CYTOTOXIC EFFECTS OF OXIDIZED PHOSPHOLIPIDS IN VASCULAR CELLS: ROLE OF CERAMIDE SYNTHASES

By

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"Science investigates; religion interprets. Science gives man knowledge which is power; religion gives man wisdom which is control. Science deals mainly with facts; religion deals mainly with values. The two are not rivals. They are complementary"

Martin Luther King Jr.,

To my family

ABSTRACT

Oxidized phospholipids (OxPLs), including 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine (PGPC) and 1-palmitoyl-2-oxovaleroyl-*sn*-glycero-3-phosphocholine (POVPC) are among several biologically active derivatives that are generated during oxidation of low-density lipoproteins (LDLs). These OxPLs are contributing factors in pro-atherogenic effects of oxidized LDLs (OxLDLs), including inflammation, proliferation and cell death in vascular cells. OxLDL also elicits formation of the lipid messenger ceramide (Cer) which plays a pivotal role in apoptotic signaling pathways. Here we report that both PGPC and POVPC are cytotoxic to cultured macrophages and induce apoptotic cell death which is also associated with increased cellular ceramide levels. Exposure of RAW 264.7 cells to POVPC and PGPC for several hours resulted in a significant increase in ceramide synthase (CerS) activity. Under the same experimental conditions, acid or neutral sphingomyelinase activities were not affected. PGPC is more toxic than POVPC and a more potent inducer of ceramide generation by activating a limited subset of CerS isoforms. The stimulated CerS activities are in line with the C_{16}-, C_{22}-, and $C_{24:0}$-Cer species that are generated under the influence of the OxPL. Fumonisin B1, a specific inhibitor of CerS, suppressed OxPL-induced ceramide generation, demonstrating that OxPL-induced toxicity in macrophages is associated with the accumulation of ceramide via stimulation of CerS activity. OxLDL elicits the same cellular ceramide effects. Thus, it is concluded that PGPC and POVPC are active components that contribute to the toxic effects of this lipoprotein.

KURZFASSUNG

Oxidierte Phospholipide (OxPL) im Allgemeinen sowie ihre fragmentierten Species 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine (PGPC) und 1-palmitoyl-2-oxovaleroyl-*sn*-glycero-3-phosphocholine (POVPC) im Besonderen gehören zu einer Reihe biologisch aktiver Verbidungen, die bei der Oxidation von Low-Density Lipoprotein (LDL) gebildet werden. Diese Substanzen tragen zu den proatherogenen Effekten von oxidiertem LDL (OxLDL) bei, wie Entzündungen, Stimulierung der Proliferation und Apoptose in vaskulären Zellen. OxLDL stimuliert auch die Bildung von Ceramiden . Diese Lipide sind second messenger, die eine wichtige Rolle bei der apoptotischen Signalvermittlung spielen. In der vorliegenden Studie haben wir gefunden, dass PGPC und POVPC in kultivierten Makrophagen toxisch sind und Apoptose induzieren, die mit einem Anstieg des intrazellulären Ceramidlevels einhergeht. Bei mehrstündiger Inkubation stimulieren diese OxPL in den Zellen die Aktivität von Ceramidsynthasen (CerS), die Enzyme der Ceramid *de novo* Synthese darstellen. Unter denselben Bedingungen wurde keine Aktivierung von Sphingomyelinasen beobachtet, die die Ceramidbildung aus membranständigem Sphingomyelin katalysieren. PGPC ist toxischer als POVPC. Es stimuliert auch die Ceramidbildung effizienter, die kausal mit einer Aktivierung spezifischer CerS-Isoformen zusammenhängt und zur Bildung von C_{16}-, C_{22}- und C_{24}-Cer Species führt. Vorinkubation der Zellen mit dem CerS-spezifischen Inhibitor Fumonisin B1 erniedrigte die Aktivität der beteiligten Enzyme und die Bildung der entsprechenden Ceramidspecies unter dem Einfluss von OXPL. OxLDL zeigt die gleichen zellulären Effekte wie beide OxPL. Wir schließen daraus, dass PGPC und POVPC aktive Komponenten sind, die zu den toxischen Wirkungen von OxLDL beitragen.

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CHAPTER 1

Introduction and Outline of the Study

1.1 Abbreviations

ARE	antioxidant response element
aSMase	acid sphingomyelinase
ATF	activating transcription factor
EC	endothelial cell
eIF2α	α-subunit of eukaryotic initiation factor 2
GCLM	glutamate-cystein ligase modifier
HO-1	heme oxygenase-1
KLF4	Kruppel-like transcription factor
LDL	low density lipoprotein
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MCP	monocyte chemotactic protein
NQO1	NADP(H):quinine oxidoreductase-1
NRF2	nuclearfactor-E2-related factor 2
nSMase	neutral sphingomyelinase
OxLDL	oxidized low density lipoprotein
OxPL	oxidized phospholipid
PAF	platelet activating factor

PAPC	1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine
PC	phosphotidylcholine
PE	phosphotidylethanolamine
PEIPC	1-palmitoyl-2-(5, 6-epoxyisoprostaneE2)-*sn*-3-glycero-phosphocholine
PERK	PKR-like ER-localized kinase
PGPC	1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine
POVPC	1-palmitoyl-2-oxovaleroyl-*sn*-glycero-3-phosphocholine
PS	phosphatidylserine
siRNA	small interfering RNA
SM MHC	smooth muscle myosin heavy chain
SM α-actin	smooth muscle α-actin
SMase	Sphingomyelinase
TLR	Toll like receptor
UPR	unfolded protein response
VEGF-A	vascular endothelial growth factor-A
VSMC	vascular smooth muscle cell
XBP-1	X-box binding protein-1

2. INTRODUCTION

Atherosclerosis is a chronic inflammatory disease which is initiated by the oxidation of low-density lipoproteins (LDL) in the sub-endothelial space of the arteries. In the vascular system, the primary targets of oxidizing compounds are the esterified polyunsaturated fatty acids in the phospholipid shell and the cholesterol esters of the lipoprotein core. Oxidation of LDL leads to formation of numerous biologically active oxidized phospholipids (OxPLs) that have been shown to accumulate in atherosclerotic lesions and contribute to the cytotoxicity of OxLDL towards vascular cells (1, 2). Thus, OxPLs are considered to play a significant role not only in atherogenesis but also in the entire process of atherosclerosis, from the initial fatty streak formation to thrombosis (3). Oxidative modification of polyunsaturated phospholipids in lipoproteins and cell membranes leads to generation of a plethora of lipid oxidation products. During this process, *sn-2* fatty acyl chains may fragment to yield oxidatively truncated biologically active molecules carrying methyl, hydroxyl, aldehydic or carboxylic groups (4, 5). Both enzymatic, including lipoxygenase, myeloperoxidase and NADPH oxidase, and non-enzymatic free radical-mediated reactions may be involved in the generation of OxPLs. *In vivo* considerable amounts of phospholipid oxidation products are formed by non-enzymatic reactions under oxidative stress, since the identified lipids containing chiral side chains are mostly racemic. Phosphatidylcholines (PCs) and phosphatidylethanolamines (PEs) are the most abundant species, while phosphatidylserines (PS) are less abundant (4). There is increasing evidence that phospholipid oxidation products are formed *in vivo* and accumulate in a variety of inflammatory diseases such as atherosclerosis, neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, as well as, multiple sclerosis, rheumatoid arthritis, diabetes and systemic lupus erythematosus (6, 7).

The oxidative modification of LDL is well characterized as a risk factor in the initiation and progression of atherosclerosis and most attention has been focused on atherosclerosis in terms of identifying specific OxPLs, as this disease involves both dyslipidemia and chronic inflammation (8, 9). Various OxPLs have been found in atherosclerotic lesions and plaques. Specific bioactive OxPLs that are formed from PUFAs at the *sn-2* position of glycerol have been shown to modulate various cellular functions of vascular cells including smooth muscle cells (SMCs), endothelial cells and macrophages (10). One of the earliest reports showed that the major bioactive lipids in mm-LDL were derived from oxidation of arachidonoyl and linoleoyl phospholipids in the aorta of rabbits fed on a high-fat diet. The major fractions of OxPLs present in oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (Ox-PAPC) are identified as 1-palmitoyl-2-oxovaleroyl-*sn*-glycero-3-phosphocholine (POVPC), 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine (PGPC) (Fig. 1), and 1-palmitoyl-2-(5,6-epoxyisoprostane E2)-*sn*-glycero-3-phosphocholine (PEIPC) (11, 12). The respective oxidation products of palmitoyl- and linoloyl-PC namely Poxno-PC and Paze-PC contain longer truncated acyl chains at *sn-2* position. The levels of these lipids are increased in atherosclerotic lesions and have been shown to differentially regulate many genes in the vascular cells. The respective activities of the individual OxPL species are mostly influenced by the chemical nature of their truncated *sn*-2 chains and to a lesser extent by their head groups (2, 13, 14).

2.1. Patho-physiological actions of oxidized phospholipids

OxPLs have been shown to modulate the behavior of various cell types *in vivo* and *in vitro* (4, 7). They have been identified as essential molecular components that are responsible for the pathophysiological actions of OxLDL in the vascular system.

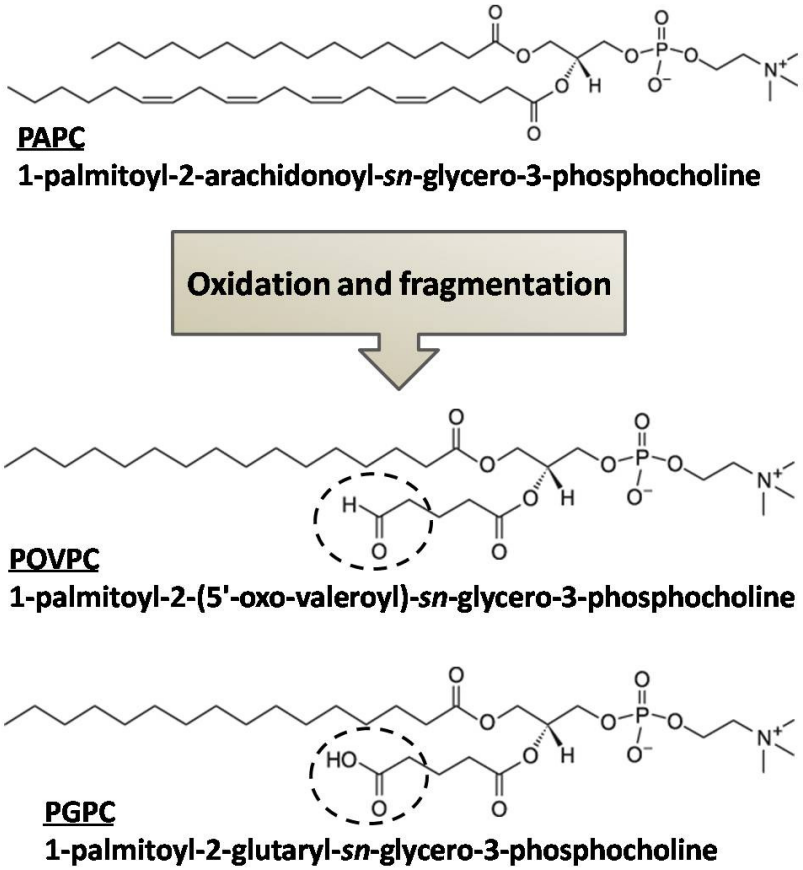

FIGURE 1. Major forms of oxidatively modified lipids identified in OxPAPC. Oxidation and successive fragmentation of *sn*-2 chain of PAPC leads to the generation of structurally very similar truncated OxPLs namely POVPC and PGPC. Dotted circle represents the functional group at *sn*-2 position.

Evidence is available from many studies that OxPAPC contains a mixture of OxPLs that mimics the activity of OxLDL by inducing a plethora of biological effects. During early atherogenesis, OxPLs have been shown to shift the phenotype of vascular smooth muscle cells (VSMCs) to an inflammatory state *in vivo*. OxPAPC and its oxidatively fragmented component POVPC reduced protein expression of smooth muscle α-actin (SM α-actin) and smooth muscle myosin heavy chain (SM MHC), while simultaneously elevating expression of monocyte chemotactic proteins (MCP-1, MCP-3) mediated through Kruppel-like transcription factor 4 (KLF4) (15). In addition, POVPC and PGPC can induce VSMC

migration and type VIII collagen expression contributing to the pathogenesis of atherosclerosis (16, 17). OxPLs have been shown to play a critical role in the progression of atherosclerotic lesions by regulating connexin expression and function in endothelial cells and SMCs, the latter effect being responsible for SMC interaction with the endothelium in the fibrous plaque (18). OxPLs were shown to serve as ligands for various receptors including scavenger receptor-CD36, TLRs, CD14, PAF-receptor, pattern recognition receptors, and LPS binding protein in vascular cells. They can activate macrophages to take up apoptotic cells that are enriched in OxPLs on their surface and stimulate signaling pathways in these cells leading to foam cell formation (19–21).

Increasing evidence shows that OxPLs are likely to play an active role in modulation of various cellular stress response pathways. OxPLs activate the antioxidant response through transcriptional induction of genes containing the antioxidant response element (ARE) in their promoters. Under oxidative stress, transcription factor nuclearfactor-E2-related factor 2 (NRF2) translocates to the nucleus, and binds to the ARE thereby inducing antioxidant genes expression. One of the earliest reports showed that expression of heme oxygenase-1 (HO-1) was regulated by OxPAPC (22). Later on, silencing of NRF2 expression by siRNA reduced HO-1 expression as well as the expression of glutamate-cysteine ligase modifier (GCLM) and NAD(P)H:quinine oxidoreductase-1 (NQO1) in OxPAPC-exposed endothelial cells (ECs) confirming that OxPLs evoke a general NRF2-mediated response and induce antioxidant genes (23). There are several reports indicating the role of OxPL induced unfolded protein response (UPR) in the atherogenic process and inflammation. OxPAPC and other classes of OxPLs were shown to activate all branches of UPR in ECs including cleavage and nuclear translocation of ATF6, splicing of XBP1 mediated by IRE1, and phosphorylation of eIF2α by the action of PERK (24). In addition, it has been shown that several different OxPLs having varying oxidative modifications in their sn-2 positions were

able to induce a critical proangiogenic growth factor VEGF-A and ATF3, emphasizing the prerequisite of oxidized *sn-2* residues in OxPLs (25).

Atherosclerosis is a chronic inflammatory disease which is initiated by endothelial dysfunction, penetration and oxidation of low-density lipoproteins (LDL) in the sub-endothelial space. Oxidized LDLs (OxLDLs) play a critical role in the formation and progression of atherosclerotic lesions, by triggering local inflammation, stress responses that contribute to vascular wall injury, plaque formation/rupture and subsequent atherothrombosis. At low concentrations, OxLDLs exhibit a variety of atherogenic activities including foam cell formation and fatty streak formation, cell proliferation, and inflammatory response. At higher concentrations and after long exposure times they can induce apoptotic cell death (26, 27). OxLDL-induced cell death is mediated through a complex sequence of signaling cascades that lead to the activation of both caspase-dependent and/or caspase-independent pathways (28–30). In the vascular system, the primary targets of oxidizing compounds are the esterified polyunsaturated fatty acids in the phospholipid shell and the neutral lipids in the core of the lipoprotein. Oxidation of LDL leads to formation of numerous biologically active oxidized phospholipids (OxPLs) that have been shown to accumulate in atherosclerotic lesions and have been demonstrated to contribute to the cytotoxicity of OxLDL towards vascular cells (1, 2). Thus OxPLs are considered to play a significant role not only in atherogenesis but also in the entire process of atherosclerosis, from the initial fatty streak formation to thrombosis (3).

