Oxidized phospholipids, including 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC) and 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC), typically present in minimally modified low density lipoprotein, have been found in atherosclerotic lesions. These compounds are gaining increasing importance as inducers of different cellular responses (inflammation, proliferation, or cell death). It was the aim of this study to understand their impact on intracellular signal transduction pathways that are responsible for these biological effects. We found that in arterial smooth muscle cells, PGPC and POVPC activated sphingomyelinases, in particular the acid isoform, which is known to participate in the very early phase of apoptotic stress responses. In addition, mitogen-activated protein kinases, which are involved in induction of stress response and apoptosis were phosphorylated (activated). Finally, activation of caspase 3 was observed, showing that stimulation of smooth muscle cells with POVPC and PGPC is associated with apoptosis. Stimulation of all these enzymes by the oxidized phospholipids almost perfectly matched their activation by minimally modified LDL. Consequently, these phospholipids seem to be responsible for the effect of this particle on cell signaling. Survival and proliferation pathways including NF-κB or AKT kinase were not induced by POVPC and PGPC. Experiments with a specific inhibitor of acid sphingomyelinase named NB6 showed that this enzyme plays a central role in mediating the apoptotic effects of the oxidized lipids. Thus, we conclude that modified phospholipids induce signal cascades via activation of acid sphingomyelinase finally leading to apoptosis of smooth muscle cells, which is a detrimental process in the development of atherosclerosis.

Oxidation of LDL is a key process in the development of atherosclerosis (1, 2). Oxidized LDL exerts multiple effects on the cells of the arterial wall, including inflammation, proliferation, and programmed cell death (3). Especially apoptosis of smooth muscle cells (SMCs) is a prominent feature of the late phase of atherosclerosis (4) and can be triggered by oxidative stress (5). Interaction of SMC with oxidized LDL and the biological consequences thereof are an important issue and much is known about the effects of highly oxidized LDL as a whole particle (6, 7). LDL can be oxidized to different degrees. Whereas highly oxidized LDL is modified at both its lipid and protein part, minimally modified LDL (mmLDL) is mainly oxidized at its lipid part, and thus exhibits unique properties, which are different from those of oxidized LDL (8). These features might be due to its content in biologically active lipids mainly originating from oxidation of arachidonic acid of the surface phospholipids (9). Among a large number of degradation products oxidized phospholipids including 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC) and 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC) are present in mmLDL. These compounds are characterized by short polar fatty acyl chains in position 2 and a single hydrophobic fatty acid in position 1 of glycerol (10). As a consequence they are highly exchangeable between cells, tissues, and lipoproteins. Oxidized phospholipids have been found in atherosclerotic lesions and are responsible for multiple phenomena in endothelial cells leading to the progression of atherosclerosis including monocyte adhesion, integrin activation, and tissue factor expression (11–13). The effects of oxidized phospholipids present in mmLDL on signaling in vascular smooth muscle cells have not been investigated to date.

Sphingomyelinases are very early upstream elements of stress-induced cell signaling (14). They generate ceramide from sphingomyelin affecting several downstream targets, including the family of mitogen-activated protein kinases (MAPK), which are activated by phosphorylation (15). Especially, c-Jun N-terminal kinase (JNK) and p38 MAPK have been reported to be involved in apoptotic stress responses (16, 17). At the very end of the apoptotic signaling cascade, caspase 3 is activated, this being responsible for the execution of apoptosis (18).

