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Effects of Oxidized Phospholipids on Gene Expression and Sphingolipid Metabolism in RAW 264.7 macrophages

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Abstract

The truncated oxidized phospholipids (oxPLs) 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine (PGPC) and 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphocholine (POVPC) are biologically active components of oxidized low-density lipoprotein (oxLDL) and largely contribute to its atherogenic activity in vascular cells (macrophages, smooth muscle cells, endothelial cells). Ceramide is an important mediator of these effects.

It was the aim of the first part of this doctoral thesis, to investigate the effects of PGPC and POVPC on enzymes of sphingolipid metabolism. We studied the toxicities of oxLDL and oxPL in cultured in RAW 264.7 macrophages and determined the formation of ceramides under the influence of these toxic agents. We found that PGPC and POVPC increased activities of acid and neutral sphingomyelinase in a concentration- and time-dependent manner. In addition, enzymes catalyzing *de novo* sphingolipid synthesis were also stimulated by the oxPLs. The latter enzyme effects correlated with results from sphingolipidome analysis. POVPC induced a faster ceramide response, whereas PGPC elicited a slower but persistent stimulatory effect on ceramide formation. Both oxPLs did not affect the activities of ceramidases. Thus, ceramide degradation is likely not to be influenced by PGPC and POVPC.

The second part of this doctoral thesis was devoted to the effects of PGPC and POVPC on gene expression in RAW 264.7 macrophages. PGPC affected the expression of many genes, whereas POVPC showed only minor effects. PGPC mainly influenced expression of genes related to cell death, angiogenesis, cholesterol efflux, procoagulant mechanisms, atherogenesis, inflammation and cell cycle. Many of these effects have already been observed with oxLDL or oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (oxPAPC), suggesting that PGPC is at least in part responsible for the atherogenic effects of their oxidized phospholipid components. In contrast to PGPC, POVPC contains an aldehyde

function and chemically reacts with proteins by Schiff base formation. Since transcriptional effects of POVPC are small, it looks as if this lipid affects cell physiology mainly on the protein level. Thus, we conclude that both, PGPC and POVPC contribute to the atherogenic properties of oxLDL but the mechanisms of their toxicities on the transcriptome, enzyme and lipidome level are entirely different.

Zusammenfassung

Die oxidierten Phospholipide PGPC und POVPC sind biologisch aktive Bestandteile von oxidiertem LDL und tragen zu dessen atherogener Aktivität in vaskulären Zellen bei (Makrophagen, glatte Muskelzellen, Endothelzellen). Ceramid ist ein wichtiger Mediator dieser Aktivität.

Der erste Teil dieser Doktorarbeit untersucht die Effekte von PGPC und POVPC auf Enzyme des Sphingolipidmetabolismus. Wir untersuchten die Lipoprotein- und Phospholipidtoxizität in kultivierten RAW 264.7 Makrophagen und bestimmten den Einfluss dieser toxischen Verbindungen auf den Ceramidgehalt. Wir konnten nachweisen, dass PGPC und POVPC die Aktivitäten der sauren und der neutralen Sphingomyelinase konzentrations- und zeitabhängig erhöht. Weiters wurden Enzyme der *de novo* Sphingolipidsynthese stimuliert. Die Induktion dieser Enzyme korrelierte mit dem Ergebnis der Sphingolipidomanalyse. POVPC induzierte eine schnelle Ceramidantwort, während PGPC eine langsamere, aber anhaltendere Stimulation der Ceramidbildung verursachte. Beide oxidierte Phospholipide zeigten keine Wirkung auf die Aktivitäten der Ceramidasen. Daher ist ein Einfluss von PGPC und POVPC auf den Ceramidabbau unwahrscheinlich.

Im zweiten Teil dieser Doktorarbeit wurden die Effekte von PGPC und POVPC auf die Genexpression in RAW 264.7 Makrophagen untersucht. PGPC veränderte die Expression vieler Gene, während POVPC nur geringe Effekte zeigte. PGPC induzierte die Expression von Genen, die in Zusammenhang mit Zelltod, Angiogenese, Cholesterolefflux, Gerinnungsmechanismen, Atherosklerose, Entzündung und Zellzyklus stehen. Viele dieser Effekte wurden bereits bei oxLDL oder oxPAPC beobachtet. Daraus kann der Schluss gezogen werden, dass PGPC zumindest teilweise für die atherogene Wirkung deren oxidierter Lipidbestandteile verantwortlich ist. Im Gegensatz zu PGPC enthält POVPC eine

Aldehydgruppe und kann mit Proteinen mittels Schiffbasenbildung chemisch reagieren. Da die Effekte von POVPC auf das Transkriptom gering sind, scheint dieses Lipid die Zellphysiologie hauptsächlich auf Proteinebene zu beeinflussen. Wir kommen zum Schluss, dass beide, PGPC und POVPC zur atherogenen Eigenschaft von oxLDL beitragen, aber die Mechanismen deren Toxizität auf Transkriptom-, Enzym- und Lipidomebene unterschiedlich sind.

Inhaltsverzeichnis

1 Oxidized phospholipids and sphingolipid mediators in vascular cells – A Review

Here we report on the effects of the oxidized phospholipids PGPC and POVPC on the RAW 264.7 macrophage like cell line. Oxidized phospholipids are bioactive compounds of oxLDL and play a crucial and complex role in atherosclerosis.

1.1 Atherosclerosis, LDL modification and macrophages

Atherosclerosis is a chronic inflammatory disease leading to most common death causes in western societies. It affects the vascular system and leads to gradual thickening of the intima causing decreased elasticity and reduced blood supply. The blood vessels mostly affected and clinically relevant include the aorta and coronary, carotid and cerebral arteries. Atherosclerosis leads to coronary artery disease, myocardial infarction and stroke. The oxidation of LDL triggers the generation of bioactive oxidation products that play important roles in the initiation of atherosclerosis and also represent a driving force of chronic inflammation. This disease comprises complex interactions between modified lipoproteins, monocyte-derived macrophages, T cells and other cellular components of the arterial wall such as endothelial cells. The inflammatory process can lead to the development of lesions or plaques. Plaque rupture and thrombosis result in acute clinical complications [1], [2], [3].

1.1.1 Oxidative LDL modification

In humans, the majority of serum cholesterol is carried by LDL particles. Under normal conditions LDL plays an essential role in cholesterol transport to peripheral tissues, but increased LDL cholesterol levels are associated with the risk of cardiovascular disease. LDL circulating in blood or more importantly after infiltrating the arterial wall, becomes oxidized via enzymatic or non-enzymatic mechanisms [4]. Oxidative stress is part of the normal host response to a variety of stimuli. Chronic vascular inflammation may result in chronic oxidative stress leading to excessive LDL oxidation [1]. This LDL oxidation occurs in animals and humans, and several studies show that antioxidant treatment has a protective effect against atherosclerosis [3]. However, clinical trials with antioxidant vitamins in patients with preexisting atherosclerosis were disappointing [5]. The specific properties of oxLDL depend on the extent of modification ranging from minimal modification, where the apolipoprotein B (apoB) remains intact, to extensive oxidation. In the latter case the apoB is fragmented and lysine residues are covalently modified with reactive breakdown products of oxidized lipids [3]. While LDL and minimally modified LDL (mmLDL) is taken up by cells via LDL receptors that recognize an N-terminal domain of apo B-100, oxLDL is internalized via so called scavenger receptors (SRs) [6]. The latter receptors have heterogeneous molecular structures, and are divided in various classes A, B, C, D, E and F [7].