Many studies have been carried out to elucidate the signaling pathways involved in mediating the toxic effects of OxLDL. For this purpose, mostly OxLDL have been used. These particles contain complex mixtures of both oxidized lipids and modified apo-protein. Thus a specific role of the oxidatively modified lipid and protein components in OxLDL could not be clearly identified. Progress over the past years has led to the recognition that

the biological activity of OxLDL is largely due to the presence of OxPLs and other lipid oxidation products. This assumption was supported by the fact that the deleterious effects of OxLDL could be abolished by enzymes with phospholipid hydrolytic activity (31). Several reports have shown that OxPLs from OxLDL induce modifications of protein structure, elicit ROS generation and alter the regulation of various stress response signaling pathways and gene expression. This necessitated many researchers to look into the biological role of OxPLs. By examining the differences or similarities between the biological activities of OxLDL and chemically defined OxPLs, it may be possible to make a good guess of the active component from a complex mixture in the OxLDL.

2.2. Cytotoxic effects of OxLDL and OxPLs: plausible roles of ceramide

Ceramide (Cer) is a sphingolipid second messenger that is involved in the regulation of various cellular stress response pathways. To date, several phenomena of cellular stress response have been described to induce Cer accumulation followed by cell proliferation, differentiation or apoptosis (32). Under the influence of various external stress stimuli Cer is generated through activation of different pathways. In the sphingomyelin-ceramide pathway, sphingomyelin is hydrolysed by sphingomyelinase (SMase) (33, 34). In the *de novo* pathway Cer synthesis starts from serine and palmitoyl CoA. A critical step is the acylation of sphinganine by various ceramide synthase (CerS) isoforms leading to the formation of distinct Cer species (35). Finally, in the salvage pathway, Cer is formed by CerS-catalyzed acylation of sphingosine. Since, the role of Cer has been linked to many patho-physiological conditions; several studies focused on the role of Cer in regulating the cytotoxic effects induced by both OxLDL and OxPL in inflammatory diseases. This study aims to provide a summary of the current knowledge with a focus on the involvement of Cer in regulating the various biological effects of both OxLDL and OxPLs in vascular cells.

2.2.1. Physiological effects of OxLDL and the role of ceramide

OxLDL has been characterized as a complex mixture of toxic oxidation products. Thus its biological effects are diverse and involve a complex network of molecular signaling mechanisms. Several studies investigated the role of Cer in various responses of vascular cells under the influence of OxLDL. In one of the earlier studies, it has been shown that effective doses of OxLDL (100 μg of apoB/mL) induced a time-dependent degradation of sphingomyelin (SM) accompanied by a concomitant increase of cellular Cer levels (36). Furthermore, it was found that hydrolysis of SM activates MAPK and thereby triggers a mitogenic signal in vascular smooth muscle cells (VSMC), suggesting Cer as an effective mediator of OxLDL induced proliferation (37). OxLDL-induced activation of the PI-3K/Akt pathway was reported in VSMC proliferation involving the SM/ceramide pathway leading to the activation of extracellular-signal-regulated kinase 1/2 (ERK 1/2) (38). OxLDL activated both neutral SMase (nSMase) and acidic SMase (aSMase) in human blood monocytes derived macrophages (HBMC), suggesting that the effects of OxLDL are at least partially due to an increase in cellular Cer levels (39).

Other reports suggest that OxLDL also induces apoptosis in vascular cells under certain conditions. OxLDL-induced apoptotic death of human umbilical vein endothelial cells (HUVEC) was attributed to activation of aSMase in a superoxide-dependent manner followed by Cer-mediated activation of caspases. OxLDL-induced apoptosis could be abrogated by two different inhibitors of SMase, desipramine and chloropromazine, suggesting a role for Cer in OxLDL toxicity (40). Stimulation of apoptosis in human macrophages and fibroblasts is also associated with an elevation of Cer levels due to aSMase activation. This process is regulated by a positive feedback mechanism since aSMase expression is increased by the elevated Cer concentration (41). In the other study, Cer has been shown to mediate OxLDL-induced apoptosis of VSMC by activation of p38 MAPK and

JNK. These effects were abolished by aSMase inhibitor NB6, suggesting an essential and specific role for aSMase in apoptotic signaling of VSMC under the influence of OxLDL (42). Interestingly, this OxLDL effect could be mimicked by OxPL indicating that the latter compounds confer toxicity to the lipoprotein particles. Truman *et. al.* showed that OxLDL and OxLDL containing immune complexes (OxLDL-IC) stimulated lysosomal aSMase (L-aSMase). In addition OxLDL-IC but not OxLDL induced early and continuous release of active secretory aSMase (S-aSMase). The authors further demonstrated that OxLDL-IC-induced activation of aSMase is responsible for the formation and release of HSP70'B- and IL-1β-containing exosomes (43). OxLDL also exerts detrimental effects in the eyes, where it is responsible for the apoptotic death of retinal pigmented epithelial (RPE) cells that promote age-related macular degeneration (AMD). This process is associated with an increase in ceramide levels, too (44).

OxLDL has been shown to increase membrane surface ceramide levels and thus influence the generation of ceramide-rich microdomains in human monocyte-derived macrophages. OxLDL-induced ceramide enrichment of raft domains in the membrane do not only alter the biophysical property of the local environment but also influence changes in clustering patterns of proteins within these domains (45). It has been reported that OxLDL led to a significant increase in ceramide levels on the cellular surface that induced apoptosis in human umbilical vein endothelial cells (HUVEC). And α-lipoic acid exhibited cytoprotective effects against OxLDL by decreasing cellular surface ceramide content (46).

In summary, OxLDL may induce multiple effects on vascular cells, and most of these effects are likely to be mediated by an increase in the concentration of the lipid second messenger ceramide. Ceramide mediates the toxic effects of OxLDL by regulating multiple components of the signaling network.

2.2.2. Toxic effects of OxPLs and the role of Ceramide

Apart from activating various stress signaling pathways, OxPLs have been demonstrated to induce apoptotic signaling pathways in vascular cells such as VSMCs and macrophages. POVPC and PGPC, the truncated products of OxPAPC, have been reported to inhibit growth and induce apoptosis in VSMCs (47). Both OxPL species were able to activate sphingomyelinase (SMase), in particular the acid isoform (aSMase) that is known to be involved in the initial stages of apoptotic signaling and phosphorylation of MAPKs. Ceramide, a bioactive sphingolipid reaction product of aSMase, induced apoptosis through the caspase-3 signaling pathway, and inhibition of aSMase activity suppressed caspase-3 activation (42, 48). In addition, in rat oligodendrocytes, POVPC was found to activate caspase-3 and -8 by increasing ceramide levels, but in this cell type neutral SMase (nSMase), not aSMase, was activated (49). Moreover, other OxPL species have been reported to damage mitochondrial membranes leading to the release of cytochrome-c and trigger apoptosis via the intrinsic pathway (50). Recent work from our laboratory showed that POVPC, PGPC and their 1-O-alkyl analogs induce apoptotic cell death in cultured macrophages, PGPC and ether-PGPC being more toxic than their POVPC counterparts. Further results demonstrated that NB19, an aSMase inhibitor, significantly reduced the activation of aSMase by POVPC and its ether analogs as well as induction of apoptosis (51). This data clearly show that aSMase activation and ceramide production are causally related to OxPL-mediated cell death.

One of the first studies related to this subject was performed using cultured arterial smooth muscle cells (SMC). POVPC and PGPC activated aSMase which was causally involved in apoptosis of these cells. Downstream of aSMase, JNK and p38 MAPK were phosporylated and activity of caspase-3 was elevated under the influence of both OxPLs. This mechanism was inferred from the fact that inhibition of aSMase by NB6 abrogated phosphorylation of

JNK and p38 MAPK and decreased caspase-3 activity (48). Walton *et al.* showed that OxPAPC activated both acid and neutral SMases in human aortic endothelial cells (HAECs). nSMase stimulation led to the formation of distinct Cer species including $C_{16:0}$-, $C_{22:0}$- and $C_{24:0}$-Cer. OxPL also showed an inhibitory effect on lipopolysaccharide (LPS)-mediated induction of interleukin-8 (IL-8) (52) in these cells, which was due to activation of nSMase. The nSMse inhibitor, GW4869, reduced the inhibitory activity of OxPAPC on LPS induction of IL-8. In contrast, the aSMase inhibitor, desipramine was unable to reduce inhibitory effect of OxPAPC's on LPS actvity. The following mechanism was suggested for the signaling of ceramide generated by nSMase under the influence of OxPL. Ceramide was reported to alter the composition of the caveolar lipid raft, which influences assembly of the LPS receptor complex and as a consequence reduce its activity.

In neonatal rat oligodendrocytes (NRO), POVPC has been shown to increase both transcription and activity of nSMase leading to an increase in $C_{18:0}$- and $C_{24:1}$-Cer levels. The authors demonstrated that nSMase is recruited by POVPC into detergent-rich microdomains (DRMs). The nSMase inhibitor GW4689, could abrogate activation of caspase-3 and apoptosis induced by POVPC (49). Recent work from our laboratory showed that POVPC, PGPC and their 1-O-alkyl analogs induce apoptotic cell death in cultured macrophages, PGPC and ether-PGPC being more toxic than the respective POVPC counterparts. The aSMase inhibitor NB19 significantly reduced induction of apoptosis by POVPC and its ether analogs in these cells pointing to a causal role of aSMase in OxPL toxicity (51).

In summary, OxPLs are biologically active components of OxLDL that exhibit a wide range of biological properties. OxPLs can activate an array of molecular signaling mechanisms, which are similar to that of induced by OxLDL.

3. SUMMARY AND PERSPECTIVES

Oxidized LDL and its constituent OxPLs are cytotoxic to many cells thus initiating and enhancing pathological conditions. Accumulating data support the view that the biological effects of OxLDL and OxPLs are mediated by the second messenger ceramide (Fig. 2). The physiological consequences depend on the cell type, the structure of OxPLs, the enzyme responsible for ceramide formation and the specific ceramide patterns (species) that are formed. The available data on the physiological effects of OxLDL/OxPLs and the role of Cer in mediating these effects were mainly obtained *in vitro* after treatment of cells with artificially oxidized LDL and/or chemically defined individual OxPL species. In this context, it has to be emphasized that composition and physical states of *ex vivo* oxidized LDL from that in physiologically oxidized particles. Nevertheless, increasing evidence suggests that effects of specific OxPL species mimic the actions of OxLDL prepared *in vitro* by various methods as well as OxPL mixtures isolated from biological sources (atherosclerotic lesions).

Nowadays, there is considerable interest in studies with pure preparations of OxPLs to confirm the causative agents and to elucidate molecular mechanisms involved in OxPL activities. As mentioned in the introduction, OxLDL contains a mixture of toxic compounds. Therefore, the physiological effects observed with pure OxPLs should be combined with studies using more biologically relevant mixtures to provide information on the likely balance of response to human pathology. Even though it has been established that OxPLs can efficiently mimic the effects of OxLDL, it has to be clarified to what extent the structural variability of the large number of different OxPL species can modulate signaling mechanisms.

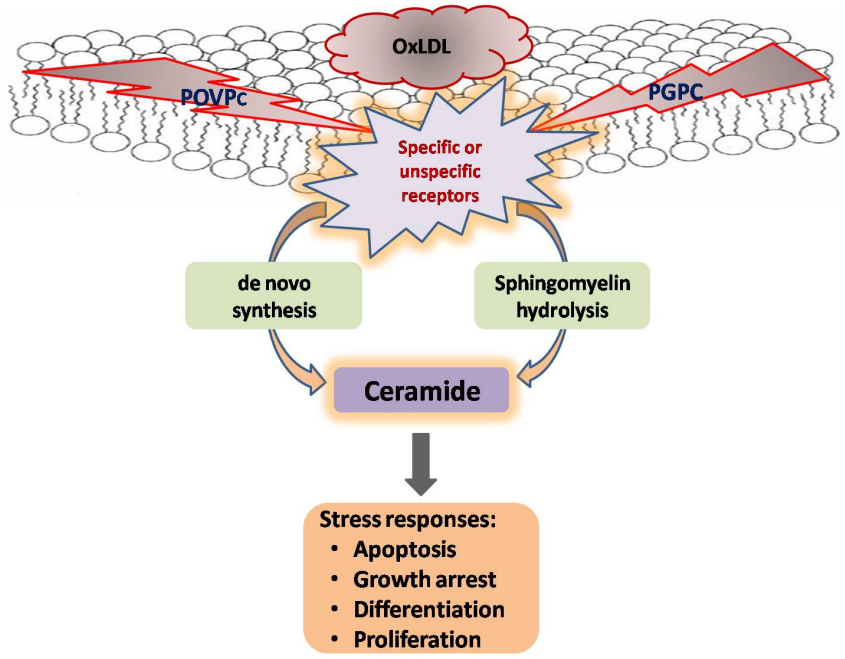

FIGURE 2. Schematic diagram showing possible mechanisms of OxLDL-, POVPC- or PGPC-induced ceramide generation and the effects mediated by ceramide.

The available evidence suggests the involvement of both SMase isoforms in mediating the effects of OxLDL and OxPL depending on the cells or tissues examined. However, another important pathway of ceramide generation i.e. *de novo* synthesis mediated by a class of six ceramide synthase (CerS) isoforms remains to be explored. Therefore, further analysis of ceramide generation and signaling mechanisms with individual and well defined mixture of OxPL species is required in order to understand the biological role of these lipid mediators influencing the effects of OxLDL/OxPLs.

In summary, accumulating data from *in vitro* experiments supports the assumption that OxLDL/OxPLs modulate cellular functions and are of relevance to human pathology. However, our current knowledge on molecular mechanisms involved in OxLDL/OxPLs-induced signaling by ceramide is just the tip of the iceberg, and that future studies with modern techniques of cell biology will improve our knowledge of OxLDL/OxPL

pathophysiology in human health and disease and perhaps unravel novel functions of these compounds.

4. OUTLINE OF THE PRESENT STUDY

A considerable amount of data points to OxPLs as markers and pathogenic factors in a variety of inflammatory diseases. Various studies identified OxPLs as the major active components of OxLDL for their ability to induce cytotoxic effects in vascular cells during the development and progression of atherosclerosis. Later investigations showed potential relevance of OxPLs to a number of other patho-physiological conditions. Oxidative modification of LDL leads to generation of various bioactive OxPLs modulating numerous physiological processes such as stress response signaling, differentiation, inflammation, migration, and apoptosis in vascular cells. These studies demonstrated that different molecular species and concentrations of OxPLs are required for the activity of particular phospholipid oxidation products. Thus, there is a considerable interest in defining molecular mechanisms underlying signal initiation and propagation, involving lipid mediators and complex enzyme networks, under the influence of specific OxPLs. To identify the contributions of the individual OxPL classes, subclasses and species, future studies should be based on well defined synthetic and labeled OxPLs. The use of metabolic or genetic manipulations, and the application of modern omics, techniques of systems biology will help to establish molecular networks of the cellular signaling responsible for the patho-physiological effects of OxPLs. The results from these studies can be the basis for the development of approaches for the diagnosis and treatment of chronic medical conditions.