Activation of extracellular regulated protein kinase (ERK), which also belongs to the MAPK family, leads to proliferation (19). Proliferation and survival pathways may also be activated independently by AKT-kinase/protein kinase B or the transcription factor NF-κB (20, 21).
It was the aim of this study to identify the effects of POVPVC and PGPC on signal transduction pathways involved in proliferative and apoptotic stress responses of vascular SMC and find out how these phenomena induced by chemically defined compounds are related to the parent (minimally oxidized) lipoprotein particle. We found that POVPVC and, to a lesser extent, PGPC activated apoptotic signaling pathways finally leading to caspase 3 activation. They did not stimulate survival and proliferation pathways. More specifically, acid sphingomyelinase represents a central element in the POVPVC- and PGPC-induced apoptotic signaling cascade. Collectively, our studies suggest that PGPC and especially POVPIC are the components that are responsible for the stress effects of mmLDL on SMCs as they mimicked mmLDL in activating the same signaling components.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were purchased from Merck (Darmstadt, Germany). Plastic containers for cell culture were obtained from Sarstedt (Nümbrecht, Germany). Media and supplements for cell culture were purchased from Pan Laboratories (Linz, Austria). Micro well plates for fluorescence and luminence assays were from Falcon (Innsbruck, Austria). TNF-α was from Calbiochem (La Jolla, CA). NB6 was provided by Hans-Peter Deigner (Clinic for Anaesthesiology and Intensive Care Therapy, FSU Jena, Germany). Chemicals for gel electrophoresis were obtained from Bio-Rad Laboratories (Heracles, CA). Nitrilcellulose membranes were from Schleicher and Schuell (Dassel, Germany). Primary polyclonal antibodies against phosphorylated forms of JNK/SAPK, p38 MAPK, ERK1/2, AKT/PKB, and caspase 3 were from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies were from Dako (Carpinteria, CA). Nitrocellulose membranes were from Schleicher and Schuell (Dassel, Germany). Primary polyclonal antibodies against phosphorylated forms of JNK/SA PK, p38 MAPK, ERK1/2, AKT/PKB, and caspase 3 were from Santa Cruz Biotechnology (Santa Cruz, CA).

ApO-1B-homogenous caspase 3/7 assay (including cell lysis/activity buffer and (Z-DEVD)-Rhodamine 110 substrate), 2 TjgTM-50 reaction buffer for the transfection of eucaryotic cells, and pSV/H9252 supplemented with 10% FCS, 2 mM L-glutamine, 100 units/ml penicillin/streptomycin and 25 mM Hepes at 37 °C was used.

The Western blot analysis of JNK, p38 MAPK, ERK1/2, AKT/PKB, and caspase 3 was performed as described by Hermetter et al. (23). Briefly, cells were seeded into six-well plates and grown for 24 h. For termination, stimulated cells were washed twice with ice-cold PBS, harvested by scraping, and lysed. The protein content of cell lysates was determined by the method of Bradford (27), proteins were transferred electrophoretically to nitrocellulose membranes. Unspecific binding was blocked by incubation of the blotting membranes with 1% bovine serum albumin in incubation buffer (50 mM Tris, 150 mM NaCl, 0.1% volume % Tween 20, pH 8.0). Blots were then incubated with primary antibodies against phosphorylated forms of JNK, p38 MAPK, ERK1/2, AKT/PKB, or activated caspase 3 in incubation buffer (dilution 1:4000) followed by incubation with alkaline phosphatase-conjugated anti-rabbit immunoglobulin (dilution 1:1500). Blots were developed with BCIP/NBT liquid substrate system.

Quantification of Caspase 3 Activity—A7r5 cells were seeded into 96 micro well plates employing a cell number of 15,000 cells/well and grown for 24 h in 100 μl DMEM + 10% FCS. Subsequently, the medium was removed and cells were incubated for 2–8 h with a 100 μM suspension of either POVPIC or PGPC corresponding to final lipid concentrations of 1, 10, or 50 μM. For inhibition experiments, cells were preincubated with 10 μM NB6 for 30 min prior to addition of oxidized lipid. A 100 μl solution of (Z-DEVD)-rhodamine 110 in cell lysis/activity buffer (50 mM Tris, 150 mM NaCl, 0.1% volume % Tween 20, pH 8.0) was then added with primary antibodies against phosphorylated forms of JNK, p38 MAPK, ERK1/2, AKT/PKB, or activated caspase 3 in incubation buffer (dilution 1:4000) followed by incubation with alkaline phosphatase-conjugated anti-rabbit immunoglobulin (dilution 1:1500). Blots were developed with BCIP/NBT liquid substrate system.