A large number of proinflammatory and proatherogenic properties have been reported for mmLDL, oxLDL and their lipid components [8]. The formation of atherosclerotic lesions begins with plasma lipoproteins infiltrating the arterial wall, which accumulate in the arterial wall. In response to lipid accumulation, monocytes migrate into the intima and differentiate into macrophages which subsequently differentiate into foam cells and accumulate lipids in the cytoplasm. The accumulation of macrophage foam cells leads to the development of

fatty streak lesions, accompanied by migration of vascular smooth muscle cells from the media to the intima, and their subsequent proliferation and transformation into foam cells. Some macrophages also proliferate *in loco,* but if lipid accumulation increases, apoptosis and necrosis become predominant. The center of the atherosclerotic plaques becomes necrotic and consists of amorphous necrotic lipid materials and cholesterol crystals. Advanced lesions are covered by a fibrous capsule called „fibrous cap“. Finally disruption in the shoulder region of the plaques can occur, leading to ulceration, thrombus formation followed by arterial occlusion [2], [9], [10].

1.1.2 The role of macrophages in atherosclerosis

Products of oxLDL serve as chemoattractants for monocytes and T cells and promote their adhesion to vascular endothelial cells. Neutrophils, which are normally involved in most inflammatory responses, are absent in atherosclerotic lesions [11]. Although the recruitment of monocytes and their subsequent differentiation into macrophages may initially have a protective function by removing cytotoxic and proinflammatory oxLDL or apoptotic cells, progressive accumulation of macrophages and the persistent uptake of oxLDL lead to the development of the atherosclerotic lesion. Monocyte recruitment is regulated by cell adhesion molecules that are expressed on the surface of endothelial cells in response to inflammatory stimuli [12]. Many proinflammatory cytokines, including interleukin 1 (IL-1), IL-6, IL-8, IL-10, IL-12 and tumor necrosis factor-α (TNF-α), are produced by macrophages and activate circulating blood monocytes in response to lipoproteins [2], [10], [9]. Many chemotactic factors for monocytes are known to be produced at atherosclerotic lesions, including monocyte chemoattractant protein 1 (MCP-1), MCP-2, MCP-3, MCP-4, MCP-5, granulocyte macrophage colony stimulating factor (GM-CSF), migration inflammatory

protein 1 (MIP-1), TNF-α, transforming growth factor-β (TGF-β), RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted) and endothelin-1 [13], [14]. To some extent, migration of monocytes into the arterial wall is stimulated directly by oxLDL [15]. Especially MCP-1 is expressed in vascular endothelial cells, macrophages, smooth muscle cells, foam cells and T lymphocytes in response to oxLDL [16], [17]. In atherosclerotic lesions, vascular endothelial cells produce MCP-1 and expose it on their cell surface to the peripheral blood [18]. Monocytes and activated T cells express C-C chemokine receptor-2 (CCR2), a receptor for MCP-1. MCP-1 and CCR2 are important for monocyte migration and recruitment into atherosclerotic lesions, but atherosclerosis is not completely blocked in absence of either MCP-1 or CCR2 [19], [20], [21].

In response to the chemotactic factors released into the blood stream, circulating monocytes move to the peripheral capillary bloodstream, are activated and express cluster of differentiation 14 (CD14) on the cell surface [22]. Activated monocytes roll on the surface of vascular endothelial cells and adhere to the endothelial cells. Finally, the monocytes pass through the vascular endothelial layer and migrate into the subendothelial space of the intima followed by differentiation into macrophages.

For monocyte/macrophage differentiation in the atherosclerotic lesion, macrophage colony-stimulating factor (M-CSF) plays a key role, even if the development of atherosclerosis is not completely inhibited in M-CSF/apoE double-knockout mice [23], [24]. This factor is involved in the production and development of a monocytic cell lineage in bone marrow, the differentiation of monocytes into macrophages in tissues and the proliferation and survival of macrophages *in loco*. The expression of M-CSF induced by oxLDL was demonstrated in various vascular cells, including macrophages [25], [26], [27]. It is responsible for macrophage survival, DNA synthesis and proliferative responses [28]. M-CSF also selectively

increases the synthesis of type I and types II class A macrophage scavenger receptors (MSR-A I, II). Thus M-CSF enhances oxLDL uptake of macrophages [16]. In contrast, GM-CSF downregulated the expression of these receptors and prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic (WHHL) rabbits, showing that uptake of oxLDL by scavenger receptors is a key element of macrophage differentiation into foam cells [29], [30].

1.1.2.1 Foam cell formation

The transformation of macrophages into foam cells is a hallmark of atherosclerosis. The driving force for this process is a massive lipid uptake and cholesterol accumulation in macrophages. Whereas LDL and mmLDL is recognized by LDL receptors via apolipoprotein B-100, oxidized LDL with an oxidatively modified apoB-moiety can only be transported into the cells via SRs. As mentioned before, oxLDL selectively upregulates the expression of some types of scavenger receptors and thus represents an essential element in macrophage progression to foam cells [31]. Recognition of oxLDL by scavenger receptors is mediated in part by oxidized phospholipids. Modified Lipid particles, including oxLDL, remnant lipoproteins (including β- very low-density lipoprotein), enzymatically degraded LDL and aggregated LDL, can be taken up through various routes of endocytosis. Proteins and transporters involved in this pathways include lipoprotein lipase (LPL), endothelin receptors (macrophage express ET_B receptors) [32], apolipoprotein E (apoE), SRs class A, SRs class B (e. g. CD36), CD68, lectin like oxLDL receptor 1 (LOX-1), scavenger receptors that bind to phosphatidylserine and LDL receptor like protein [33], [34], [35], [36]. Although several proteins may contribute to this overall process, scavenger receptors class A and CD36 (belongs to the SR class B) are quantitatively most important [37]. Scavenger receptors in

general are unspecific and recognize a broad variety of ligands. For example, SRs class A bind acetyl LDL, oxLDL, polyinosinic acid, dextran sulfate, fucoidan, lipid A and advanced glycated end products. In mice with a pro-atherogenic apoE$^{-/-}$ and LDL-receptor$^{-/-}$ (LDL-R) genetic background, SR class A null alleles result in a significant decrease in atherosclerotic lesions [37]. Peritoneal macrophages of CD36$^{-/-}$ mice showed a 60-80 % decrease in oxLDL binding and oxLDL uptake [38].