The present study was motivated by the current paucity in experimental data regarding the molecular mechanisms underlying the biological activities of ceramide in response to defined OxPLs. To address these issues, the present study is focused on the cytotoxic effects of truncated OxPLs, namely POVPC and PGPC which differ only in their reactive groups at

sn-2 position. PGPC contains ω-COOH (carboxylic) in *sn-2* chain. POVOC contains ω-CHO (aldehyde) which is chemically reactive and can form covalent Schiff bases with other biomolecules. These compounds elicit a fast ceramide response due to activation of aSMase. It is not known whether this mechanism or other (*de novo*) pathways generate ceramide under conditions of longer exposure to these OxPLs. Thus, we studied the response of *de novo* ceramide synthesis to OxPLs with emphasis on ceramide synthase isoforms that are responsible for the synthesis of distinct ceramide species with specific biological activities.

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CHAPTER 2

Oxidized Phospholipids induce Activation of Ceramide Synthases in Murine Macrophages

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1.1 Abbreviations

aSMase	acid sphingomyelinase
Cer	ceramide
CerS	ceramide synthase
FB1	fumonisin B1
LC-MS/MS	liquid chromatography tandem mass spectrometry
LDL	low density lipoprotein
mmLDL	minimally modified low density lipoprotein
nSMase	neutral sphingomyelinase
OxLDL	oxidized low density lipoprotein
OxPL	oxidized phospholipid
PGPC	1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine
POVPC	1-palmitoyl-2-oxovaleroyl-*sn*-glycero-3-phosphocholine
SMase	sphingomyelinase

1.2 ABSTRACT

Oxidized phospholipids (OxPLs), including 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine (PGPC) and 1-palmitoyl-2-oxovaleroyl-*sn*-glycero-3-phosphocholine (POVPC) are among several biologically active derivatives that are generated during oxidation of low-density lipoproteins (LDLs). These OxPLs are contributing factors in pro-atherogenic effects of oxidized LDLs (OxLDLs), including inflammation, proliferation and cell death in vascular cells. OxLDL also elicits formation of of the lipid messenger ceramide (Cer) which plays a pivotal role in apoptotic signaling pathways. Here we report that both PGPC and POVPC are cytotoxic to cultured macrophages and induce apoptotic cell death which is also associated with increased cellular ceramide levels. Exposure of RAW 264.7 cells to POVPC and PGPC for several hours resulted in a significant increase in ceramide synthase activity. Under the same experimental conditions, acid or neutral sphingomyelinase activities were not affected. PGPC is more toxic than POVPC and a more potent inducer of ceramide generation by activating a limited subset of CerS isoforms. The stimulated CerS activities are in line with the C_{16}-, C_{22}-, and $C_{24:0}$-Cer species that are generated under the influence of the OxPL. Fumonisin B1, a specific inhibitor of CerS, suppressed OxPL-induced ceramide generation, demonstrating that OxPL-induced toxicity in macrophages is associated with the accumulation of ceramide via stimulation of CerS activity. OxLDL elicits the same cellular ceramide effects. Thus, it is concluded that PGPC and POVPC are active components that contribute to the toxic effects of this lipoprotein.

2. INTRODUCTION

Macrophage apoptosis is a prominent feature of advanced plaques. Previous studies have identified apoptotic macrophages in animal and human atherosclerotic lesions, along with other vascular cells (1, 2). There is a strong correlation between apoptotic macrophages and acute vascular events including plaque rupture, indicating macrophages as the key determinants of plaque instability (3). However, the significance and the factors influencing macrophage apoptosis in atherosclerosis remain poorly understood. Oxidative modification of low-density lipoprotein (LDL) is an initiating process during atherosclerosis and thought to play a critical role in oxidant injury leading to local inflammation and apoptotic events in the vascular cells (4–7). LDL is readily oxidized at the protein and lipid moieties leading to the generation of oxidized LDL (OxLDL), which is characterized by complex mixtures of toxic oxidation products. The cytotoxicity of OxLDL has been largely attributed to the modified lipid fractions including oxidized phospholipids (OxPL) and oxysterols. OxPLs, including 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine (PGPC) and 1-palmitoyl-2-oxovaleroyl-*sn*-glycero-3-phosphocholine (POVPC) are present in OxLDL and atherosclerotic lesions and are contributing factors mediating the detrimental effects of OxLDL (8). There are many studies explaining the mechanism of OxLDL cytotoxicity, however, the contribution of the OxPLs remain unclear. They are often associated with various stress response mechanisms including inflammatory conditions, oxidative stress, and apoptosis. Although the importance of OxPLs is increasingly established, understanding of the initial interaction with the cells and signaling mechanisms is limited.

The sphingolipid ceramide (Cer) is a bioactive lipid mediator regulating many cellular stress response pathways under the influence of various external stimuli (9). Various cellular and environmental stresses such as chemotherapeutics (10), ischemia-reperfusion (11), ultraviolet radiation (12) and ionizing radiation (13) can induce ceramide generation either

by enzyme-catalyzed hydrolysis of complex sphingolipids, sphingomyelin, by sphingomyelinase (SMase) or by the acylation of the long chain sphingoid bases sphinganine and sphingosine by ceramide synthase (CerS) enzymes via *de novo* or salvage pathways, respectively. Recently identified six CerS isoforms (Fig. 1) regulate *de novo* generation of distinct ceramide species, each using a restricted set of fatty acyl-CoA substrates of specific chain length (14–18). Increasing evidence suggests that different CerS isoforms are activated under the influence of various stress stimuli in a cell-specific manner. There is also a report suggesting an interplay among CerS isoforms and the balance between the generated Cer species in deciding the fate of a cell under particular stress stimuli (19, 20).

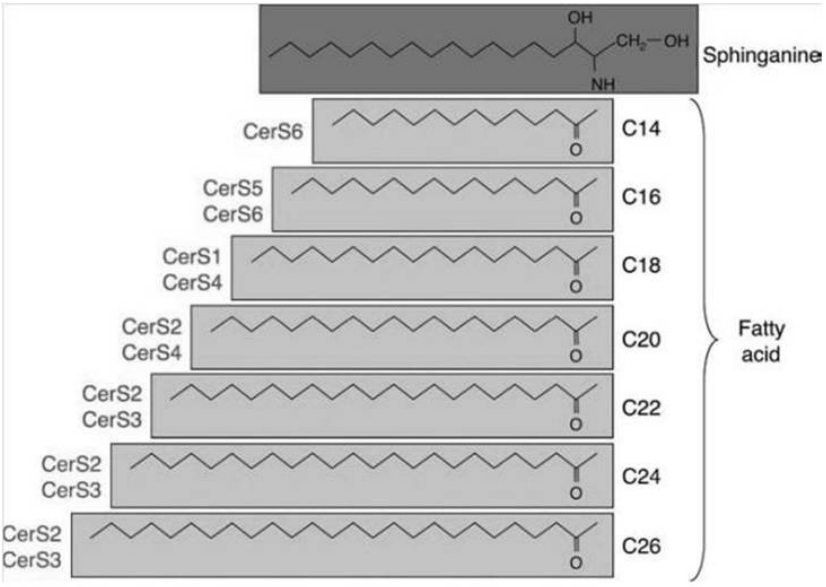

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FIGURE 1. Ceramide synthase isoforms and specificity toward the acyl CoA chain lengths for *N*-acylation of sphingoid long chain base. Sphinganine is shown in dark, the acyl chain in gray color.

It has been demonstrated that POVPC and PGPC can activate caspase-3 and 8 leading to apoptotic cell death by increasing cellular ceramide levels by activating SMase (21, 22). Another study showed that OxPAPC induced activation of nSMase/ceramide leads to the

inhibition of LPS action in human aortic endothelial cells (HAEC) (23). Perhaps surprisingly, given the pivotal roles of ceramide signaling in various stress response pathways, information is lacking on CerS-mediated Cer generation and signaling under the influence of OxPLs.

Here we report that oxidized phospholipids (OxPLs) including 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphocholine (POVPC) and 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphochloine (PGPC) stimulate *de novo* synthesis of ceramide to influence RAW 264.7 macrophage cell apoptosis by activating a limited subset of CerS isoforms. We found that PGPC is a more toxic and a potent inducer of ceramide generation by specifically enhancing the activities of CerS2 and CerS5/6 in a time-dependent manner. Concomitantly, PGPC increased ceramide levels significantly, including long chain C_{16}-, C_{22}-, and $C_{24:0}$-Cer. In contrast, POVPC treatment influenced the ceramide levels to a lesser extent. These results provide evidence for the differential regulation of CerS isoforms by OxPLs. Our data contribute to the growing body of knowledge elucidating the novel roles of specific ceramide species and CerS proteins in stress stimuli and cell type specific manner.

3 EXPERIMENTAL PROCEDURES

3.1 Reagents

Cell culture materials were obtained from Sarstedt (Numberecht, Germany) or Greiner (Kremsmunster, Austria). Dulbecco's modified Eagle's medium and heat-inactivated fetal bovine serum were from Invitrogen (Leek, The Netherlands). PBS and other cell culture supplements were obtained from PAA (Linz, Austria), unless otherwise indicated. Oxidized phospholipids (POVPC and PGPC) were synthesized in our laboratory or purchased from Avanti Polar Lipids (Alabaster, USA). Fumonisin B1 was from Calbiochem (Darmstadt, Germany).

3.2 Cell culture

The murine macrophage-like cell line RAW 264.7 (ATCC No. TIB-71, American Type Culture collection, Rockville, MD, USA) was a kind gift from Prof. Dagmar Kratky, Medical University of Graz, Austria. Cells were maintained in DMEM (4.5 g/l glucose, 25 mM HEPES, 4 mM L-glutamine, without sodium pyruvate) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 IU/mL), and streptomycin (100 µg/mL) at 37 °C in a humidified atmosphere of 5% CO_2. Incubations with OxPLs were conducted with DMEM with or without phenol red supplemented with 0.1% FCS. Incubation mixtures were prepared by adding ethanol stock solutions of lipids to the culture medium. The ethanol concentration was below 1% (v/v) and control experiments were performed using only ethanol without lipid additives.

3.3 Isolation and oxidative modification of LDL

Human LDL was isolated by density ultracentrifugation in OptiSeal tubes using a Beckman NVT65 Rotor at 4 °C (24) from pooled healthy fresh plasma (a kind gift of Dr. Gholam Ali Khoschsorur, University Hospital, Graz). The LDL fraction was collected and stored at 4 °C for up to 8 days prior to use. Lipoprotein concentration is expressed by protein measured by the method of Bradford (25). LDL was desalted using PD 10 columns (GE Healthcare, Munich, Germany) prior to oxidative modification. Oxidation of LDL was performed by incubating with 1 mM $CuSO_4$ in sterile H_2O for 48 h protected from light at 37 °C. OxLDL was desalted prior to use.

3.4 Cell viability assay

Cells were treated with OxPLs in 96 well plates and cellular viability was analyzed by the Vybrant® MTT cell proliferation Assay kit according to the manufacturer's protocol. Briefly, after removal of the medium, 100 µL of MTT (5 mg/10 mL of PBS) was added to each well

and incubated at 37 ºC for 4 h The MTT solution was removed, and 50 µL of dimethyl sulfoxide (DMSO) was added. The color intensity of the soluble formazan was measured at 570 nm after 10 minutes.

3.5 Flow cytometric apoptosis assay

Cells were incubated for 4 h with OxPLs, washed twice in cold PBS and stained for surface phosphatidylserine with Alexa Fluor®488 fluorescent annexin V and propidium iodide. Samples were then analyzed using a FACSCalibur flow cytometer (BD Bioscience, Heidelberg, Germany).

3.6 RNA isolation and cDNA synthesis

Total RNA was isolated using RNeasy® mini kit (Qiagen) according to the manufacturer's protocol. The concentration and quality of RNA samples were evaluated spectrophotometrically. Complementary DNA was synthesized from 1 µg of the total RNA using Verso cDNA kit (Thermo Scientific).

3.7 Real-time quantitative PCR (RT-qPCR) analysis of CerS expression

RT-qPCR reactions were performed using TaqMan® Gene Expression assays and Universal PCR Master Mix using a 7300 Sequence Detection System (Applied Biosystems). Quantitative analysis was performed using a standard curve generated by the serial dilutions of cDNA for each CerS gene. The relative quantitative mRNA level was determined using the comparative *Ct* method using Hprt and Gusb as the reference genes. Primer sequences were as follows (Applied Biosystems): [Mm00433562_m1 (CerS1), Mm00504086_m1 (CerS2), Mm03990709_m1 (CerS3), Mm01212479_m1 (CerS4), Mm00510991_m1 (CerS5), Mm00556165_m1 (CerS6), Mm01545399_m1 (Hprt), Mm00446953_m1 (Gusb)]. PCR reaction conditions included initial denaturation at 95 °C for

10 min, followed by 40 cycles of 15 s at 95 °C, 30 s at 56 °C and 30 s at 68 °C. The data were normalized to an internal control gene, GAPDH.

3.8 In vitro ceramide synthase assay

Cells were harvested and homogenized in HEPES buffer [20 mM HEPES-KOH (pH 7.2), 25 mM KCl, 2mM $MgCl_2$, and 250 mM sucrose] containing a protease inhibitor cocktail-AEBSF, 104 mM; Aprotinin, 80 μM; Bestatin, 4 mM; E-64, 1.4 mM; Leupeptin, 2 mM and Pepstatin A, 1.5 mM (SIGMA-ALDRICH). Protein concentrations were measured using the Bradford method (Bio-Rad). CerS activity was assayed as described previously (14). Briefly, cell homogenates were incubated in a final volume of 250 μL HEPES buffer with 0.25 μCi of [4,5-^{3}H]sphinganine/15 μM sphinganine/20 μM defatted-bovine serum albumin and 50 μM of different fatty acyl-CoAs in accordance with the substrate specificity of each CerS at 37 °C for 20 min. Reactions were terminated by addition of three volumes of chloroform/methanol (1/2; v/v). Lipids were extracted (26) and separated by thin layer chromatography (TLC) plate using chloroform/methanol/2M ammonium hydroxide (40/10/1; v/v/v) as solvent. [^{3}H] Lipids were visualized using a phosphor imaging screen (Fuji, Tokyo, Japan), recovered from TLC plates by scraping the silica directly into scintillation vials, and quantified by liquid scintillation counting.

3.9 Sphingomyelinase assay

SMase activity was measured as previously described (27) with some modifications. Briefly, cell homogenates containing 50 μg protein were incubated in a final volume of 500 μL Tris-KCl buffer (25 mM KCl, 50 mM Tris pH 7.4 and 5 mM $MgCl_2$) for the neutral sphingomyelinase assay or 500 μL sodium acetate buffer (50 mM sodium acetate pH 4.5) for the acid sphingomyelinase assay. The reactions were started by addition of 1 nmol C_6-NBD-SM (ethanol stock mixed in the buffer) and incubated in the dark at 37 ºC for 20 minutes (nSMase) or 15 minutes (aSMase). Reactions were terminated by the addition of three

volumes of chloroform:methanol (1:2; v/v). Lipids were extracted as mentioned earlier and separated by TLC using chloroform:methanol:9.8 mM aqueous $CaCl_2$ (60:35:8; v/v/v). NBD-labeled sphingolipids were identified using authentic standards by Fluor-S Max device and quantified using Image Quant program.