Measurement of NF-κB Activity—For transient transfection, A7r5 cells were seeded into 6-well plates (9.6 cm²), grown to 50% confluence in DMEM supplemented with 10% FCS, and, subsequently, incubated with either POVPIC, PGPC (10 μM each), or mmLDL (60 μg apoB/ml) for 10, 50, or 90 μFCS. Media and aliquots containing 20 μg of protein were subjected to SDS-PAGE on 12% acrylamide gels using the mini-PROTEIN III electrophoresis according to Laemmli (28). Proteins were transferred electrophoretically to nitrocellulose membranes. Unspecific binding was blocked by incubation of the blotting membranes with 1% bovine serum albumin in incubation buffer (50 mM Tris, 150 mM NaCl, 0.1% volume % Tween 20, pH 8.0). Blots were then incubated with primary antibodies against phosphorylated forms of JNK, p38 MAPK, ERK1/2, AKT/PKB, or activated caspase 3 in incubation buffer (dilution 1:4000) followed by incubation with alkaline phosphatase-conjugated anti-rabbit immunoglobulin (dilution 1:1500). Blots were developed with BCIP/NBT liquid substrate system.

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After treatment of the smooth muscle cells with the respective compounds, cells were washed with cold phosphate-buffered saline, scraped, harvested, and lysed by incubation with either neutral lysis buffer for determination of neutral SMase activity or acid lysis buffer for determination of acid SMase activity as described previously (26). Cell lysates were subjected to protein quantification according to the method by Bradford (27), and aliquots containing 20 μg of protein were analyzed for acid and neutral sphingomyelinase activity using fluorescein diacetate as substrate.

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1. A. Hermetter and E. Zenzmaier, unpublished results.

2. Bis-(N-CBZ-γ-L-glutamyl-L-glutamyl-L-aspartic acid amide)rhodamine 110.

3. M. A. Hermetter and E. Zenzmaier, unpublished results.
FIG. 1. **Sphingomyelinase activities in smooth muscle cells.** Effects of POVPC and PGPC in comparison to minimally modified LDL. Rat aortic smooth muscle cells (A7r5) were incubated with either POVPC, PGPC (10 μM each), native, or minimally modified LDL (60 μg apoB/ml) for 30 or 360 min after treatment with or without 10 μM NB6 for 30 min. Cells were isolated and lysed in lysis buffer for assaying acid and neutral SMase. Enzyme activities were determined as described previously (26) and expressed as % of control. Data are means ± S.D. (n = 3). A, acid sphingomyelinase activities. B, neutral sphingomyelinase activities.
RESULTS

Activation of Sphingomyelinases by Oxidized Phospholipids—Sphingomyelinases are central elements in stress-induced signal transduction leading to apoptosis or proliferation (29). We investigated the effects of the pure oxidized phospholipids PGPC and POVPC, which are typically present in minimally oxidized LDL, on acid and neutral sphingomyelinases in smooth muscle cells using fluorescent sphingomyelin as a substrate (26). Minimally modified LDL and native LDL were applied as control stimuli. In addition, experiments were performed with NB6, which has been shown to specifically inhibit acid sphingomyelinase on the transcriptional level (30). As shown in Fig. 1A, 10 μM POVPC stimulated activity of acid sphingomyelinase to the same extent as mmLDL (50 μg apoB/ml) after 30 min (180% of control). In contrast, PGPC and native LDL showed only minor effects. The maximum effect of POVPC was reached after 30 min. However, significant activation was already detectable after 5 min (data not shown). After 360 min of incubation with POVPC, aSMase activity returned to control levels. Incubation of smooth muscle cells with 10 μM NB6 for 30 min prior to stimulation with either POVPC, PGPC, or mmLDL led to a decrease of acid sphingomyelinase activity to control levels.

