) |          |          |                            |
| 100 | None | 157 ± 12 |                            |
| 100 | Anti-PDGF, 25 | 148 ± 8 |                            |
| 0 | Anti-PDGF, 25 | 103 ± 9 |                            |
| 100 | Anti-EGF, 60 | 161 ± 10 |                            |
| 0 | Anti-EGF, 60 | 101 ± 8 |                            |
| 100 | Anti-FGF, 1 | 159 ± 11 |                            |
| 0 | Anti-FGF, 1 | 102 ± 9 |                            |

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a [3H]Thymidine incorporated into DNA ranged between 346,000 and 512,000 dpm/mg of cell protein.

b p < 0.01% (comparison to the relative controls, i.e. between assays with and without antibody or UV-ox-LDL).
Ceramide-dependent Activation of MAPK by Oxidized LDL

TABLE III
Effect of preconditioned ("chase") culture media (from AG-08133A SMC preincubated with UV-oxLDL) on MAPK activity and [3H]thymidine incorporation of reporter AG-08133A SMC

Preconditioned media were prepared as follows. SMC were preincubated (or "pulsed") for 1 or 16 h in RPMI containing 1% FCS and 100 μg of apoB/ml of UV-oxLDL, then, this medium (containing UV-oxLDL) was discarded, and SMC were incubated ("chased") for an additional 2 or 4 h in RPMI containing 1% FCS (preconditioned culture medium). The preconditioned media ("chase" media) were then transferred to reporter AG-08133A SMC. After addition of the preconditioned medium, the MAPK activities and [3H]thymidine incorporation were determined on the reporter SMC, at 1 and 3 h for MAPK (time of the MAPK peaks evoked by UV-oxLDL) and 16 h for [3H]thymidine incorporation. Mean ± S.E.M. of three to five experiments.

| Preconditioning conditions | Effect of preconditioned medium on reporter SMC |
|----------------------------|-----------------------------------------------|
| oxLDL | Pulse Time | oxLDL | Chase Time | MAPK activity<sup>a</sup> | [3H]Thymidine incorporation<sup>b</sup> |
| μg/ml h | μg/ml h | 1 h | 3 h | 16 h |
| 0 | 0 | 90 ± 12 | 112 ± 15 | 100 ± 9 |
| 0 | 0 | 110 ± 16 | 108 ± 12 | 101 ± 8 |
| 100 | 1 | 780 ± 92 | 930 ± 102<sup>c</sup> | 165 ± 9<sup>c</sup> |
| 100 | 1 | 820 ± 81<sup>c</sup> | 890 ± 97<sup>c</sup> | 167 ± 7<sup>c</sup> |
| 100 | 1 | 102 ± 18 | 97 ± 16 | 102 ± 9 |
| 100 | 1 | 102 ± 15 | 104 ± 14 | 105 ± 8 |
| 100 | 16 | 111 ± 12 | 106 ± 14 | 101 ± 10 |
| 100 | 16 | 108 ± 11 | 109 ± 16 | 106 ± 8 |

<sup>a</sup> MAPK activity is expressed as pmol/min/mg cell protein.

<sup>b</sup> [3H]Thymidine was determined under standard conditions and expressed as percent of untreated control. [3H]Thymidine incorporated into DNA ranged between 394,000 and 487,000 dpm/mg cell protein.

<sup>c</sup> p < 0.01 (comparison to the related control).

Diagram:

**Fig. 3.** Relative time course of SMase activation, SM hydrolysis, ceramide generation, and MAPK activation in AG-08133A SMC treated by UV-oxLDL (100 μg of apoB/ml) and inhibition by 10 μM serpins TPCK and DCIC (empty squares and triangles, respectively). A, time course of SMase activation elicited by UV-oxLDL. SMC were incubated for 48 h in RPMI medium containing 1% FCS. Then, the medium was replaced by fresh medium containing UV-oxLDL (100 μg of apoB/ml). At the indicated times, SMase activities were determined under the conditions described under "Experimental Procedures." Neutral SMase activity was determined at pH 7.4 in the presence of 10 mM EDTA (●), acid SMase activity at pH 5.0 (■), and alkaline SMase activity at pH 9.5 (▲). SMase activities were expressed as percent of the value at time 0 (i.e. 74 ± 6, 51 ± 4, and 2290 ± 130 pmol/h/mg cell protein for the neutral, alkaline, and acid SMases, respectively). B, inhibition by serpins of the neutral SMase activated by UV-oxLDL. SMC were starved for 48 h in RPMI medium containing 1% FCS. The medium was discarded, and cells were preincubated in fresh medium without (●) or with serpins for 2 h (10 μM TPCK (●)) or DCIC (■), just before addition (time 0) of 100 μg of apoB/ml of UV-oxLDL. At the indicated times, cells were harvested and immediately used for determining neutral SMase activity under the conditions used in A. C and D, SM hydrolysis and ceramide generation induced by UV-oxLDL is inhibited by serpins. SMC were preincubated for 48 h in RPMI medium containing 1% FCS and [methyl-3H]choline (0.5 μCi/ml) (in C) or [3H]palmitic acid (0.5 μCi/ml) (in D) and then treated under the same conditions as in B. At the indicated times, cells were harvested and lipids extracted for determining radiolabeled SM and ceramide levels as described under "Experimental Procedures." Results are expressed as percent of values at time 0. E, MBP kinase activation induced by UV-oxLDL is inhibited by serpins. SMC were treated under the conditions of B; at the indicated time, cells were harvested for determining MBP kinase activity. F, cell viability of SMC incubated for 5 h (black bars) and 24 h (gray bars) under the conditions of B, i.e. in basic culture medium (controls, Co), in media containing UV-oxLDL (oxL) or UV-oxLDL plus TPCK (oxL + T) or UV-oxLDL plus DCIC (oxL + D). Cell viability was evaluated by trypan blue exclusion and expressed as unstained cells percent. Mean ± S.E. of three to five experiments.