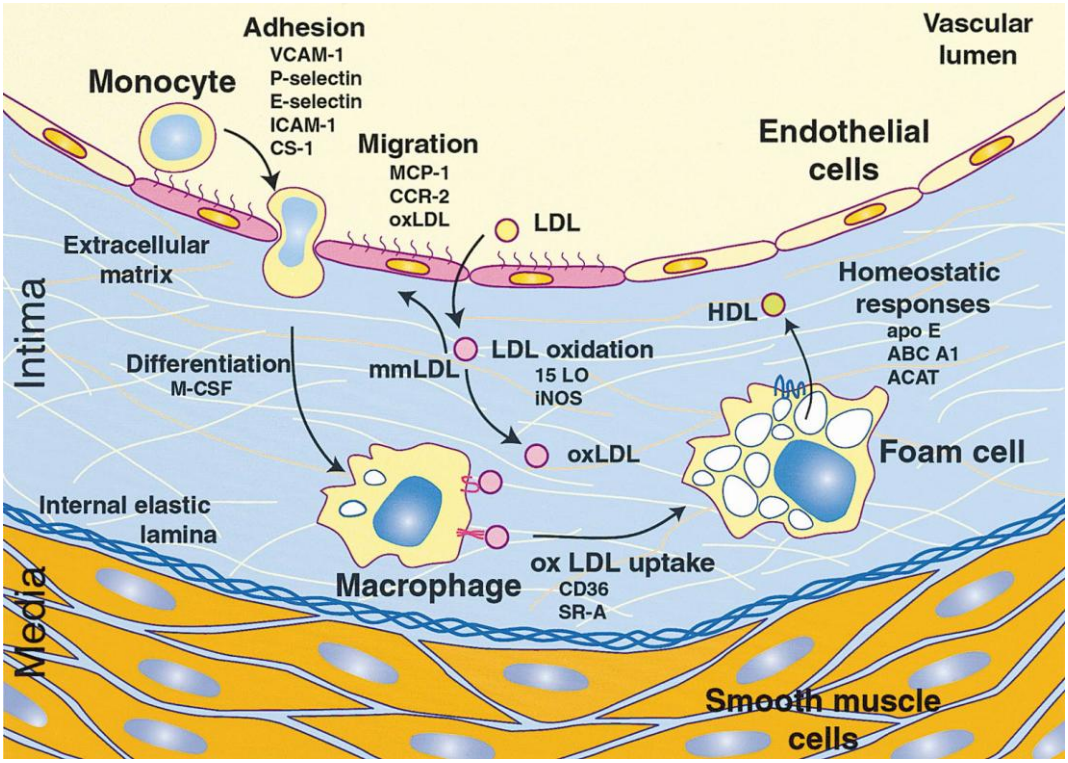

Figure 1: Initiating events in the development of a fatty streak lesion.

LDL is subject to oxidative modifications in the subendothelial space of the arterial wall. Monocytes attach to endothelial cells that have been activated by mmLDL and inflammatory cytokines to express cell adhesion molecules. Adherent monocytes migrate into the subendothelial space, differentiate into macrophages and develop to foam cells due to massive uptake of oxLDL (C. K. Glass & J. L. Witztum [39]).

After endocytotic uptake, lipoproteins are finally transported into lysosomes, where they are degraded. Cholesterol ester is hydrolyzed to free cholesterol and fatty acids [7]. High amounts of free cholesterol is toxic to cells [40]. Free cholesterol released from lysosomes by neutral cholesterol ester hydrolase, is re-esterified by acyl coenzyme A:cholesterol acyltransferase (ACAT). This cycle is called the „cholesterol ester cycle“ [41]. This detoxifying cycle is highly developed in macrophages. In absence of extracellular acceptors of free cholesterol, e. g. high-density lipoprotein (HDL) which facilitates cholesterol efflux, cholesterol ester is translocated to membrane-free lipid droplets, where it accumulates.

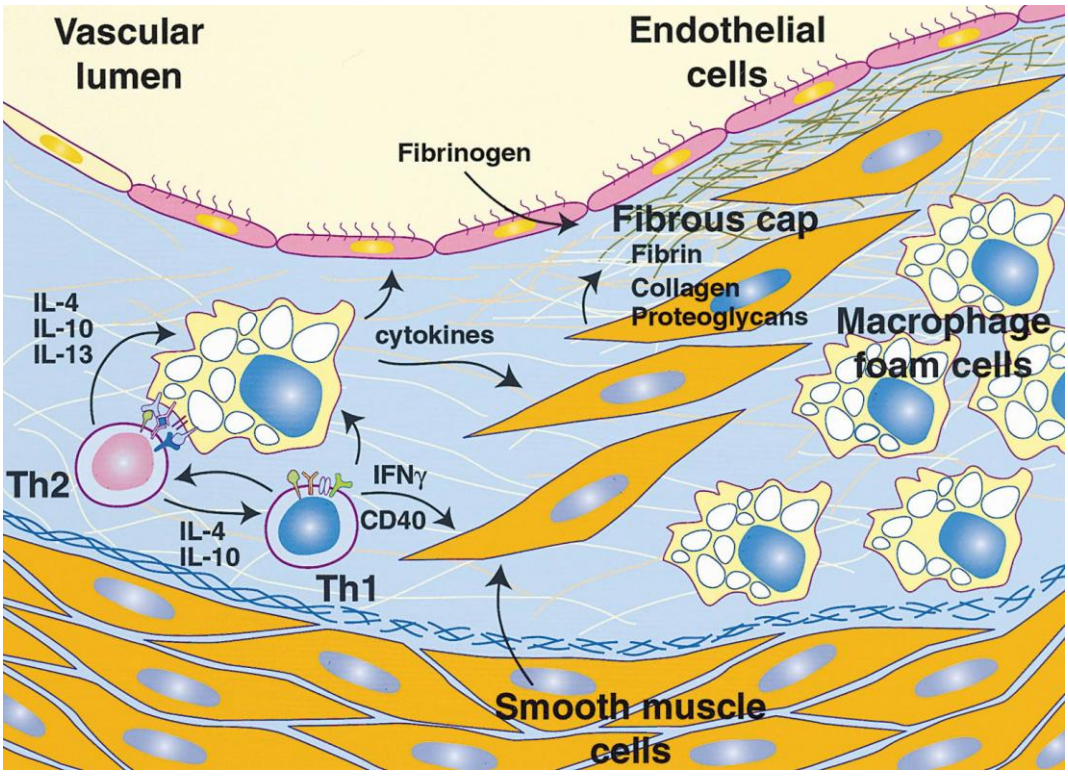

Figure 2: Lesion progression.

Interactions between macrophage foam cells, T helper cells 1 and T helper cells 2 gives rise to a chronic inflammatory process. Cytokines secreted by lymphocytes and macrophages exert both pro- and antiatherogenic effects on the cells of the vessel wall. Smooth muscle cells migrate from the media of the arterial wall to the intima, proliferate and secrete extracellular matrix proteins that form a fibrous cap (C. K. Glass & J. L. Witztum [39]).

Cholesterol esters can be mobilized from lipid droplets again by the action of hormone-sensitive lipase [39]. Cholesterol efflux from macrophages protects the cells against differentiation into foam cells. The main mechanism of cholesterol efflux is lipid transfer via ATP-binding cassette transporter (ABCA1) and ATP-binding cassette sub-family G member 1 (ABCG1) across the membrane to HDL. Free cholesterol is removed from the membranes by apolipoprotein AI (apoAI), a major protein component of HDL [42], [43]. This activity of HDL inhibits the development of atherosclerosis and explains why the risk of atherosclerosis is inversely correlated with HDL levels [44]. Cholesterol efflux is also mediated by expression and secretion of apolipoprotein E (apoE) from macrophages. Macrophages in atherosclerotic lesions synthesize and secrete apoE, which incorporates in HDL_2 and HDL_3, two small subclasses of HDL. This apoE-transfer is accompanied by the transfer of cholesterol from the macrophages to the HDL-particle. This process is dependent on lecithin:cholesterol acyltransferase (LCAT), esterifying cholesterol after transfer to the lipoprotein [45], [46]. ApoE deficiency results in severe hypercholesterolemia and atherosclerosis in humans [47]. The cholesterolester-loaded HDL is targeted to the liver for excretion.