3.10 Lipid extraction and LC/MS-MS analysis of sphingolipids

After exposure to OxPL as described above, cells were harvested with cold phosphate buffered saline. The pellet was resuspended in a mixture of 100 μL distilled water and 750 μL chloroform: methanol (1:2; v/v) along with internal standards. Samples were sonicated and incubated over-night at 48 °C. Alkaline hydrolysis was carried out by adding 75 μL 1 M KOH in methanol, sonicated and incubated for 2 h at 37 °C. Samples were then neutralized by adding 3 μL glacial acetic acid and lipids were partitioned in 3 mL chloroform:water (1:2; v/v). The upper aqueous layer was separated and the solvent was removed from the lower organic layer under N_2. Lipid extracts were analyzed for ceramide species by LC/MS-MS (28). Data are expressed as pmole of ceramide per mg of total protein.

4 RESULTS

4.1 POVPC and PGPC are cytotoxic and induce apoptosis in murine macrophages

To determine the effect of oxidized phospholipids, RAW 264.7 cells were treated with POVPC or PGPC and cell viability was measured using the photometric MTT assay. Both POVPC and PGPC reduced optical density of the marker dye in the MTT reagent to a significant level compared to untreated control cells. Fig. 2 illustrates reduced viability of macrophage cells under the influence of both OxPLs, in a time dependent manner. PGPC is more cytotoxic to the cells compared to POVPC.

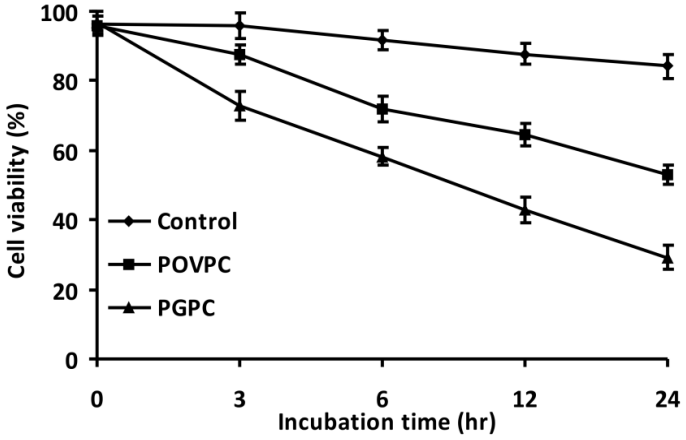

FIGURE 2. Oxidized phospholipids POVPC and PGPC are cytotoxic to cells. RAW 264.7 cells were incubated with 50 μM of POVPC and PGPC dispersions in low serum (0.1% FCS) DMEM for the indicated periods. Control cells were treated with 1% ethanol in medium. Cell viability was determined by Vybrant® MTT assay kit. Results are expressed as a percentage of viable cell number in treated cells compared to ethanol treated control cells. Data are expressed as means ± S.E. *, $p < 0.01$, $n = 8$ in each group.

Further, we treated the cells with OxPLs for 4 h and analyzed for externalization of phosphatidylserine on the outer leaflet of the plasma membrane, an early marker of apoptotic cell death. Both POVPC and PGPC increased the percent of annexin V stained cells in a concentration-dependent manner indicating the induction of apoptotic cell death (Fig. 3).

Taken together, the data suggest that both OxPLs are cytotoxic to cells, and that PGPC is a more potent inducer of RAW 264.7 cell death compared to POVPC.

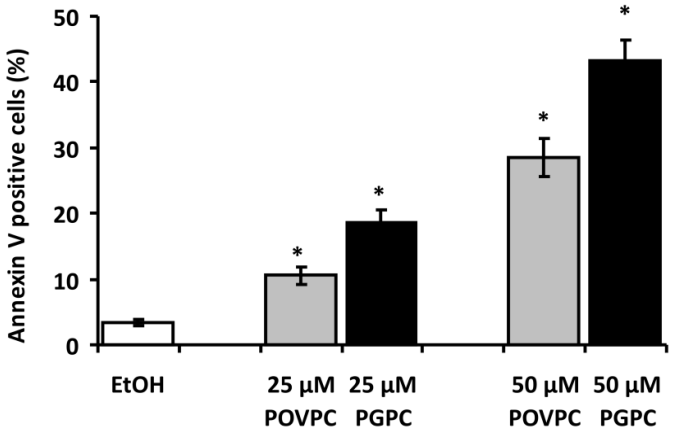

FIGURE 3. Oxidized Phospholipids POVPC and PGPC induce apoptotic cell death. RAW 264.7 cells were incubated with the indicated concentrations of POVPC and PGPC for 4 h. The cells were analyzed for fluorescent Alexa $Fluor_{488}$-annexin V staining by Flow cytometry as described under "Experimental Procedures". Results are expressed as means ± S.E. *, $p < 0.001$, $n = 8$ in each group.

4.2 CerS expression and CerS activity in cultured murine macrophages

Ceramide is a bioactive sphingolipid and an intracellular signaling molecule that plays a pivotal role in regulating various cellular signaling pathways including apoptosis. Earlier studies suggested that six CerS isoforms are differentially expressed in different tissues (16, 29). We assessed mRNA expression of CerS isoforms in cultured RAW 264.7 cells. RT-qPCR analysis revealed that CerS2 was the predominant isoform while CerS4, CerS5 and CerS6 were expressed at significant levels (Fig. 4). In contrast, CerS1 and CerS3 were virtually undetectable. These results demonstrate that CerS2 is the predominant CerS in RAW 264.7 macrophages.

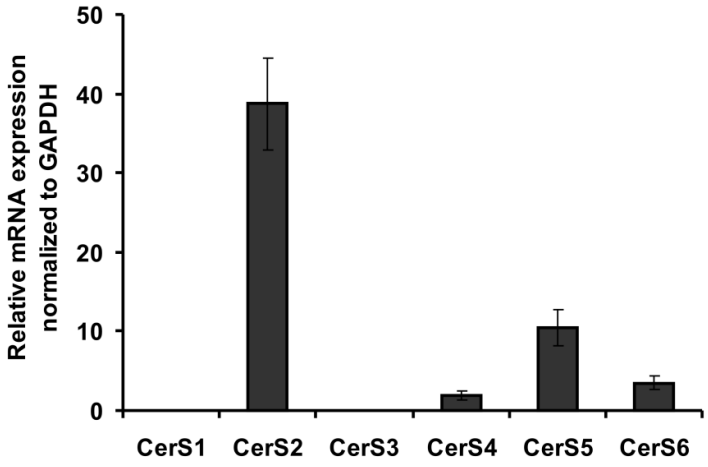

FIGURE 4. CerS expression in cultured macrophage cells. RAW 264.7 cells were harvested and cDNA was synthesized. CerS mRNA levels were measured by RT-qPCR as described under "Experimental Procedures". The data were normalized to GAPDH mRNA expression and expressed as means ± S.E. from three independent experiments performed in triplicate.

Each CerS utilizes a restricted subset of fatty acyl-CoAs to synthesize ceramides with defined acyl chains. We, therefore, next examined CerS activity in cell homogenates using a range of acyl-CoAs as enzyme substrates. Optimal assay conditions were determined by measuring the CerS activity as a function of various protein concentrations and time of the reaction (Fig. 5).

Reaction linearity was determined from product formation at various protein concentrations after 20 minutes reaction time using different fatty acyl CoA substrates (Fig. 6). Analysis of the reaction conditions and the linearity ranges of the reactions showed that 100 μg of protein was optimal for measuring the CerS activity at 37 °C for 20 minutes of reaction time.

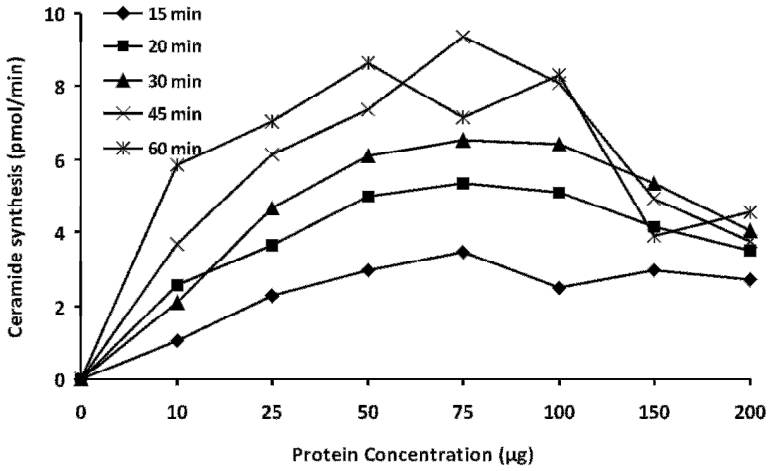

FIGURE 5. CerS activity in RAW 264.7 cell homogenates. Assays were performed using increasing amounts of protein at various reaction times. ^{3}H-sphinganine and C_{22}-CoA were used as the substrates as described under "Experimental Procedures". The auto radiogram was developed by a phosphorimager. The amounts of ceramide were quantified after scraping the ceramide bands using scintillation counter. Ceramide formed (pmol) was plotted versus protein amount (µg) used for the reaction.

Under optimal reaction conditions quantification of the reaction product (dihydro) ceramide was entirely consistent with the mRNA expression levels. $C_{22:0}$-Cer, the product of CerS2, was found to be much more efficiently synthesized than $C_{16:0}$-Cer and $C_{20:0}$-Cer (Fig. 7, 8). However, we could not detect very long-chain products ($C_{26:0}$-Cer) synthesized by CerS3 enzyme. The data suggest that CerS2 is highly expressed and likely responsible for the generation of significant levels of ceramide in RAW 264.7 cells.

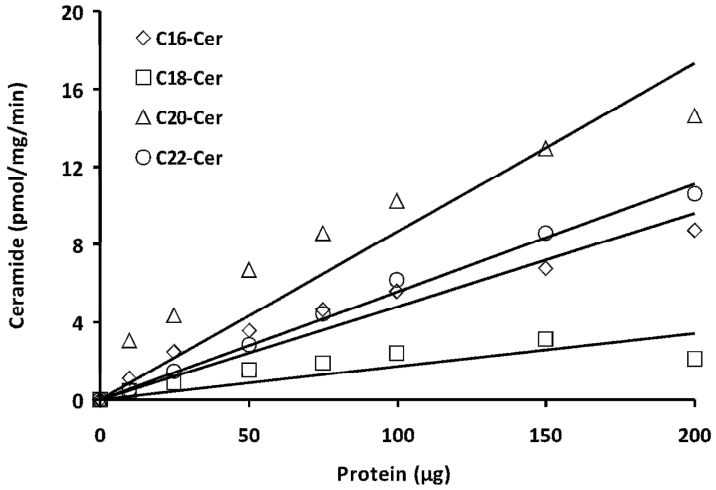

FIGURE 6. CerS activity as a function of protein concentration. Assays were performed using increasing amount of protein at 37 °C for 20 minutes. ^{3}H-sphinganine and C_{16}-CoA, C_{18}-CoA, C_{20}-CoA or C_{22}-CoAs were used as substrates. Ceramide was quantified as described earlier and CerS activities (pmol/mg/min) were plotted versus protein amount (µg).

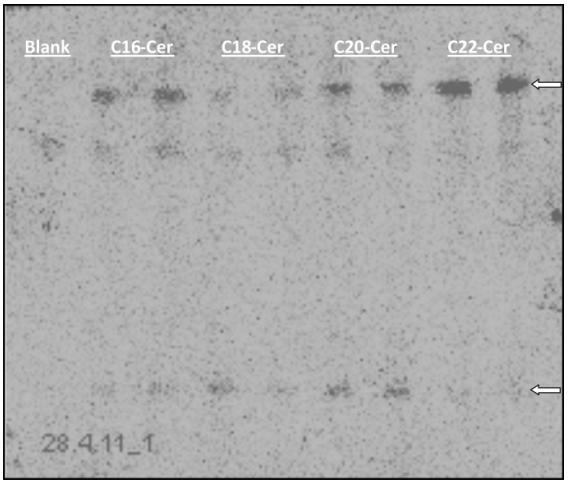

FIGURE 7. Endogenous CerS activity in RAW 264.7 cells. CerS activity was assayed in cell homogenates using C_{16}-CoA, C_{18}-CoA, C_{20}-CoA or C_{22}-CoAs and ^{3}H-sphinganine substrates as described under "Experimental Procedures". The auto radiogram was screened by a phosphorimager.

The amounts of pmol ceramide (bands shown by upper arrow) were quantified after scraping the ceramide bands using scintillation counter.

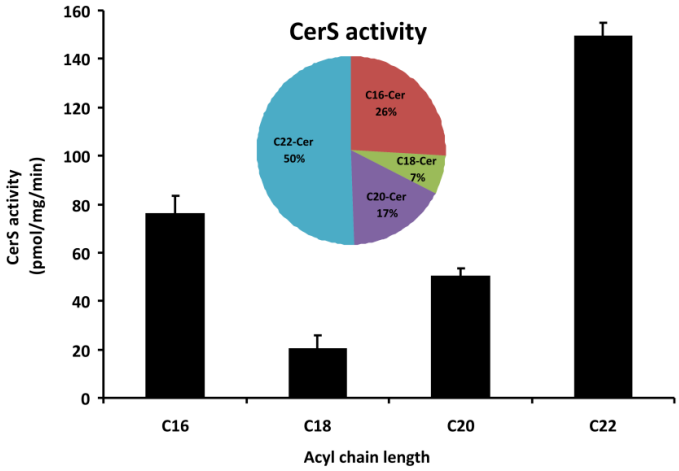

FIGURE 8. Endogenous CerS activity in RAW 264.7 cells. CerS activity was assayed in cell homogenates using C_{16}-CoA, C_{18}-CoA, C_{20}-CoA or C_{22}-CoA and ^{3}H-sphinganine substrates as described earlier. Results are expressed as means ± S.D. from four independent experiments.

4.3 OxPLs lead to changes in ceramide levels by activating CerS in macrophage cells

Previous studies demonstrate elevation of intracellular ceramide levels in arterial smooth muscle cells (VSMC) under the influence of OxPLs leading to apoptotic death (22). Therefore, we determined ceramide concentrations in untreated and OxPL-treated macrophages. Ceramide analysis by LC/MS/MS revealed a significant increase in total ceramide concentration in both POVPC- and PGPC- treated cells compared to untreated control cells (Fig. 9).

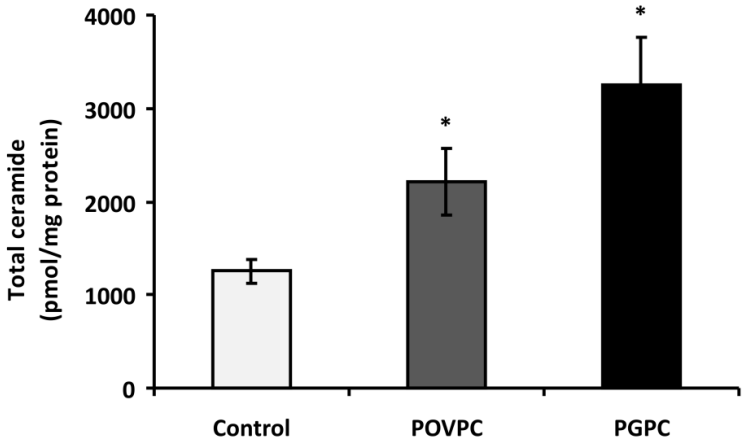

FIGURE 9. Influence of POVPC and PGPC on total ceramide levels in RAW 264.7 cells. Both POVPC and PGPC elevate the ceramide content in RAW 264.7 cells. Cells were stimulated with the respective OxPLs (50 μM) or with medium containing 1% ethanol as control for 24 h. Lipids were extracted and analyzed for ceramide levels by LC/MS-MS as described under "Experimental Procedures". The data are expressed as means ± S.E., n = 4.