ramide generated by SMase and permeant C2-ceramide was not abrogated by actinomycin D or cycloheximide (data not shown). (v) Blocking antibodies against PDGF, bFGF, and EGF (used under conditions inhibiting the response induced by the related growth factor) did not affect the MAPK activation nor the mitogenic response triggered by UV-oxLDL. Moreover, in
agreement with Stiko-Rahm et al. (49), when the preconditioned medium (obtained by preincubating SMC with oxLDL) was transferred on reporter SMC, no significant mitogenic effect was observed. Taken together, these data strongly suggest that an autocrine secretion of growth factors triggered by oxLDL plays only a minor role in our model system. Therefore, from our recently reported data (11), we hypothesized that UV-oxLDL may act directly as a mitogen to SMC by triggering membrane damages triggering the activation of the MAPK cascade, and downstream signaling, i.e. MAPK activation and DNA synthesis.

Another major question is to know whether the activation of the SM-ceramide pathway is involved in the mitogenic effect or is only a parallel consequence of the response of cells to UV-oxLDL. The former hypothesis is supported by the fact that both MAPK activation and mitogenic effect of UV-oxLDL were mimicked by exogenous short chain permeant ceramide and by ceramide generated at the plasma membrane by exogenous SMase. A nonspecific effect of these mediators (for instance membrane damages triggering the activation of the MAPK pathway) is unlikely because (i) phospholipid hydrolysis by exogenous phospholipase C did not trigger MAPK activation or DNA synthesis, although it induced probably plasma membrane structural changes similar to those elicited by SMase, and (ii) C2-dihydroceramide was ineffective, in contrast to C2-ceramide.

Together, the above reported data are consistent with the hypothesis that UV-oxLDL may induce the sequential activation of the SM-ceramide pathway, MAPK cascade, and downstream mitogenic signaling. This is consistent with Raf1 phosphorylation (59) and MAPK activation induced by endogenous or cell-permeant ceramides in HL60 leukemic cells (60, 61), only activation of a neutral SMase (peaking at 1 h), which may be similar to the enzyme described by Okazaki et al. (56). The activation of the neutral SMase was inhibited by TPCK and DCIC concomitantly with SM hydrolysis and ceramide generation, thus suggesting that the neutral SMase is involved in SM hydrolysis and mitogenic signaling triggered by UV-oxLDL. These data are consistent with the role of this SMase in the TNF-α-induced mitogenic signaling in fibroblasts (38). The molecular mechanism of the activation of this neutral SMase by oxLDL remains unknown, but its inhibition by serpins (Ref. 47 and present paper) or by caspase inhibitors (57, 58) supports the hypothesis for a role of protease(s) in SMase activation.

Furthermore, the two serpins TPCK and DCIC inhibited concomitantly ceramide generation (subsequent to SM hydrolysis), MAPK activation, and [3H]thymidine incorporation triggered by UV-oxLDL but did not inhibit the MAPK activation and mitogenic effect induced by short chain ceramide. Taken together, these data suggest that serpins act upstream from the SMase, and do not interfere, in our model system, between ceramide and downstream signaling.
fibroblasts (62, 63), and endothelial cells (64). Although MAPK activation by ceramide has been demonstrated in cellular systems undergoing apoptotic or inflammatory responses, our results strongly implicate activation of MAPK by ceramide in a proliferative signaling pathway consistent with the well-documented mitogenic role of MAPK (30, 31, 55).

In various experimental models, other sphingolipid mediators such as lactosylceramide, sphingosine 1-phosphate, or sphingosylphosphocholine have been shown to stimulate the p42/p44 MAPK or exert a proliferative effect (54, 65–67). The possibility that, in our model system, ceramide may be degraded and converted into sphingosine 1-phosphate or sphingosylphosphocholine, or utilized as a precursor for lactosylceramide biosynthesis, cannot be ruled out. To date, the respective role of the ceramide pathway and other pathways involving other sphingolipid mediators in the oxLDL-induced mitogenic effect remains to be elucidated.

In conclusion, the present paper reports several novel results concerning the mitogenic intracellular signaling triggered by oxLDL in vascular SMC. (i) The mitogenic effect of UV-oxLDL is mediated through serpin-sensitive serine proteases, which activate in turn the SM-ceramide pathway. (ii) The mitogenic effect resulting from the activation of the SM-ceramide pathway is mediated through a biphasic and sustained activation and nuclear translocation of MAPK. Finally, our data suggest that the activation of SM-ceramide pathway induces in turn a sustained activation of the MAPK pathway, associated with nuclear translocation of MAPK, which is a critical event during G1 progression (55) and seems to be sufficient for inducing cell proliferation (68). As oxLDL are present in atherosclerotic lesions, but not in normal vascular wall (7), this mitogenic stimulus is locally persistent and specific to atherosclerotic areas. It is therefore suggested that, besides the other cytokines and growth factors potentially involved in atherogenesis (6), oxLDL may play a critical role in the proliferation of SMC occurring in atherosclerotic plaque.

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