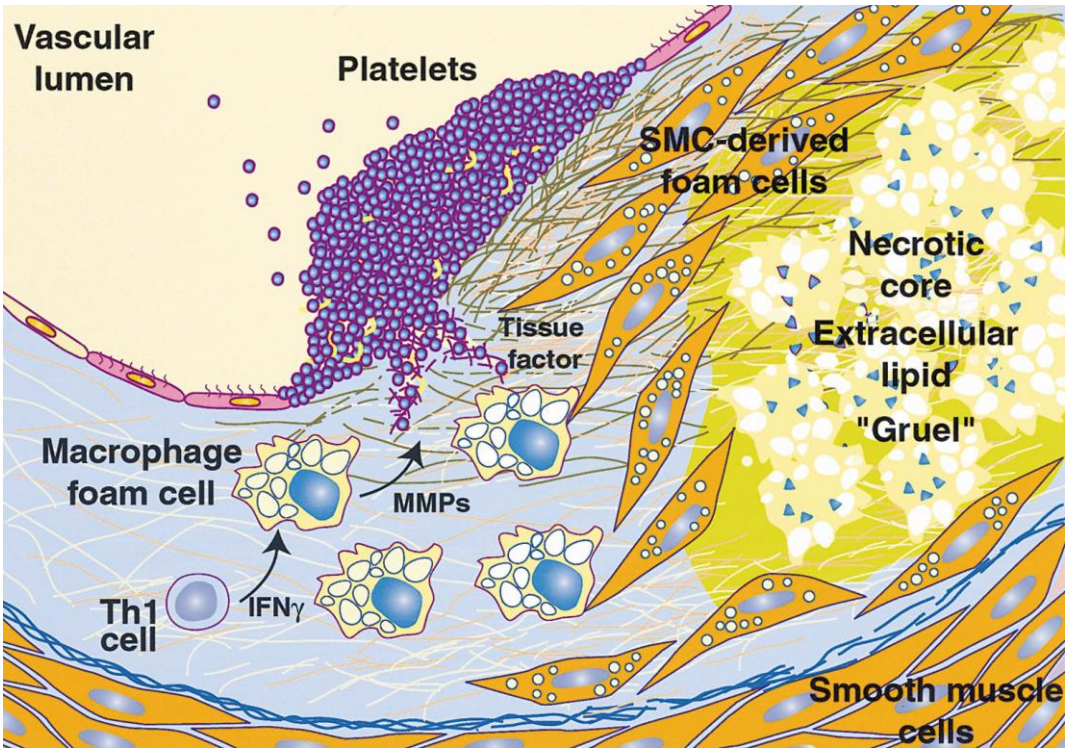

Figure 3: Plaque rupture and thrombosis.

Cell death of macrophage and smooth muscle cell-derived foam cells leads to the formation of a necrotic core and accumulation of extracellular cholesterol in atherosclerotic plaques. Secretion of matrix metalloproteinases from macrophages and neovascularization contribute to weakening of the fibrous cap. Plaque rupture exposes blood components to tissue factor, initiating coagulation, the recruitment of platelets and the formation of a thrombus (C. K. Glass & J. L. Witztum [39]).

1.1.3 Lesion progression and immunological responses

The progression of atherosclerosis and the development of fatty streaks to more complex lesions is characterized by the migration of smooth muscle cells from the medial layer of the artery wall into the intima. Intimal smooth muscle cells may proliferate, take up modified lipoproteins and contribute to foam cell formation. Furthermore, they synthesize extracellular matrix proteins such as collagen, leading to the development of the fibrous cap [2], [3], [48]. This stage of atherosclerosis is influenced by interactions between monocyte/macrophages and T cells that result in a broad range of cellular and humoral

responses. It shows the characteristics of a chronic inflammatory state with increased cytokine expression affecting the cells of the lesion [49]. The immune response may give rise atherogenic and antiatherogenic effects. Even individual immunomodulatory cytokines can elicit positive and negative effects. For example, the cytokine interferon-γ (IFN-γ) reduces scavenger receptor expression in macrophages, decreases collagen synthesis and inhibits smooth muscle cell proliferation, thus inhibiting plaque progression. However, IFN-γ also stimulates production of proinflammatory cytokines by macrophages and increases expression of major histocompatibility complex class II (MHC class II) molecules. These effects are supposed to increase accumulation of macrophages within lesions. Apo E-deficient mice, lacking the IFN-γ receptor, exhibit significantly less atherosclerosis, indicating that the net effect of IFN-γ contributes to atherosclerosis [50]. In summary, an intense immune response is ongoing in atherosclerotic lesions. The most important antigens driving activation of the immune system are not entirely known. Adducts of proteins (e. g. apoB) with aldehydolipid in oxLDL are candidates. Hörkkö et al. provided evidence for oxLDL epitopes as dominant immunogens [51].

Advanced atherosclerotic lesions can lead to ischemic conditions, plaque rupture, thrombosis and finally to myocardial infarction and stroke. Plaque rupture generally occurs in the shoulder regions of the plaque and is more frequently seen in lesions with thin fibrous caps containing high concentrations of lipid-filled macrophages and large necrotic cores [52], [53]. Studies in humans and animal models suggest that programmed cell death plays an important role in the formation of the necrotic core, as increased accumulation of apoptotic cells, decreased removal of apoptotic cells and release of insoluble oxidized lipids occur [54]. Apoptosis of macrophages and smooth muscle cells may not only be important for lesion

progression, but may also influence plaque stability, thereby increasing the risk of thrombosis.

Neovascularization is also present in human atherosclerotic lesions, associated with plaque rupture. Angiogenesis occurs in association with vascular remodeling and protease activation in surrounding tissues, suggesting that neovascularization also could contribute to plaque instability and rupture [55].

1.2 LDL oxidation and bioactive oxidation products

OxLDL exhibits a variety of biological activities related to atherosclerosis, such as induction of adhesion protein expression and subsequent recruitment of monocytes, foam cell and fatty streak formation, induction of smooth muscle cell migration and proliferation, remodeling of the extracellular matrix, changes of coagulation pathways and disturbance in the arterial tone regulation [56], [57]. Furthermore oxLDL induces apoptosis and necrosis of vascular cells [58], [59], [60]. Lipoproteins are complex particles with different susceptibility to oxidation. LDL oxidation is a continuous process ranging from minimally to extensive oxidized LDL. OxLDL particles are heterogeneous with respect to composition and biological properties. They contain many oxidized molecular species such as lipidaldehydes, oxysterols and lipid peroxides. Therefore conflicting results are often reported because the quality and composition of the lipoproteins under investigation may differ depending on experimental conditions.