Further analysis of ceramide species showed a significant increase in $C_{16:0}$, $C_{22:0}$ and $C_{24:0}$-Cer (Fig. 10) in OxPL treated cells compared to untreated cells. In contrast, we only saw a small elevation in C_{18}-Cer and C_{20}-Cer but, the changes were not statistically significant. In summary, this data indicates a selective effect of OxPLs on levels of C_{16}- and $C_{22:0}/C_{24}$-ceramides.

To determine which pathway, namely activation of ceramide synthase or hydrolysis of sphingomyelin via sphingomyelinase, is involved in OxPL-induced ceramide generation at long exposure times, we first examined the effects of both POVPC and PGPC on CerS activity in RAW 264.7 cells.

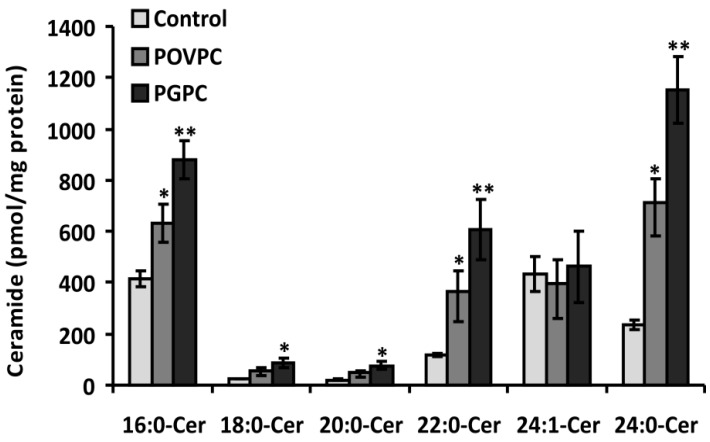

FIGURE 10. Influence of POVPC and PGPC on ceramide patterns in RAW 264.7 cells. Cells were stimulated with the respective OxPLs (50 μM) or with medium containing 1% ethanol as control for 24 h. Lipids were extracted and analyzed for ceramide levels by LC/MS-MS as described under "Experimental Procedures". Ceramide speciation was performed and data are expressed as means ± S.E. *, $p < 0.05$, **, $p < 0.01$ compared with control, n = 4.

Exposure of cells to 50 μM POVPC and 50 μM PGPC for 24 h resulted in a significant increase in CerS2 activity (45.8 ± 5.1 pmol.mg protein $^{-1}$ min^{-1} for POVPC and 57.2 ± 6.3 pmol.mg protein $^{-1}$ min^{-1} for PGPC) compared with control (26.5 ± 3.9 pmol.mg protein $^{-1}$ min^{-1}). CerS5/6 activities (33.6 ± 3.5 pmol.mg protein $^{-1}$ min^{-1} for POVPC and 38.7 ± 4.0 pmol.mg protein $^{-1}$ min^{-1} for PGPC) were also higher compared to control (22.4 ± 4.7 pmol.mg protein $^{-1}$ min^{-1}) as shown in Fig. 11.

We then measured the activity of sphingomyelinase in RAW 264.7 cells subjected to 24 h OxPL exposure. In contrast to activation of CerS, both POVPC and PGPC did not result in any significant change in neutral sphingomyelinase (nSMase) (Fig. 12) and acid sphingomyelinase (aSMase) activity (data not shown). Taken together, the data suggest that OxPL-induced ceramide generation in RAW 264.7 cells is likely to be due to the activation of distinct CerS isoforms. C_{16}-Cer, has been to shown to be the product of both CerS5 and CerS6 activities, and C_{22}- and $C_{24:0}$-Cer species are synthesized by CerS2. In summary,

elevation of specific Cer species by OxPLs perfectly correlates with OxPL-mediated stimulation of the particular CerS isoforms that catalyze the formation of the respective Cer species.

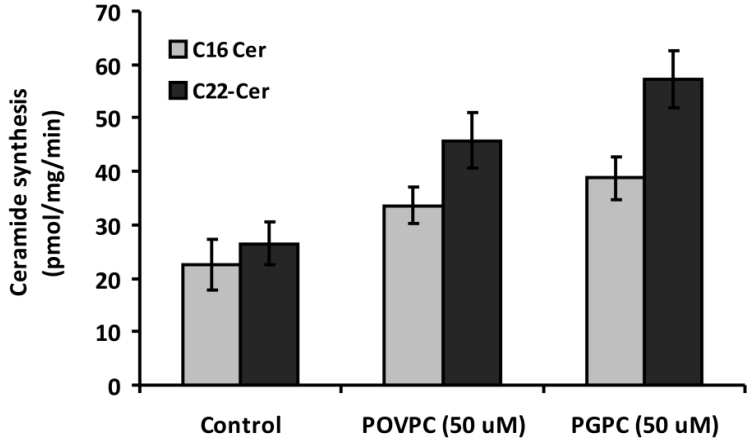

FIGURE 11. Effect of POVPC and PGPC on CerS activity. Cells were stimulated with the respective OxPLs (50 μM) or with medium containing 1% ethanol as control for 24 h. After OxPL treatment cells were harvested and CerS activity in cell homogenates was measured as described earlier. Results are expressed as means ± S.D. of four independent experiments.

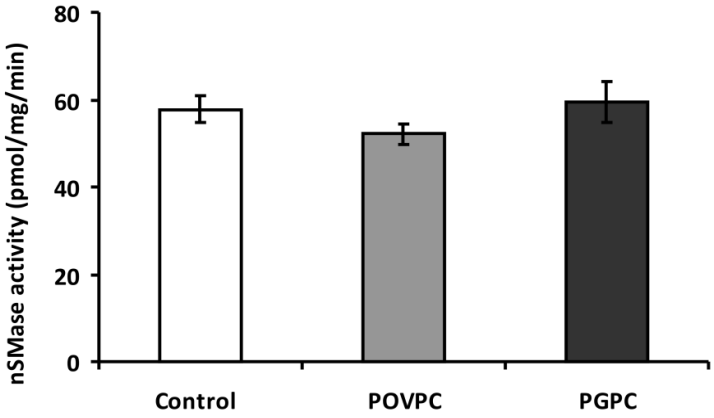

FIGURE 12. Effect of POVPC and PGPC on nSMase activity. Cells were stimulated with the respective OxPLs (50 μM) for 24 h or with medium containing 1% ethanol as control. nSMase activity

was measured in cell homogenates using C6-NBD sphingomyelin substrate as described under "Experimental Procedures". The data are expressed as means ± S. D., compared with control, n = 4.

Taken together, the data suggest that OxPL-induced ceramide generation in RAW 264.7 cells is likely to be due to the activation of LASS proteins.

4.4 Effect of OxPLs on CerS mRNA expression

To find out whether the observed increase of C_{16}-, C_{22}- and $C_{24:0}$-Cer is due to an effect on CerS gene expression, we determined mRNA levels. RAW 264.7 cells were incubated with 50 μM POVPC or PGPC for 24 h and analyzed for the changes in expression levels of CerS isoforms. PGPC treatment led to an increase of mRNA expression of CerS2 and CerS4 that are specific for the synthesis of C_{22}- and $C_{24:0}$-Cer, and C_{20}-Cer, respectively over control. POVPC also induced a similar increase in CerS4 and CerS5 mRNA levels, whereas mRNA levels of other CerS increased only slightly (Fig. 13).

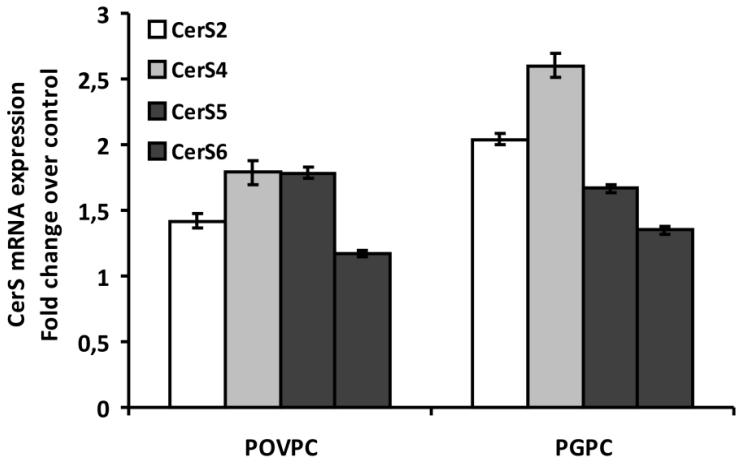

FIGURE 13. Effect of OxPLs on CerS mRNA expression in cultured macrophages. RAW 264.7 cells were harvested after treatment with OxPLs for 24 h and cDNA was prepared. CerS mRNA levels were measured by RT-qPCR as described under "Experimental Procedures". The data were

normalized relative to GAPDH mRNA expression and expressed as means ± S.E. from three independent experiments performed in triplicate.

4.5 Fumonisin B1 inhibits CerS and abrogates ceramide elevation

FB1 is a potent and specific inhibitor of the CerS. We examined the effect of FB1 on OxPL induced ceramide generation. Cells were pre-incubated with FB1 (20 μM) for 2 h and then exposed to OxPLs for 24 h. As expected, we found that ceramide content was reduced significantly in the presence of FB1 (Fig. 14A and 14B).

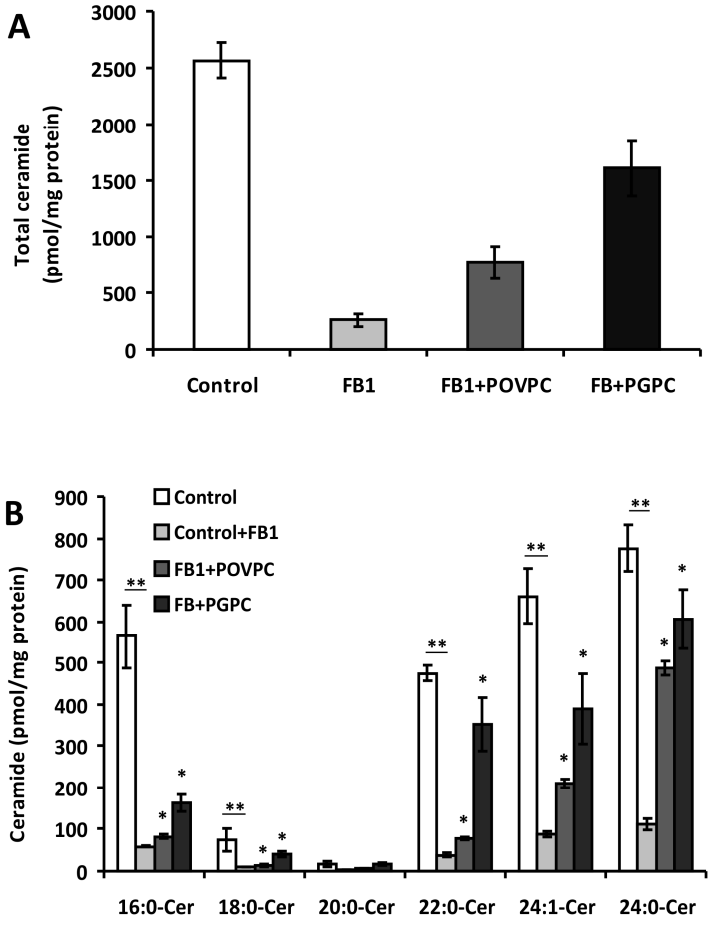

FIGURE 14. Effect of FB1 on OxPL-induced ceramide levels. A, Raw 264.7 cells were pre-incubated with FB1 (20 μM) for 2 h prior to treatment with OxPLs for 24 h. Lipids were extracted and ceramide levels were analyzed as described under "Experimental Procedures". The data are expressed as

means ± S.E., compared with control, n = 4. **B,** Ceramide species were analyzed as described in Fig. 10. The data are means ± S.E. *, $p < 0.05$, **, $p < 0.01$ compared with control, n = 4.

It is noteworthy that even though FB1 abrogated ceramide accumulation, we could observe the difference in ceramide levels under the influence of OxPLs compared to FB1 treated control cells (Fig. 15). Notably, both OxPL induced ceramide elevation and ceramide levels in the control cells were sensitive to FB1.

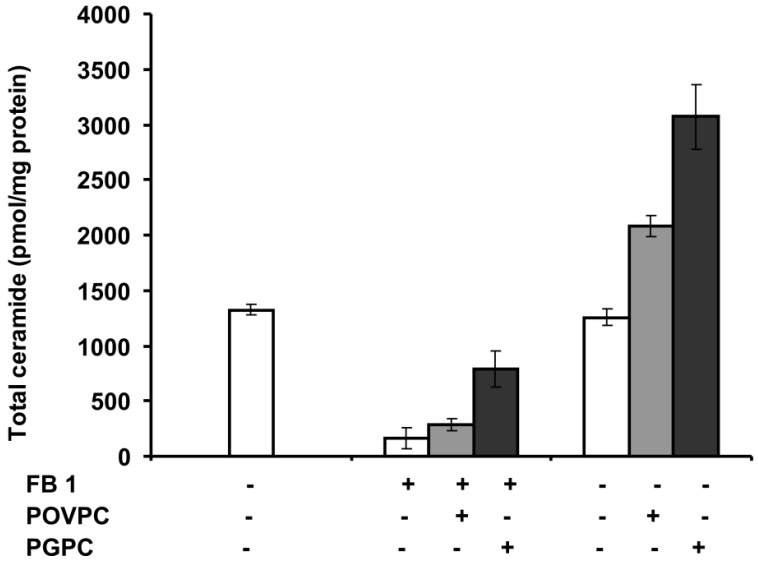

FIGURE 15. Effect of FB1 on OxPL-induced ceramide levels. Raw 264.7 cells were pre-incubated with FB1 (20 μM) for 2 h prior to treatment with OxPLs for 24 h. Lipids were extracted and ceramide levels were analyzed as described under "Experimental Procedures". The data are expressed as means ± S.E., compared with control, n = 4.

We then measured CerS activity under the influence of OxPLs with or without pretreatment with FB1. Indeed, OxPL-induced CerS activity and base-line enzyme activities were sensitive to FB1. It is noteworthy that even though FB1 abrogated CerS activity, we could still observe the difference in CerS activity under the influence of OxPLs compared to enzyme activity in only FB1 treated control cells (Fig. 16).