The neutral isomorph of this enzyme was activated by POVPC and PGPC to the same extent (Fig. 1B). Nonetheless, in contrast to the acid isoform, neutral SMase remained activated after 360 min. MmLDL caused substantially higher stimulation as compared with the oxidized phospholipids. In this context, it has to be emphasized that native LDL also strongly activated neutral SMase. Thus, the lipoprotein effect seems not to be specific for the oxidized particle. Treatment of SMC with the acid SMase inhibitor NB6 prior to stimulation with either the oxidized phospholipids or mmLDL did not affect neutral sphingomyelinase activation at all.

Influence of POVPC and PGPC on JNK and p38 MAPK—JNK and p38 MAPK belong to the family of MAPKs and mediate apoptotic stress response (31). They are supposed to be downstream targets of ceramide and, as a consequence, of sphingomyelinase activity. Thus, we investigated the role of JNK and p38 MAPK in cell signaling triggered by oxidized phospholipids and the potential involvement of acid sphingomyelinase as an upstream element. For this purpose, SMC were exposed to 10 μM POVPC or PGPC or 60 μg/ml mmLDL for 5–30 min with or without pretreatment with NB6 as an inhibitor of aSMase. Fig. 2, A and B show data from the Western blot analysis of cell extracts for phosphorylated (activated) JNK and p38 MAPK.
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FIG. 3. Caspase 3 activity in SMCs. Effects of POVPC, PGPC, and NB6. A7r5 cells were stimulated with 1, 10, or 50 μM POVPC or PGPC, respectively, for 2–8 h after treatment with or without 10 μM NB6 for 30 min. Caspase 3 activity was determined using an homogenous caspase 3 activity assay (for a detailed description see "Experimental Procedures"). Data shown are means ± S.D. (n = 4). A, effect of POVPC on caspase 3. B, effect of PGPC on caspase 3.

JNK/SAPK (A) and p38 MAPK (B), respectively. The blots clearly demonstrate that mmLDL-induced activation of both JNK/SAPK and p38 MAPK is mimicked by POVPC and PGPC, POVPC being the more efficient inducer of phosphorylation (activation). Activation was already observed after 5 min in all cases. Maximum activity was detected after 10 min of incuba-
Addition of NB6 to the cells prior to stimulation abolished POVPC-, PGPC-, and mmLDL-induced phosphorylation of JNK and p38 MAPK, indicating that acid sphingomyelinase is essential for SMC signaling initiated by mmLDL and its oxidized phospholipid constituents.

Activation of Caspase 3 by Oxidized Phospholipids—To provide more direct evidence that the incubation of smooth muscle cells with POVPC or PGPC does, in fact, lead to apoptosis we measured caspase 3 activity after stimulation of the cells with various concentrations (1–50 μM) of POVPC and PGPC for different lengths of time. According to Fig. 3, POVPC induced caspase 3 activation in a time- and concentration-dependent manner (Fig. 3A). Maximum caspase 3 activity was reached after incubation with 50 μM POVPC for 6 h. In contrast, caspase 3 was only slightly activated by PGPC. Inhibition of acid sphingomyelinase by NB6 led to a substantial decrease of POVPC-induced caspase 3 activity, providing additional evidence that acid sphingomyelinase seems to be a central element in mediating the apoptotic effect of POVPC. Data obtained from analysis of caspase 3 activity were confirmed by Western blot analysis (Fig. 4), showing activated caspase 3 in lysates of cells, which have been exposed to the oxidized phospholipids.

Lack of Activation of Proliferative and/or Salvage Pathways—Further experiments were performed to find out whether or not POVPC and PGPC affect survival and proliferation pathways in addition to apoptotic signaling cascades. In this context, activity of NF-κB was measured using a luciferase reporter gene assay. This transcription factor might play a role in atherogenesis because it mediates survival, proliferation and inflammation (21).

To make sure that the luciferase expression system worked properly, activation of NF-κB by the cytokine TNF-α was examined, this being a potent activator of NF-κB in many cell types including vascular smooth muscle cells (32). Fig. 5 shows that TNF-α induced NF-κB activity in smooth muscle cells in a time-dependent manner.