1.2.1 LDL oxidation

It is believed that LDL oxidation does not take place in the blood circulation, because serum lipoprotein lipids are well protected from oxidation by antioxidant defenses and LDL itself contains most of α-tocopherol, a lipophilic antioxidant [61], [62], [63]. LDL oxidation is supposed to occur predominantly in the arterial wall. LDL may be exposed to cell-derived oxidants in the subendothelial space of the artery. It can be oxidized by transition metal ions, hemin and other catalysts and there are many tentative mechanisms by which LDL could become oxidized through several enzymes within the arterial wall. LDL undergoes oxidative modifications by incubation with macrophages, endothelial cells and smooth muscle cells [64], [65], [66], [67].

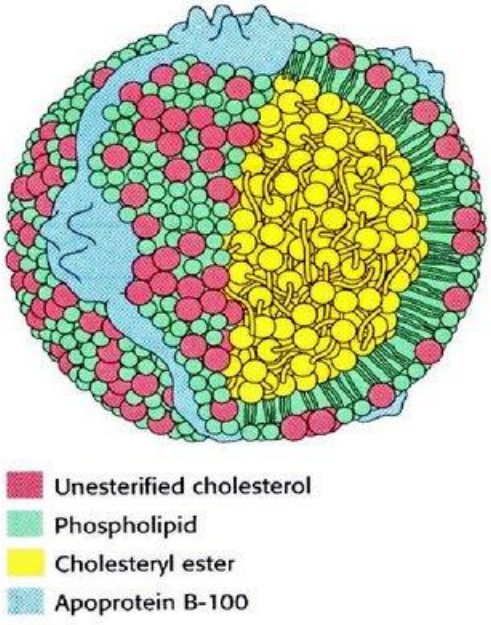

Figure 4: Composition of an unmodified LDL particle.

The core of the lipoprotein particle consists of cholesterol, triacylglycerols and some hydrophobic antioxidants. The outer monolayer contains phospholipids and cholesterol. One molecule of apolipoprotein B100 is surrounding each LDL particle, serving as a recognition site for the LDL receptor.

The core of a LDL particle contains approximately 1600 molecules cholesteryl ester (CE), mainly esterified to linoleic acid (LA), 100 molecules of triacylglycerols (TG) and only a few hydrophobic antioxidants (6 – 12 molecules of Vitamine E). The outer monolayer is composed of 600 molecules cholesterol, 700 molecules phospholipids (PL), including phosphatidylcholine (PC), sphingomyelin, 6 – 12 molecules of tocopherol and one

apolipoprotein B100 surrounding the particle [68]. The oxidizability of LDL is largely due to the presence of oxidizable lipids with polyunsaturated fatty acids (PUFAs). Large individual variations of PUFA content depending on diet are observed, ranging from 25 % to 50 % of total fatty acids [69].

1.2.2 LDL oxidation products

In the initial step of LDL oxidation, oxidants react with the most susceptible compounds, namely antioxidants, polyunsaturated fatty acids and sterols. In mammalian cells, the *sn*-2-position of diacylglycerophospholipids is frequently linked to PUFAs. Typical PUFAs containing lipids of LDL are PAPC or 1-hexadecanoyl-2-lineoyl-*sn*-glycero-3-phosphocholine. Due to the low dissociation energy of their bisallylic carbon-hydrogen bond, a hydrogen atom can easily be removed, followed by a reaction with molecular oxygen [70]. The PUFAs found in mammalian glycerophospholipids include arachidonic acid (AA), linoleic acid (LA) and longer polyunsaturated fatty acids, including docosahexaenoic or eicosapentaeonic acid. The oxidation of a single PUFA can lead to a broad variety of different reaction products, due to many different possible reaction mechanisms [71].

The first steps of PUFA peroxidation include hydrogen abstraction, rearrangement of double bonds and addition of triplet oxygen [72], [73], leading to a peroxyl radical. This radical can subsequently react in different ways, including further hydrogen abstraction, fragmentation generating truncated phospholipids and different types of low molecular weight aldehydes like 4-hydroxy-trans-2-nonenal (HNE) and malondialdehyde, or intramolecular cyclization leads to different possible isoprostanes. The oxidatively modified phospholipid acyl chains can be released from the glycerol backbone by phospholipases including platelet-activating factor acetylhydrolase or paraoxonase [71], [74]. The initial hydrogen abstraction leads to

the formation of hydroperoxide fatty acyl chains, that are unstable and reactive. Nevertheless, they can be found in human atherosclerotic plaques. The hydroperoxy fatty acyl residues can decompose, leading to various products including hydroxyeicosatetraenoic acid (11-HETE or 15-HETE) (from arachidonic acid) or hydroxyoctadecanoic acid (9-HODE or 13-HODE) (from linoleic acid). Fragmentation of HETE and HODE phospholipids leads to the formation of truncated phospholipids (PLs) such as POVPC, PGPC and the so-called γ-hydroxyalkenal PLs. Furthermore, HNE or 4-oxo-(2E)-nonenal (ONE) are released from the ω-end of the oxidized acyl chains [75]. These respective fragmentation products contain terminal carboxy or aldehyde group. In γ-hydroxyalkenal PCs the terminal oxygenated group is adjacent to an unsaturated C-C double bond [71].

Instead of formation of hydroperoxydienoyl-PLs and fragmentation to truncated PLs, peroxyl radicals can undergo cyclization leading to different isoprostanes and isolevuglandins. Free and esterified PUFAs can serve as substrates for non-enzymatic cyclisation *in vivo*, whereas the cyclization of free fatty acids can be catalyzed by cyclooxygenase [76], [77], [71].

Derivatives of oxidized cholesterol may also contribute to oxLDL bioactivity. The most abundant oxysterols found in atherosclerotic plaques are either free or esterified 7-keto- and 27-hydroxycholesterol and lower levels of 7-carboxy-, 7-hydroxycholesterol, which are mainly esterified to fatty acids. Oxysterols can also be generated through enzymatic or non-enzymatic oxidation [78]. 7-carboxycholesterol is more toxic to cultured cells than 27-hydroxycholesterol, which is more toxic than keto-cholesterol [79], [80].