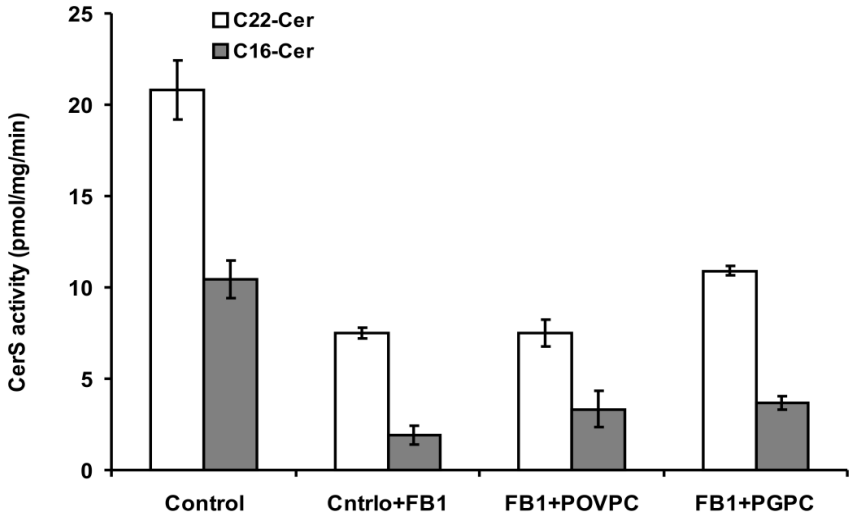

FIGURE 16. Effect of FB1 on OxPL-induced CerS activity. Raw 264.7 cells were pre-incubated with FB1 (20 μM) for 2 h prior to treatment with OxPLs for 24 h. Cells were harvested and CerS activity in cell homogenates was measured using C_{16}-CoA and C_{22}-CoA as substrates as described under "Experimental Procedures". Results are expressed as means ± S.D. from four independent experiments.

Taken together, the results of these studies suggest that OxPLs trigger activation of CerS in macrophage cells to elevate ceramide levels.

4.6 Oxidized LDL activates CerS in RAW 264.7 cells

Human LDL was isolated and oxidation was performed as described under "Experimental Procedure" and analyzed for oxidative modification by gel shift assay (Fig. 17). To determine the influence of oxidized LDL on CerS activity, RAW 264.7 cells were treated with 50 μg/mL of LDL, mmLDL or OxLDL for 24 h and CerS5/6 and CerS2 activities were measured using C_{16}- and C_{22}-CoAs as substrates respectively.

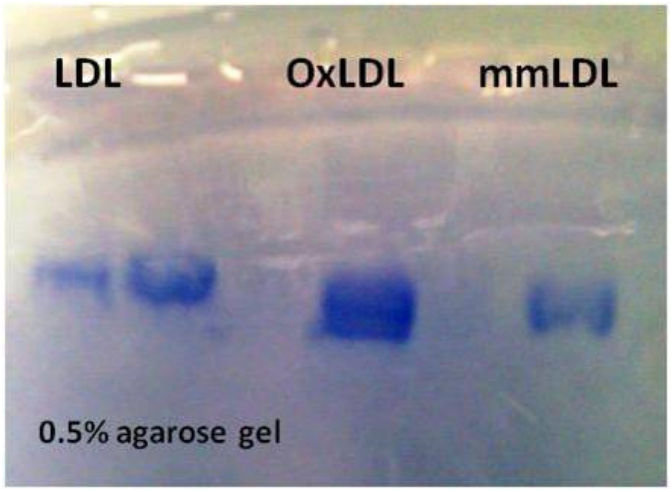

FIGURE 17. Gel shift analysis of oxidatively modified LDL. LDL was oxidized using 25 μM $CuSO_4$ (OxLDL) and 10 μM $FeSO_4$ (mmLDL) as described under "Experimental Procedures". Native and oxidized LDLs (40 μg protein) were applied onto 0.5 % agarose gel and separated by electrophoresis. Lipoprotein bands were visualized by staining with Coomassie blue.

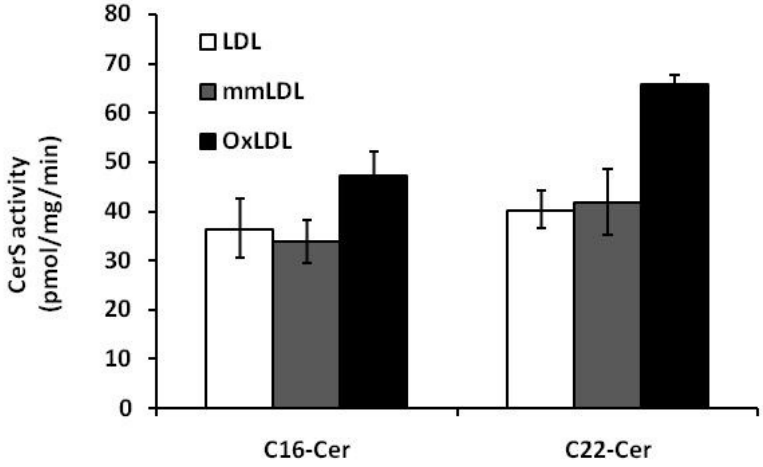

FIGURE 18. Effect of native and oxidized LDL on CerS activity. Cells were grown to confluency and stimulated with LDL, mmLDL or OxLDL (50 μg/mL) for 24 h. Cells were harvested and CerS activity was measured in cell homogenates using C_{16}-CoA (for CerS5/6) or C_{22}-CoA (for CerS2) and ^{3}H-sphinganine as substrates as described earlier. Results are expressed as means ± S.D. from four independent experiments.

Fig. 18 shows a significant increase in CerS2 activity in OxLDL-treated cells compared to LDL and mmLDL treated cells, whereas CerS5/6 activity was slightly increased compared to the cells exposed to LDL and mmLDL.

These results indicate that oxidation products present in the OxLDL are likely to be responsible for the increased CerS activity.

4.7 Lipid extracts from LDL and OxLDL elevate CerS activity in macrophage cells

It has been shown that POVPC and PGPC are present in OxLDL and are responsible for the deleterious effects of OxLDL. To demonstrate a functional role of lipid oxidation products in elevating ceramide levels, we treated macrophage cells with lipid extracts from LDL and OxLDL and examined changes in CerS activity.

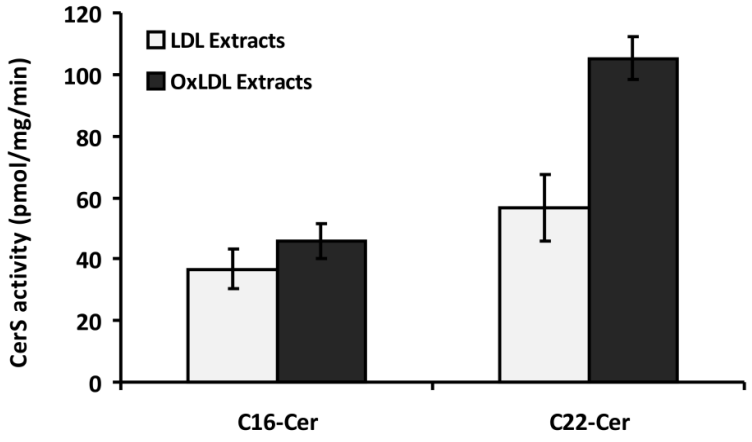

FIGURE 19. Effect of lipid extracts from LDL and OxLDL on CerS activity. Oxidation of LDL was performed as described under "Experimental Procedures". RAW 264.7 cells were stimulated with lipid extracts from native LDL and OxLDL (50 μg/mL protein respectively) for 24 h. CerS activity in cell homogenates was measured using C_{16}-CoA (for CerS5/6) and C_{22}-CoA (for CerS2) and ^{3}H-sphinganine as substrates. Results are expressed as means ± S.D. from three independent experiments.

As shown in Fig. 19, after 24 h of treatment, lipid extracts from OxLDL increased CerS2 activity to a significant level, whereas CerS5/6 activity was slightly elevated compared to the cells treated with lipid extracts from native LDL.

These results indicate that lipid oxidation products such as POVPC and PGPC present in the OxLDL are likely to be responsible for the increased activation of CerS enzymes.

We then analyzed ceramide levels in the cells treated with total lipid extracts from LDL and OxLDL. As expected, total ceramide content was elevated significantly in cells treated with the lipid extracts from OxLDL compared to the cells treated with lipid extracts from native LDL (Fig. 20).

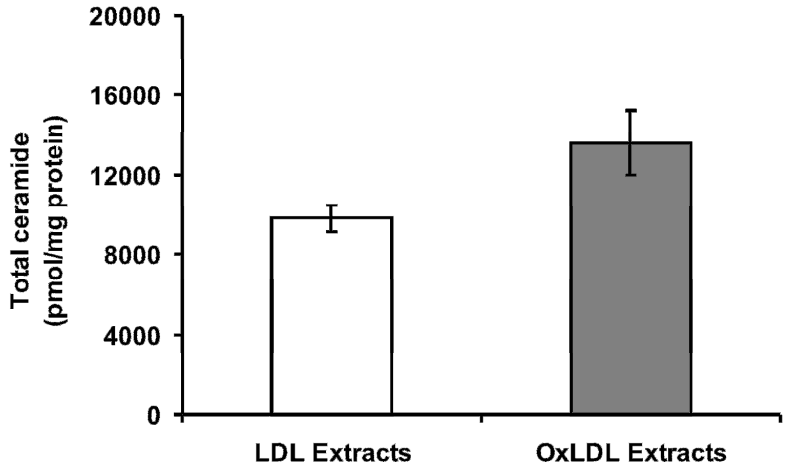

FIGURE 20. Effect of lipid extracts from LDL and OxLDL on total ceramide levels. Oxidation of LDL was performed and lipids were extracted as described under "Experimental Procedures". RAW 264.7 cells were treated with lipid extracts from native LDL and OxLDL (50 μg/mL protein, respectively) for 24 h. Lipids were extracted and analyzed for ceramide levels by LC/MS-MS as described under "Experimental Procedures". The data are expressed as means ± S.E., n = 4.

In agreement with the data shown (Fig. 19), further analysis of ceramide species revealed a significant elevation in C_{16}-, C_{22}-, $C_{24:0}$- and $C_{24:1}$-Cer levels (Fig. 21).

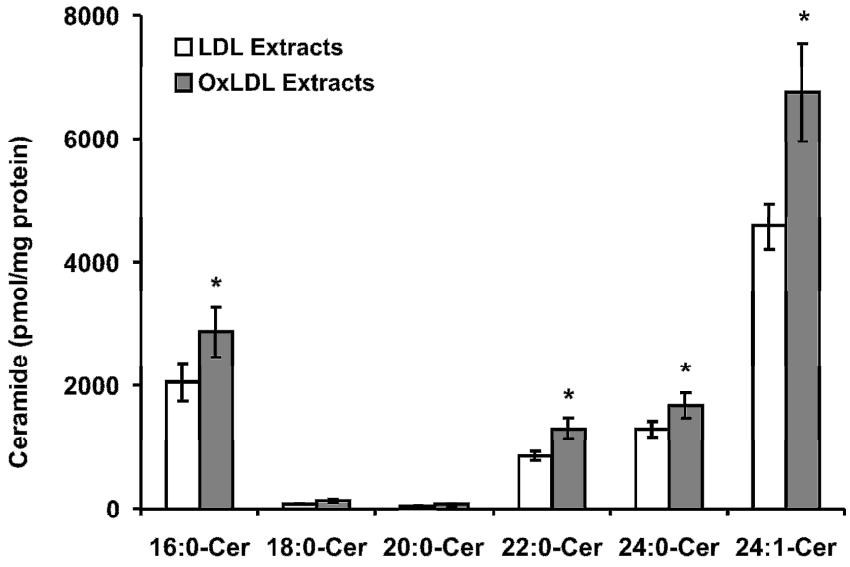

FIGURE 21. Influence of lipid extracts from LDL and OxLDL on ceramide patterns. Oxidation of LDL was performed and lipids were extracted as described under "Experimental Procedures". RAW 264.7 cells were treated with lipid extracts from native LDL and OxLDL (50 μg/mL protein, respectively) for 24 h. Lipids were extracted and analyzed for ceramide levels by LC/MS-MS as described under "Experimental Procedures". The data are means ± S.E., n = 4.

Taken together, the results suggest that the increase in ceramide content under the influence of total lipid extracts from OxLDL is likely due to activation of CerS enzymes by lipid oxidation products such as POVPC and PGPC present in the OxLDL.

5. DISCUSSION

The present study demonstrates that POVPC, PGPC and OxLDL trigger ceramide accumulation in RAW 264.7 cells. The results support the assumption that the elevation in ceramide content is mediated by the OxPL-induced activation of the key enzyme of the biosynthetic ceramide pathway, CerS. Both POVPC and PGPC activated particular CerS isoforms catalyzing the synthesis of ceramide species containing specific length fatty acyl-chains. We demonstrate that PGPC is a potent activator of CerS2 whereas CerS5/6 is activated to a lesser extent. Since both OxPLs and OxLDL show the same effect on Cer formation, we conclude that POVPC and PGPC are the active components of the modified lipoproteins that contribute to its toxic properties. These studies highlight the induction of ceramide generation by oxidized phospholipids and the activation of specific enzymes involved in ceramide biosynthesis.

Under various stress-induced responses, ceramide can be generated by two major pathways, namely, sphingomyelin hydrolysis and *de novo* synthesis. Depending on stimuli or cell type, both pathways may be activated (30, 31). In earlier studies, a correlation was established between apoptotic signaling and the fast POVPC- and PGPC-induced activation of aSMase in vascular smooth muscle cells (22), and RAW 264.7 macrophages (32), POVPC being more potent than PGPC. The present study suggests that POVPC and PGPC also activate CerS after long incubation times, thereby leading to accumulation of distinct ceramide species. The suppression of OxPL-induced ceramide formation by FB1 supports the assumption that CerS catalyze the formation of these lipids and therefore are key modulators of OxPL toxicity in cultured macrophages. This data is in line with our observation that PGPC and to a lesser extent POVPC are activators of CerS enzymes catalyzing the synthesis of these lipid species. This effect correlates with the tiny structural difference between both OxPLs (Fig. 1A, dotted circles) which determine their cellular localization. POVPC, containing a reactive

aldehyde group at the *sn*-2 position can form covalent Schiff-bases with the free amino groups of proteins and amino-phospholipids. Thus, it is retained in the plasma membrane for longer periods where it may modify SMase (33). In contrast, PGPC contains a ω-carboxylic group and can not form any bonds with other biomolecules. As a consequence, it is rapidly internalized (34) and can influence the activities of enzymes (such as CerS) inside the cells. Fluorescence microscopy studies revealed that labeled PGPC becomes enriched in intracellular membranes. As a consequence, the OxPL can modulate intracellular membrane protein (e. g. CerS localized to the ER) activities either indirectly by affecting membrane lipid organization or by direct lipid-protein interaction. Thus it can be speculated that these enzymes are activated by OxPLs in the above defined fashion.

Both POVPC and PGPC have been identified as major biologically active lipids present in oxidatively modified LDL (35). It has been shown that the respective lipids (21, 22), as well as the entire OxLDL particle, trigger an early activation of sphingomyelinase and ceramide formation to propagate its biological effects in various cells (36–38). In this study we found that, over a long incubation period, POVPC, PGPC and total lipid extracts from OxLDL activate CerS. Obviously, POVPC and PGPC present in the extracts of OxLDL are likely responsible for the increased activation of CerS enzymes in RAW 264.7 cells as well.