Stimulation of cells with mmLDL (60 μg apoB/ml) for 60 or 240 min did not lead to any NF-κB-induced luciferase expression. Under the same conditions, NF-κB levels were neither affected by POVPC nor PGPC (10 μM each), coinciding with results obtained by stimulation of the cells with mmLDL. Consequently, we may conclude that the NF-κB pathway is not involved in POVPC- and PGPC-induced signaling.

In addition to NF-κB, there are two other components important for survival and proliferation signaling pathways. AKT-kinase/PKB and ERK are protein kinases that participate in cytokine- and growth factor-induced signal transduction leading to proliferation of most cells (19, 20). Exposure of cells to 10 μM of the oxidized lipids or mmLDL (60 μg apoB/ml) neither stimulated ERK nor AKT/PKB. Western blot analysis (Fig. 6) of stimulated cell extracts for the phosphorylated (activated) proteins did not show any differences compared with the controls. These results again confirm the assumption that mmLDL and its biologically active components POVPC and PGPC do not activate signaling pathways responsible for proliferation and/or survival of vascular smooth muscle cells.

![Graph](image_url)

**Fig. 5.** Effect of POVPC and PGPC on NF-κB activity in SMCs. A7r5 cells were transfected with a reporter plasmid construct for determination of NF-κB activation followed by incubation with either TNF-α (3 ng/ml) as positive control, POVPC, PGPC (10 μM each), or mmLDL (60 μg apoB/ml) for 60–240 min. Cells were collected and lysed and NF-κB activity was determined using a luciferase assay system as described under “Experimental Procedures.” Data represent mean values ± S.D. of at least 4 independent measurements.
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DISCUSSION

This work reports on the role of POVPC and PGPC in mmLDL-induced signaling of vascular SMC. We demonstrate that both phospholipids under consideration evoked apoptotic cell death by time- and concentration-dependent induction of caspase 3 activity, albeit to a different extent. POVPC was the more potent apoptotic lipid. Both POVPC and PGPC activated the sphingomyelinase pathway, particularly acid sphingomyelinase, to which an important role in stress-induced apoptosis has been assigned (33). We found a maximum of SMase activity in SMCs after 30 min of incubation with POVPC and PGPC. A significant increase of aSMase activity (150% of control) was already found after 5–10 min (data not shown). POVPC and PGPC also activated p38 MAPK and JNK, both playing an eminent part in apoptotic signaling. Phosphorylation of JNK and p38 MAPK due to cell stimulation with POVPC and PGPC was again very fast (within 5–30 min), POVPC being more potent than PGPC.

POVPC- and PGPC-induced activation of acid SMase could be abrogated by a specific inhibitor of acid sphingomyelinase (NB6). NB6 also prevented activation of the downstream targets p38 MAPK and JNK. Finally, the execution of apoptosis by caspase 3 could be inhibited by NB6 as well. These results indicate that acid sphingomyelinase is a central mediator in the oxidized phospholipid-triggered apoptotic signaling.

Survival pathways, based on activation of ERK, NF-κB and AKT-kinase/PKB, were neither activated by POVPC nor PGPC. Obviously, the SMCs are bound to death if these compounds, which are present in sufficient amounts, e.g. in an atheroma. Remarkably, POVPC and, to a minor extent, PGPC closely mimic the biological activity of mmLDL. They activate the same signaling pathways leading to apoptosis. POVPC might be a component in the oxidized particle, which determines its properties to a major extent.

Activation of aSMase and its downstream elements occurs extremely fast (within a few minutes). This might be due to the rapid transfer of the respective lipids into the plasma membrane of the cell. The oxidized phospholipids have only one long hydrophobic side chain in position 1 of glycerol. The fatty acyl chain at position 2 is short and contains either a polar (C = O) or a charged polar (COO−) group. Hence, this chain becomes a part of a tremendously large head group. Consequently, these lipids may be considered highly surface active substances, similar to lysophosphatidylcholine, which is also known for its potency to activate signaling and apoptosis (34, 35).

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