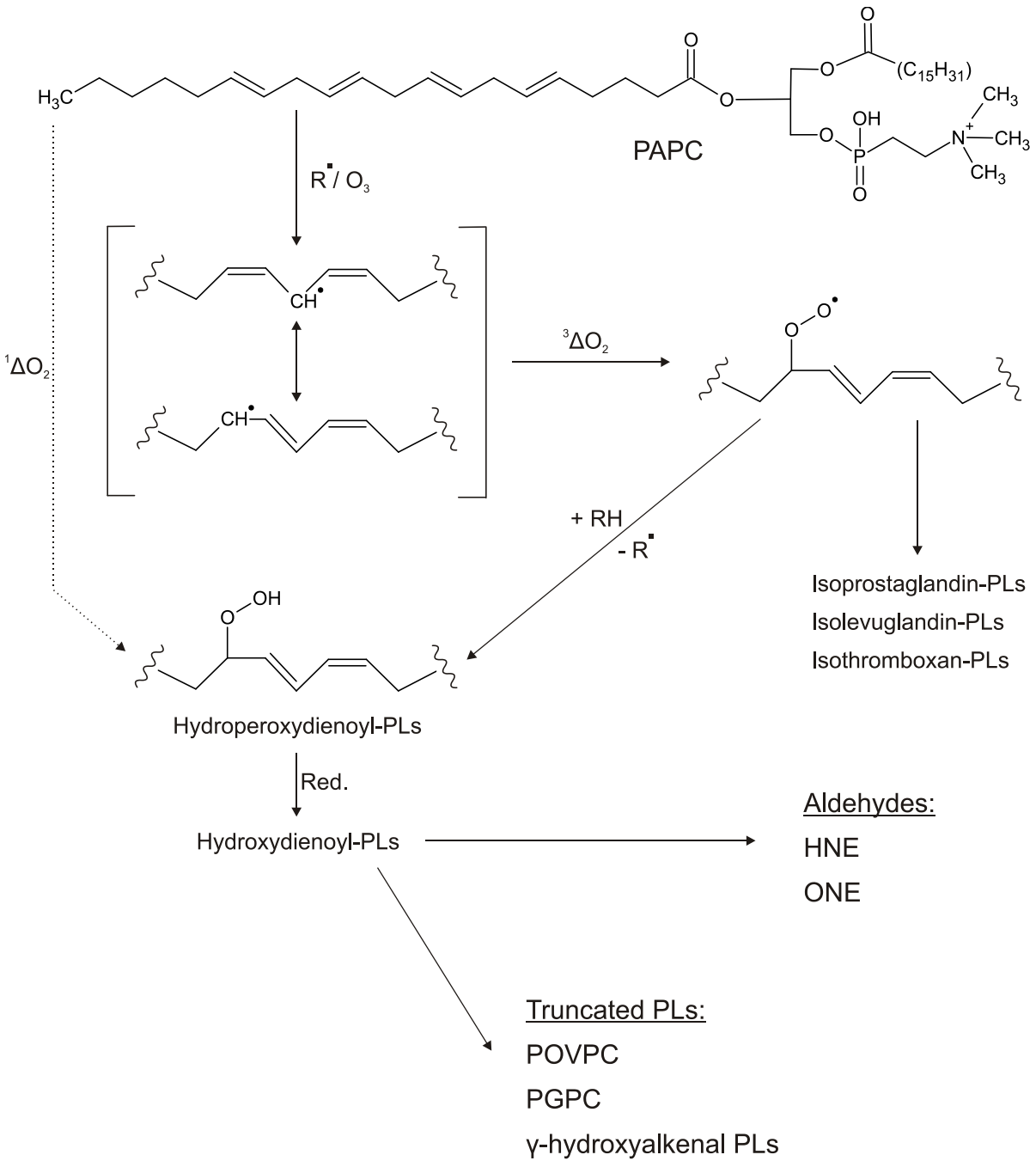

Figure 5: Free radical-induced oxidation of PAPC.

Oxidation of PLs with PUFAs leads to a big variety of oxidation products such as truncated oxidized phospholipids, short chain aldehydes, isoprostanes, isolevuglandins and isothromboxanes [71].

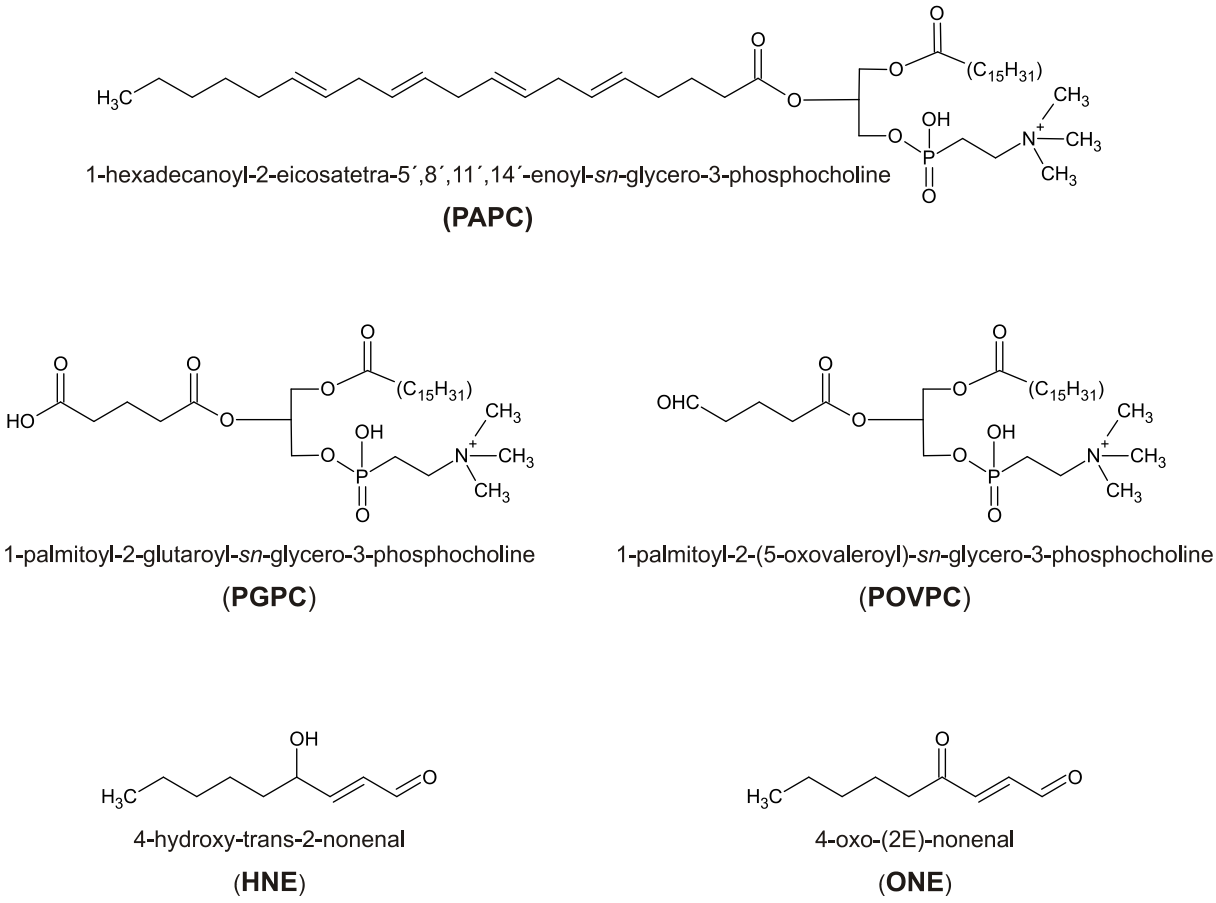

Figure 6: End products of PAPC oxidation: PGPC, POVPC, HNE and ONE.

1.3 Biological effects of oxidized phospholipids

The uptake of oxidized LDL compounds has been extensively investigated in terms of receptor- and non-receptor-mediated processes. Scavenger receptors, in particular CD36, mediate the cellular uptake of oxLDL via endocytosis. Other receptors, like platelet activating factor (PAF) receptor or toll-like receptor 2 recognize oxPLs [81], [82], [83]. OxPLs show similar molecular features with ligands of the PAF receptor [84]. It´s ligands are biologically active phospholipids, namely lysolecithin and platelet activating factor. CD36 recognizes negatively charged motifs. Therefore PGPC, a truncated oxPL with a carboxylic group in *sn*-2 position, is a candidate for recognition [85]. The group of Berliner found that the biological activities of truncated phospholipids are mainly determined by the polar *sn*-2 chain and to a much lesser extent by the structure of the polar head [86]. This underlines the Whisker

model proposed by Greenberg et al., suggesting the polar acyl chains of oxPLs protrude into the aqueous phase [87]. This tendency is much more pronounced in the PGPC than in the POVPC molecule. This Greenberg model has also been supported by molecular dynamic studies on 1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine (Paze-PC) and 1-palmitoyl-2-(9'-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine (Poxno-PC), that are homologs of PGPC and POVPC with longer acyl chain-lengths in position *sn*-2 [84]. Unlike entirely water-soluble receptor ligands, oxPLs can influence the functions of membrane-bound proteins either by direct chemical interactions (Schiff base formation) or indirect by modulating lipid dynamics and organization in the environment of membrane-bound proteins.