Activation of distinct CerS isoforms has been shown in different cell types under various stress conditions (12, 19, 39–41). Our studies demonstrate that POVPC and PGPC activate specific sub-sets of CerS isoforms in RAW 264.7 cells to a different extent and as a consequence the profiles of ceramide species that accumulate under the influence of both OxPLs are consistent with the activation of CerS2 and CerS5/6, which have been shown to be responsible for the generation of $C_{22:0}$-, $C_{24:0}$-, and $C_{16:0}$-ceramide respectively. It will be the aim of future studies to determine the biophysical and biochemical effects of the formed ceramides on the (sub)cellular membranes and their role in lipid-mediated cell death.

Both OxPLs closely mimic the toxic properties of OxLDL in cultured vascular cells. Chronic exposure to μM concentrations of POVPC and PGPC induce apoptosis in cultured SMC and macrophages. Lipid-induced cell death is associated with the formation of ceramide which is considered as an apoptotic messenger mediating extracellular physical, chemical and biochemical stresses. However, the induction of ceramide generation pathway seems to be different. Whereas POVPC is a more effective activator of aSMase at shorter exposure times, PGPC elicits a much more pronounced ceramide response by the FB1-sensitive CerS activity after several hours of incubation. Thus, initiation of ceramide formation by OxPLs is biphasic, showing an initial peak of aSMase activity under the influence of POVPC and a late persistent activation of CerS by PGPC. Thus, it can be speculated that OxLDL containing both compounds efficiently and persistently activates ceramide formation in the cells of the vascular wall. Therefore, CerS- and Cer-associated cell death is a biologically relevant phenomenon that is critical to the pathobiochemistry of atherosclerosis. It remains to be clarified what are the biophysical and biochemical mechanisms of CerS-catalyzed ceramide formation and its consequences for programmed cell death on the cellular and molecular level.

In summary, the present study demonstrates that the OxLDL components POVPC and PGPC trigger activation of ceramide synthases, but not sphingomyelinases, after prolonged incubation resulting in accumulation of ceramide in RAW 264.7 cells. PGPC is a more potent inducer of ceramide generation compared to the structurally similar but chemically different POVPC.

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CHAPTER 3

Influence of Oxidized Phospholipids on Ceramide Synthases in Vascular Smooth Muscle Cells

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1.1 Abbreviations

aSMase	acid sphingomyelinase
Cer	ceramide
CerS	ceramide synthase
FB1	fumonisin B1
LC-MS/MS	liquid chromatography tandem mass spectrometry
LDL	low density lipoprotein
mmLDL	minimally modified low density lipoprotein
nSMase	neutral sphingomyelinase
OxLDL	oxidized low density lipoprotein
OxPL	oxidized phospholipid
PGPC	1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine
POVPC	1-palmitoyl-2-oxovaleroyl-*sn*-glycero-3-phosphocholine
SMase	sphingomyelinase
VSMC	vascular smooth muscle cells

1.2 ABSTRACT

Oxidized phospholipids (OxPLs), including 1-palmitoyl-2-oxovaleroyl-sn-glycero-3-phosphocholine (POVPC) and 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC), present in OxLDL, have been shown to be responsible for the toxic effects of OxLDL on the cells of the vascular wall. These compounds mimic the biological effects of OxLDL by inducing different cellular responses including proliferation, inflammation or apoptosis. It was the aim of this study to investigate the impact of OxPLs on intracellular signaling pathways responsible for the detrimental effects of OxLDL in vascular smooth muscle cells (VSMC). We found that prolonged stimulation of cultured A7r5 cells with POVPC or PGPC led to an increase in ceramide synthase (CerS) activity, CerS5/6 and CerS2 being the most active isoforms. PGPC more efficiently stimulated CerS activity than its structurally similar counterpart, POVPC. Stimulation of CerS enzymes by the OxPLs matched their activation by lipid extracts of OxLDL. Thus, it is concluded that POVPC and PGPC are likely to contribute to the toxic OxLDL effects in VSMCs. In contrast to cultured macrophages, CerS activation by OxPLs does not correlate with a significant increase in Cer levels. Obviously, POVPC and PGPC operate in a cell-specific manner to induce Cer formation and cell death in VSMC and macrophages.

2. INTRODUCTION

Atherosclerosis is a chronic inflammatory disease which is initiated by the oxidation of low-density lipoproteins (LDL) and accumulation of the oxidized LDL (OxLDL) in the sub-endothelial space. OxLDLs play a critical role in the formation and progression of atherosclerotic lesions, by triggering various stress response events in the vascular cells that participate in vascular wall injury, plaque formation/rupture and subsequent atherothrombosis. At low concentrations, OxLDLs exhibit a variety of atherogenic properties including foam cell formation and fatty streaks, cell proliferation, and inflammatory responses, while at higher concentrations they can induce apoptotic cell death (1, 2). Vascular smooth muscle cells (VSMC) play a critical role in atherogenesis by switching their phenotype to an inflammatory state involving increased proliferation, enhanced migration and down regulation of SMC differentiation marker genes. In addition, cell death of SMC during the progression of atherosclerosis involving both apoptosis and necrosis contributes to plaque instability (3). Interaction of OxLDL with SMC and the resultant biological consequences are an important issue during atherosclerosis and little knowledge is available in understanding the molecular mechanisms involved in the biological effects of OxLDL.

A growing body of evidence suggests that the cytotoxicity of OxLDLs is largely due to the oxidatively modified lipid fractions including oxidized phospholipids (OxPL) and oxysterols. OxPLs, including 1-palmitoyl-2-oxovaleroyl-sn-glycero-3-phosphorylcholine (POVPC) and 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphorylcholine (PGPC), have been shown to be responsible for the detrimental effects of OxLDL (4). So far, several studies have been carried out using OxLDL which contains a complex mixture of lipid oxidation products. The lipid complexity of phospholipid oxidation products in OxLDL presents a challenge to the investigators to reach a better understanding of the effects of OxPLs generated under (patho)physiological situations. There is a considerable interest in using

individual isolated or chemically synthesized OxPL species in elucidating their contribution to the cytotoxicity of the modified lipoproteins and determining the molecular signaling mechanisms that underlie this biological effects.

Ceramide (Cer) has emerged as a key signaling molecule involved in the regulation of many cellular stress response pathways under the influence of various external stimuli (5–9). This sphingolipid can be generated by sphingomyelinase (SMase)-catalyzed hydrolysis of sphingomyelin, by, via the *de novo* synthesis or the salvage pathway involving ceramide synthases (CerS), responsible for acylation of sphinganine/sphingosine. Several studies have reported on the role of ceramide in mediating the biological effects of OxLDL on various vascular cells including SMC (10–13). It is also known that POVPC and PGPC induce apoptotic cell death in VSMCs by stimulating SMase activity and elevating cellular ceramide levels (14, 15). Recent work from our laboratory demonstrated similar effects of POVPC, PGPC and their ether analogs in cultured macrophages. Since the aSMase inhibitors NB6 and NB19 abrogated the apoptotic effects of OxPLs in the respective cells it was concluded that activation of aSMase is causally related to OxPL toxicity in VSMC and MQs (16). While, aSMase-catalyzed Cer formation is rapidly triggerd by OxPLs, stimulation of *de novo* synthesis is much slower. We found that POVPC and PGPC stimulated CerS activity in MQs after several hours leading to an increase in Cer levels. The response of CerS and Cer levels to chronic exposure of other vascular cells has not been studied in detail. Thus, it was the aim of this study to investigate the effects of POVPC and PGPC on CerS-mediated formation of Cer in VSMCs and to compare them to the effects of the parent OxLDL. We found that, under long incubation conditions, both POVPC and PGPC activate a limited set of CerS isoforms. PGPC is the more potent OxPL preferentially activating CerS5/6 whereas, CerS2 was only slightly activated. In contrast to the OxPL effects in cultured macrophages, POVPC and PGPC had little influence on Cer levels under the same conditions.

3. EXPERIMENTAL PROCEDURES

3.1 Reagents

Cell culture materials were obtained from Sarstedt (Numberecht, Germany) and Greiner (Kremsmunster, Austria). Dulbecco's modified Eagle's medium and heat-inactivated fetal bovine serum were from Invitrogen (Leek, The Netherlands). PBS and other cell culture supplements were obtained from PAA (Linz, Austria), unless otherwise indicated. Oxidized phospholipids (POVPC and PGPC) were synthesized in our laboratory or purchased from Avanti Polar Lipids (Alabaster, USA). Fumonisin B1 was from Calbiochem (Darmstadt, Germany).

3.2 Cell culture

Smooth muscle cells from Rat thorax aorta (A7r5) were a kind gift from Prof. K Groschner, University of Graz, Austria. Cells were maintained in DMEM (4.5 g/l glucose, 25 mM HEPES, 4 mM L-glutamine, without sodium pyruvate) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 IU/mL), and streptomycin (100 µg/mL) at 37 °C in a humidified atmosphere containing 5% CO_2. Incubations of cells with OxPLs were conducted under same conditions using DMEM with or without phenol red supplemented with 0.1% FCS. Incubation mixtures were prepared by adding ethanol stock solutions of lipids to the culture medium. The ethanol concentration was below 1% (v/v) and control experiments were performed using medium containing only ethanol without lipid additives.

3.3 Isolation and oxidative modification of LDL

Human LDL was isolated by density ultracentrifugation in OptiSeal tubes using a Beckman NVT65 Rotor at 4 °C (17) from pooled healthy fresh plasma (a kind gift of Dr. Gholam Ali Khoschsorur, University Hospital, Graz). The LDL fraction was collected and stored at 4 °C for up to 8 days prior to use. Protein concentrations of samples were measured by the

method of Bradford (18). LDL was desalted using PD 10 columns (GE Healthcare, Munich, Germany) prior to oxidative modification. For LDL oxidation the lipoprotein was performed by incubated with 1 mM $CuSO_4$ in sterile H_2O for 48 h protected from light at 37 °C. OxLDL was desalted prior to use as described above.

3.4 Fluorescence ceramide synthase *In vitro* assay

A7r5 cells were harvested and homogenized in HEPES buffer [20 mM HEPES-KOH (pH 7.2), 25 mM KCl, 2mM $MgCl_2$, and 250 mM sucrose] containing a protease inhibitor cocktail (AEBSF, 104 mM; Aprotinin, 80 μM; Bestatin, 4 mM; E-64, 1.4 mM; Leupeptin, 2 mM and Pepstatin A, 1.5 mM) from SIGMA-ALDRICH. Protein concentrations were measured using the Bradford method (Bio-Rad). CerS activity was assayed as described previously (19) except using NBD-sphinganine (Avanti Polar Lipids) instead of unlabeled sphinganine spiked with tritiated sphinganine. Briefly, cell homogenates containing 50 μg protein were incubated in a final volume of 125 μL HEPES buffer containing 10 μM NBD-sphinganine, 20 μM defatted-bovine serum albumin. The reaction was started by addition of 50 μM of fatty acyl-CoAs (Avanti Polar Lipids) at 37 °C for 20 min, depending on the substrate specificity of each CerS. Reactions were terminated by addition of three volumes of chloroform/methanol (1/2; v/v). Lipids were extracted (20) and separated by thin-layer chromatography (TLC) on silica plates using chloroform/methanol/water (8/1/0.1; v/v/v) as solvent (21). Fluorescent lipids were visualized and fluorescence intensities were determined with a Herolab CCD camera (excitation at 365 nm) using EasyWin®-software for data acquisition and processing (Fig. 1).

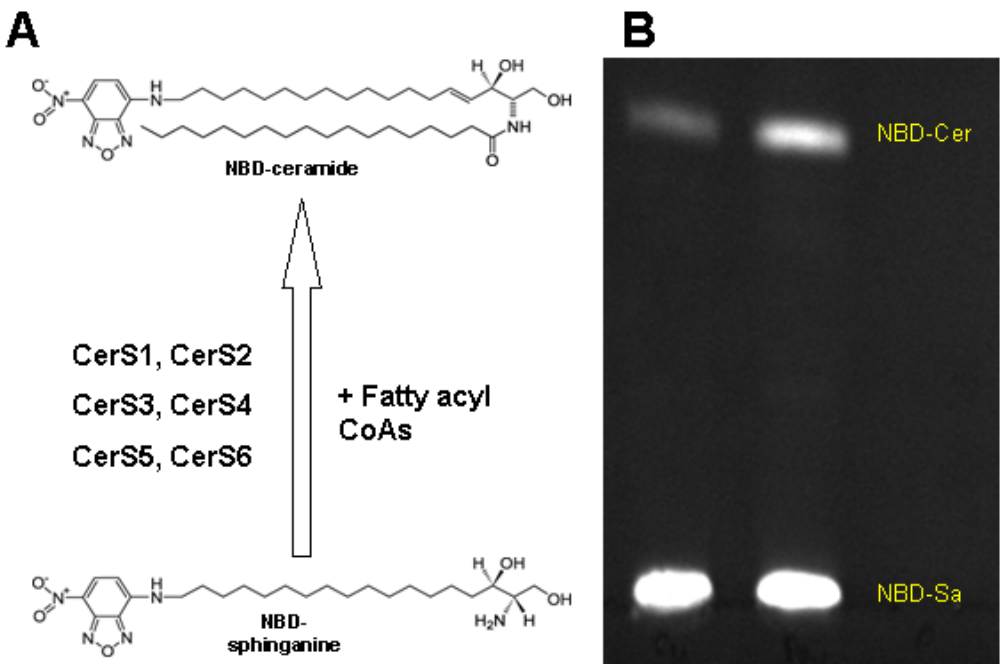

FIGURE 1. Fluorescence CerS assay: formation of NBD-ceramide. A, CerS-catalyzed acylation of NBD-sphinganine generates NBD-ceramide. The utilized fatty acyl CoA depends on the individual CerS isoform. **B,** TLC separation of NBD-sphinganine (NBD-Sa) and NBD-ceramide (NBD-Cer) on silica plate using chloroform/methanol/water (8/1/0.1; v/v/v) as solvent.

3.5 Lipid extraction and LC/MS-MS analysis of sphingolipids

Cells were incubated with OxPLs as described previously and were harvested with cold phosphate-buffered saline. The cell pellet was re-suspended in a mixture of 100 µL distilled water and 750 µL chloroform: methanol (1:2; v/v) containing internal standards. Samples were sonicated and incubated over-night at 48 °C. For alkaline hydrolysis of ester lipids 75 µL 1 M KOH in methanol was added, followed by sonication and incubation for 2 h at 37 °C. Samples were neutralized by adding 3 µL glacial acetic acid and lipids were partitioned in 3 mL chloroform:water (1:2; v/v). The upper aqueous layer was separated and the solvent was removed from the lower organic layer under a stream of N_2. Lipid extracts were analyzed for ceramide species by LC/MS-MS (22). Data are expressed as pmole ceramide per mg of total protein.

4. RESULTS

4.1 CerS activity in vascular smooth muscle cells

Ceramide is an important signaling and structural lipid which is synthesized by a family of six ceramide synthases. Each isoform displays a certain specificity toward the fatty acyl-CoA chain used for *N*-acylation of the sphingoid base to generate respective ceramide species (23, 24). We examined CerS activity in cell homogenates using a range of acyl-CoAs with NBD-sphinganine as substrates. Quantification of the fluorescent reaction product (dihydro)ceramide showed that $C_{16:0}$-Cer, the product of CerS5/6, was abundantly synthesized in VSMC homogenates (39.94 ± 6.45 pmol/ min/ mg protein) whereas the amount of $C_{22:0}$-Cer generated by CerS2 was slightly lower (34.24 ± 3.45 pmol/ min/ mg protein). $C_{18:0}$- and $C_{20:0}$-Cer species, which are synthesized by CerS1 and CerS4 respectively, were also in the detectable range. The respective activities were about half of CerS5/6 (Fig. 2).