Furthermore, recent studies by Stemmer et al. revealed that the amphipathic character of oxPLs does not only affect their bioactivity, but also their exchangeability between extracellular donors such as oxidized LDL, albumin and cell membranes [88]. Experiments with BODIBY labeled POVPC (aldehydo-phospholipid) and PGPC (carboxylic-phospholipid) showed that LDL and albumin are able to form stable complexes with fluorescent oxidized phospholipids. Complexes with albumin and LDL are the relevant donors of oxPLs *in vivo*. LDL is a site of oxPL formation and Albumin may extract oxPLs from LDL. This property is in line with its binding affinities for other amphipathic phospholipids such as lysolecithin, which contain only one long hydrophobic fatty acid. Due to their structural differences in the *sn*-2 acyl chain, the binding mechanisms of PGPC and POVPC differ to a great extent. POVPC can form Schiff bases with amino groups of proteins and aminophospholipids, whereas PGPC can only physically interact with LDL and albumin. It is important to note that POVPC can easily exchange between albumin, LDL or cell membranes, irrespective of its covalent binding to these molecule particles. The exchangeability of POVPC is due to the instability of its covalent lipid adducts. The pK-values for Schiff bases are around 7 [68] and make the imines

prone to protonation and nucleophilic substitution by other amines, thus resulting in a very dynamic character of this covalent linkage. This underlines the notion that short-chain oxPLs including phospholipid aldehydes do not only locate to the site of their formation, but may also spread as “signal transducers” to the near cellular environment by diffusion within the tissues or to tissues far distant from the site of formation in the circulation [88].

Intracellular transport of oxidized phospholipids is also determined by their amphipathic character and the capability of aldehyde-phospholipids to form Schiff bases. Stemmer et al. showed that PGPC is quickly internalized by RAW 264.7 macrophages, whereas POVPC retained in the plasma membrane. Using single molecule microscopy, Rhode et al. showed that fluorescent PGPC partitions into endocytotic raft-like membrane domains (caveolae) for subsequent uptake [89].

1.3.1 Protein modification and molecular targets

Covalent protein modification by oxPLs, represents an important feature of their biological activity. OxPLs containing aldehyde or electrophilic α,β-unsaturated carbonyl groups react with nucleophilic groups such as thiol and amino groups (cysteinyl and lysyl residues) to from Michael adducts or Schiff bases, respectively, thus modulating protein activity. Secondary reactions of Schiff bases can produce pyrroles, lactams and hydroxylactams that are stable protein-lipid adducts, which can be detected *in vivo* [90]. Covalent modification by oxPLs can also induce polymerization of proteins resulting in protein dysfunction. Brame et al. showed that formation of isolevuglandin-PL-protein adducts impair function of the cardiac K^+ channel [91]. Furthermore γ-hydroxy-α,β-unsaturated aldehyde-PCs react with cysteinyl residues of cathepsin B, forming Michael adducts, reducing its protolytic activity [92]. Formation of covalent adducts with oxPLs also plays a key role in activation of the redox-

sensitive transcription factor, nuclear factor erythroid 2-related factor 2 (NRF2). Covalent binding of cyclopentenone isoprostanes (isoPs) to cysteines of NRF2 partner protein kelch-like ECH-associated protein 1 (KEAP-1) results in escape of NRF2 from degradation and mediates translocation to the nucleus, thus promoting transcription of NRF2 target genes [93]. For phospholipid-linked isoPs, such a covalent reaction has not been shown yet. Some protein-oxPL adducts are stable and serve as biomarkers of oxidative stress [94]. A further example for carbonylation as a biological mechanism, is the change in activation of phospholipase A_2 by Poxno-PC, a longer chain homologue of POVPC. Lipid binding abolished the lag time of phospholipase A_2, which is usually observed with this and other interfacially active enzymes on lipid surfaces [95].

Molecular targets of the truncated PL POVPC, was identified by Stemmer et al. and Gugiu et al. [88], [85], [96]. Stemmer et al. used a 2-dimensional gel electrophoresis (2-DGE) approach to label and detect target proteins of BODIBY-labeled POVPC in RAW 264.7 macrophages. For this purpose, RAW 264.7 macrophages were incubated with fluorescent POVPC and the resulting labile adducts were stabilized by chemical reduction. A comparison of the lipidated proteins pattern with total protein pattern revealed that only a selective set of cellular proteins formed Schiff base adducts with BY-POVPC. This selectivity is likely not to be due to specific molecular recognition, but rather due to differences in pK values of the individual amino groups of the target proteins. This value is influenced by surface exposure, interaction with membrane lipids and proteins in the immediate vicinity of the amino group. A detailed analysis of labeled proteins led to the identifications of proteins involved in cell death and survival, stress response, transport and lipid metabolism [88]. An analogue experiment which was performed by the same group with vascular smooth muscle cells, also showed selectivity in protein modification by BY-POVPE [97]. Guigu et al. used biotinylated oxPAPC in

human aortic endothelial cells (HAECs). Labeled proteins were separated from unlabeled proteins by affinity purification with avidin beads followed by identification of proteins by Western blotting. Selectivity of oxPL-protein adduct formation was also demonstrated with this approach [96]. Some of the identified targets were identical to the proteins identified in RAW 264.7 macrophages.

In summary, oxidized phospholipids can interact with membrane components two fold. They can bind to proteins or interact with the lipid phase of the membrane, thus leading to activation or inactivation of proteins in a more unspecific manner. Aldehydo-phospholipids can covalently link to specific proteins via Schiff base formation, or via Michael addition, if the *sn*-2 acyl chain contains α,β-unsaturated aldehydes.

1.3.2 Effects of oxidized phospholipids on cell signaling

Effects of oxPLs on several receptors on the cell surface and in the cytoplasm have been reported [94], PAF receptors, Scavenger receptors, Vascular endothelial growth factor (VEGF) receptors, Sphingosine-1-phosphate receptor, Toll-like receptors and nuclear ligand-activated transcription factors such as peroxisome proliferator-activated receptors (PPARs).