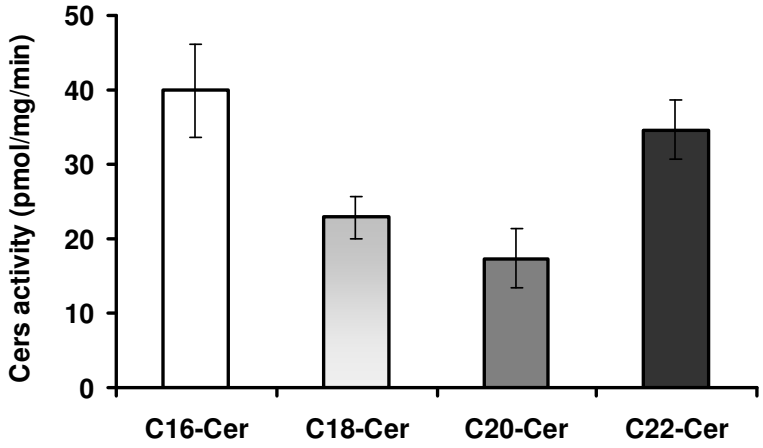

Figure 2. Endogenous CerS activity in VSMC homogenates. CerS activity was measured in cell homogenates using C_{16}-, $C_{18:0}$-, $C_{20:0}$- or $C_{22:0}$-CoA and NBD-sphinganine as substrates as described under "Experimental Procedures". Lipids were extracted and separated by thin layer chromatography (TLC) on silica plates using chloroform/methanol/water (8/1/0.1; v/v/v) as

solvent. Fluorescent lipids were visualized and intensities were determined with a Herolab CCD camera (excitation at 365 nm) using EasyWin®-software for data acquisition and processing. Results are expressed as means ± S. D. from four independent experiments.

However, very long-chain products ($C_{26:0}$-Cer) synthesized by CerS3 enzyme were not detectable. These data suggest that CerS5/6 and CerS2 are highly active in VSMC and are likely to be responsible for the generation of the major ceramide formation.

4.2 Influence of OxPLs on Cer levels and CerS activity

Earlier studies from our group demonstrate an elevation of intracellular ceramide levels in VSMC under the influence of OxPLs at shorter incubation times leading to apoptotic cell death (14). To determine the influence of OxPLs on ceramide levels at long exposure times, we incubated the cells for 24 h and analyzed the ceramide content. There was no significant effect of OxPLs on the Cer levels. Instead, we observed a decrease in long and very long chain Cer species (Fig. 3).

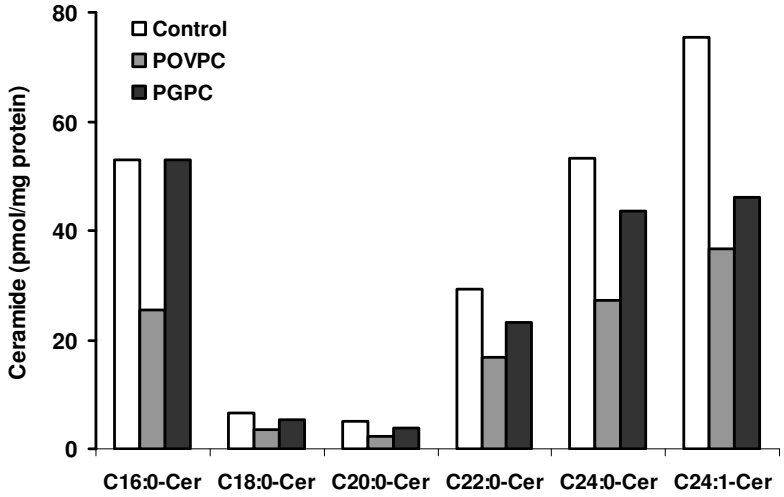

FIGURE 3. Influence of POVPC and PGPC on ceramide patterns. VSMC cells were treated with POVPC or PGPC (50 μM respectively) in parallel to ethanol-treated cells for 24 h. Lipids were

extracted and analyzed for ceramide levels by LC/MS-MS as described under "Experimental Procedures". The data are expressed as mean from two experiments.

In addition, we examined the effects of both POVPC and PGPC on CerS activity in VSMC at long exposure time (24 h). Exposure of cells to 50 μM PGPC resulted in a 1.5 fold increase in CerS5/6 activities whereas POVPC had no significant effect. There was only a slight increase in CerS2 activity under the influence of both OxPLs as compared to ethanol-treated control cells (Fig. 4 & 5).

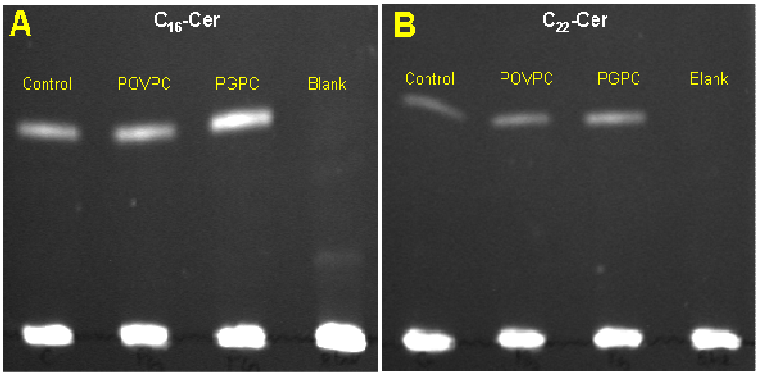

FIGURE 4. CerS activity in VSMC cells under the influence of POVPC and PGPC: Fluorescence assay. Cells were stimulated with the respective OxPLs (50 μM) in parallel to ethanol treated control cells for 24 h. After OxPL treatment cells were harvested and CerS activity was measured in cell homogenates using NBD-sphinganine and fatty acyl CoAs as substrates as described in Fig. 2. **A,** CerS5/6 activity was measured in cell homogenates using C_{16}-CoA as substrate. **B,** CerS2 activity measured in the presence of C_{22}-CoA as substrate. The results are representative for four independent experiments.

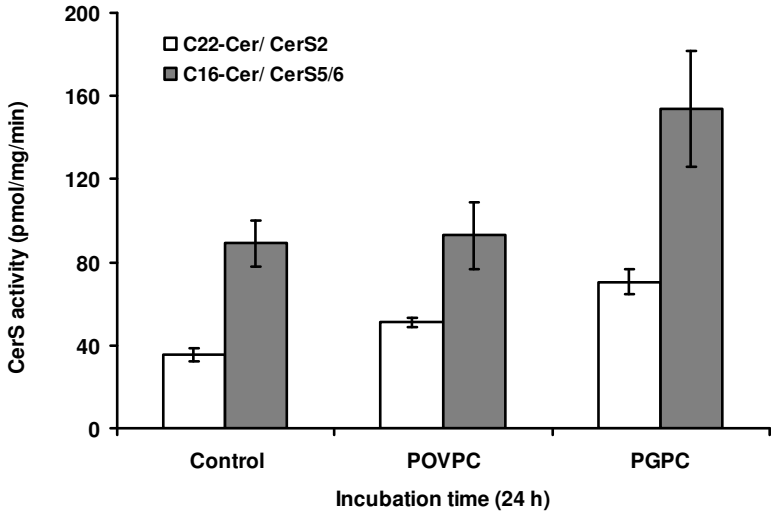

FIGURE 5. Effect of POVPC and PGPC on CerS activity in VSMC homogenates. Cells were stimulated with the respective OxPLs (50 μM) in parallel to ethanol treated control cells for 24 h. After OxPL treatment cells were harvested and CerS activity was measured in cell homogenates using C_{16}-CoA, C_{22}-CoA and NBD-sphinganine as substrates as described in Fig. 2. Results are expressed as means ± S.D. from four independent experiments.

Together, the results indicate that PGPC and to a lesser extent POVPC stimulated CerS activity in VSMC at long incubation times but this did not lead to an increase in Cer levels. These results suggest for a role of Cer consuming enzymes under the chosen experimental conditions.

4.3 Influence of lipid extracts from LDL and OxLDL on CerS activity and Cer levels

To investigate the role of OxLDL on Cer generating enzymes, we treated VSMCs with lipid extracts from native LDL and OxLDL for 24 h and examined for the changes in CerS activity. As shown in Fig. 6, lipid extracts from OxLDL increased CerS activity very little as compared to cells treated with lipid extracts from native LDL.

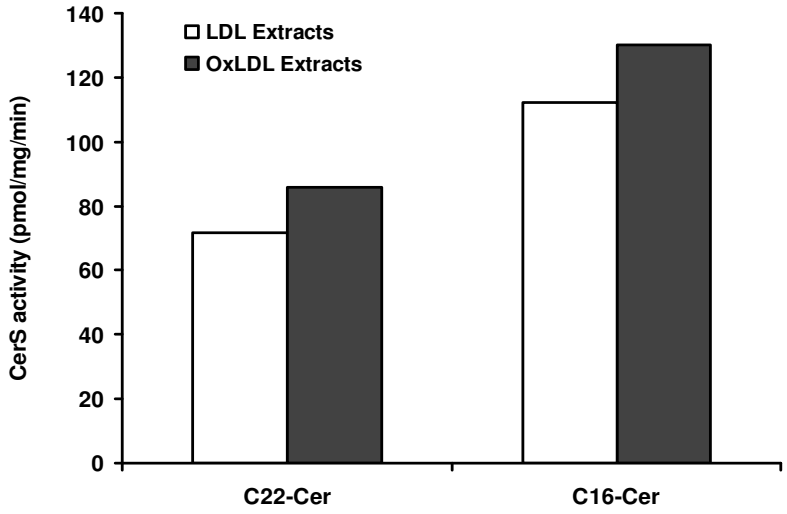

FIGURE 6. Effect of lipid extracts from LDL and OxLDL on CerS activity in VSMC. Oxidation of LDL was performed as described under "Experimental Procedures". VSMC were stimulated with lipid extracts from native LDL or OxLDL (50 μg/mL protein respectively) for 24 h. CerS activity in cell homogenates was measured using C_{16}-CoA and C_{22}-CoA substrates as described earlier. The results are expressed as means of two experiments.

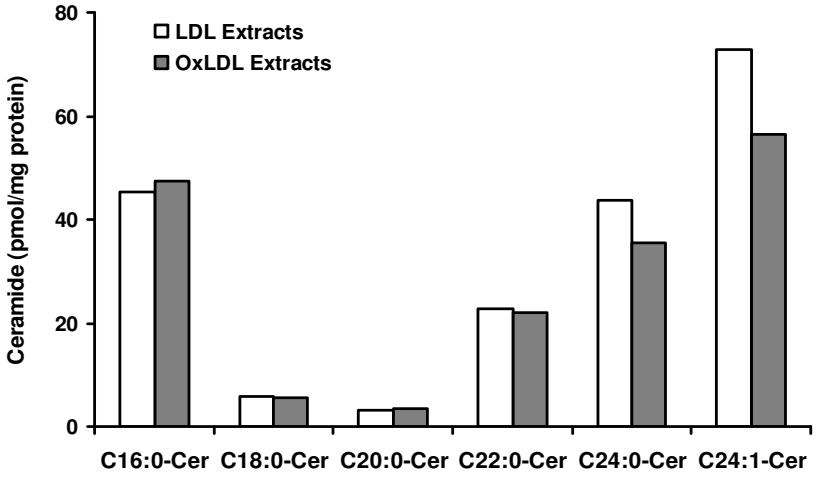

FIGURE 7. Influence of lipid extracts from LDL and OxLDL on ceramide patterns. Oxidation of LDL was performed as described under "Experimental Procedures". VSMC were treated with lipid extracts from native LDL and OxLDL (50 μg/mL protein respectively) for 24 h. Lipids were extracted

and analyzed for ceramide species by LC/MS-MS as described under "Experimental Procedures". The data are expressed as means from two experiments.

Analysis of ceramide levels in cells treated with lipid extracts from LDL or OxLDL did not show any effect on the amounts of $C_{16:0}$-, $C_{18:0}$-, $C_{20:0}$- and $C_{22:0}$-Cer (Fig. 7). However, the OxLDL lipids slightly reduced the levels of $C_{24:0}$- and $C_{24:1}$-Cer species.

Together, the results indicate that lipid extracts present in the OxLDL had no significant effect on CerS activity nor on most Cer species. Only the very-long chain Cer species were slightly affected.

5. DISCUSSION

In this study, we report that oxidized phospholipids including POVPC and PGPC increase CerS activity in VSMCs. We found that PGPC is the more potent inducer of CerS activity, particularly increasing CerS5/6 activities and to a lesser extent CerS2 activity.

Bioactive Cer has been shown to be elevated in many cell types under various stress-induced responses. Cer can be generated by sphingomyelin hydrolysis by SMase activity and CerS-dependent *de novo* synthesis (25, 26). We and others have shown that POVPC and PGPC (14–16), as well as OxLDL trigger an early activation of SMase to elevate cellular Cer levels (11, 27, 28). This effect is causally related to apoptosis in cultured VSMC since inhibition of aSMase by NB6 abrogated lipid toxicity (Loidl 2003). Here we found that at long incubation times, POVPC and PGPC induce CerS activity in VSMCs. However, Cer levels were not elevated under the same conditions. We even observed a decrease in cellular Cer content. These results point out towards the activation of Cer metabolizing enzymes including ceramidase (CDase), glucosylceramide synthase (GCS) and ceramide kinase (CERK) in VSMC under long exposure to either OxPL. This observation is at variance with previous studies (chapter 2) showing that the same OxPLs increased CerS activity in RAW 264.7 macrophages after several hours and led to an increase in Cer levels. In addition, the CerS isoforms activated under the influence of the same OxPLs in cultured macrophages and VSMCs are different. PGPC significantly elevated CerS2 activity compared to CerS5/6 activities in cultured macrophages. In VSMCs, CerS5/6 activities are significantly higher than CerS2 activity. Nevertheless, prolonged incubation with OxPLs activated CerS isoforms in both cell types. In summary, our data obtained with cultured VSMC and MQ support the growing body of evidence suggesting that CerS isoforms are differentially expressed and specific CerS are activated in a cell type and stress stimuli-specific manner.

In contrast to cultured macrophages, activation of CerS enzymes by OxPLs in VSMC did not result in Cer accumulation. In the latter cells, aSMase appears to be the major enzyme catalyzing the formation of Cer in response to OxPL (14). This supports the assumption that different signaling pathways are stimulated by OxPLs in both cell types leading however to the same biological consequences. POVPC and PGPC are toxic to VSMC and MQ and induce apoptosis in these cells. Since large fractions of OxPL, OxLDL as well as apoptotic VSMC and MQs are found in atherosclerotic lesions, it can be speculated, that the above described Cer-associated effects of OxPLs are physiologically relevant. Thus, the same stress response pathways as pertinent to apoptosis may be mediated through increased levels of the lipid second messenger Cer also in the cells of the vascular wall and contribute to the development of atherosclerosis. It will be the aim of future studies to determine the role of other sphingolipid metabolizing enzymes in vascular cells in response to OxPLs/OxLDL.

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