1.3.2.1 Platelet-activating factor receptor

The receptor specific for PAF is an important phospholipid mediator of inflammation and platelet aggregation. It receptor recognizes alkyl-acyl-PCs containing long alkyl chains linked by ether bonds to the *sn*-1 position in combination with acetyl residues at *sn*-2 position. The ligand with the highest receptor affinity is 1-O-alkyl-2-acetyl-sn-glycero-3-PC, which is generated from Lyso-PAF by acetylation. Oxidative fragmentation of PCs with *sn*-2-PUFAs generates compounds which also serve as ligands for the PAF-receptor. These and other

analogs of PAF were found in atherosclerotic lesions and are also formed upon LDL oxidation *in vitro* [98], [99], [100]. Although the affinity of the receptor for these ligands is 10-fold lower than for PAF, PAF-receptor stimulating PCs can reach concentrations in oxLDL, sufficient to activate the receptor [99]. PAF-like lipids can activate all major types of PAF-receptor expressing cells [101]. POVPC carries a carboxyacyl residue linked at *sn*-2 position, and a long-chain fatty acid at the *sn*-1 position. Despite this considerable structural differences between POVPC and PAF, POVPC also stimulates adhesion of neutrophils through PAF-receptor activation. This effect was inhibited by three different PAF-receptor antagonists [83]. In addition, POVPC competed with PAF for binding to macrophages and mimicked some effects of PAF in these cells. The overall biological importance of PAF receptor activation by oxPLs is not completely known. OxPL and PAF effects are cell-specific. Many effects of oxPLs on endothelial cells are not induced by PAF nor inhibited by PAF receptor antagonists [102], [103].

1.3.2.2 Scavenger receptors

The role of scavenger receptors in oxLDL uptake was already discussed in previous chapters. CD36 recognizes fragmented oxPLs [104]. CD36 has signaling activity together with proto-oncogene tyrosine-protein kinase Src (SRC) kinases FYN and LYN (tyrosine kinases), CD9 and JNK2. FYN, LYN and CD9 are recruited due to oxLDL-mediated stimulation of CD36 to a CD36-CD9 signaling complex, followed by activation of mitogen-activated kinase kinase 4 (MKK4) and JNK2 protein kinases [105]. C-Jun, a transcription factor activated by JNK2, is responsible for expression of several proinflammatory and proapoptotic genes. It was hypothesized that CD36-dependent signaling stimulated by fragmented oxPLs is responsible for hyperactivity of platelets in patients with dyslipidemia [106]. Macrophages from CD36 knockout mice

showed a high decrease of IFN-γ, MCP-1, MIP-1α and TNF-α expression in response to oxLDL, suggesting that oxPLs activate a proinflammatory CD36/CD9/Scr-kinases/JNK-pathway [107].

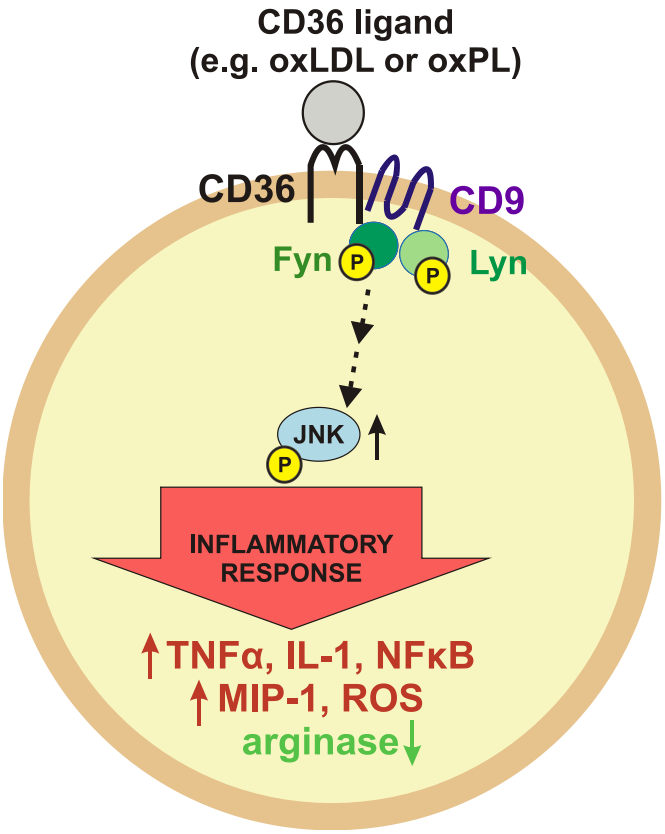

Figure 7: CD36 signaling contributes to inflammation.
Binding of oxLDL at CD36 induces the association of CD9, followed by the recruitment of the tyrosine kinases Fyn and Lyn. This protein complex serves as signaling platform, inducing the activation of inflammatory genes like TNFα, IL-1, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), MIP-1 and the generation of reactive oxygen species (ROS) (D. J. Kennedy et al. [107]).

1.3.2.3 Vascular endothelial growth factor (VEGF) receptors

OxPAPC increases phosphorylation (activation) of VEGF receptor 2 (VEGFR2) within minutes. Blocking antibodies against VEGF-A (vascular endothelial growth factor A) inhibited activation of VEGFR2 by exogenous VEGF-A [108], but did not affect the effects of oxPAPC. This result suggests that receptor activation by oxPAPC was independent from VEGF-A. It was hypothesized that trans-activation of VEGFR2 in oxPAPC-treated cells was mediated by SRC. Inactivation of VEGFR2 by siRNA and chemical inhibitors showed that this receptor plays a role in several oxPAPC-mediated effects in endothelial cells, including activation of signaling pathways (SREBP, ERK1/2) and expression of chemokine IL-8 and LDL receptor

[108]. In addition, oxPAPC stimulated VGEF-A production in HUVECs, thus stimulating angiogenesis, which is also important for the formation of atherosclerotic plaques [102].

1.3.2.4 Sphingosine-1-phosphate receptor (S1P-R)

It was shown, that oxPAPC stimulates recruitment of sphingosine-1-phosphate receptor 1 (S1P-R1) to caveolin-enriched membrane microdomains, leading to its phosphorylation (activation) by PKB. These processes were important for oxPAPC-induced activation of small GTPase RAC-1, leading to cytoskeleton reorganization and enhancement of the endothelial barrier [109].

1.3.2.5 Toll-like receptor 4

Several publications suggest that oxPLs may activate Toll-like receptor 4 (TLR4). In particular, oxPAPC mediates induction of IL-8 production in HeLa cells via TLR4 [110] and IL-6 production in macrophages via TLR4-TRIF-TRAF6 pathway [111]. The involvement of TLR4 in these processes was demonstrated by knockout or knockdown experiments. Nevertheless, the mechanisms of oxPL interaction with TLR4 remain controversial. Several groups showed that lipopolysaccharide (LPS) induced activation of TLR4 leads to increased levels of cell adhesion molecules E-selectin, ICAM-1, VCAM-1, the cytokine TNF-α, the chemokines IL-6, IL-1α, IL-1β and cyclooxygenase-2 in vascular cells, including human umbilical vein endothelial cells (HUVEC), blood monocytes, macrophages or fibroblasts. These targets are not induced by oxPLs via TLR4 [112], [113], [114]. Whether oxPLs act as direct ligands of TLR4 is still a matter of debate. It is hypothesized that a whole complex of receptors is involved in the recognition of oxPLs. Thus, oxPLs are unlikely to be canonical TLR4 ligands and do not activate the inflammatory targets of LPS in the same way. In several *in vitro* and

in vivo models, oxPLs were shown to even counteract acute inflammation induced by LPS [113]. However, this data do not exclude a role of TLR4 in oxPL-induced inflammation, and it is possible that oxPLs stimulate TLR4 only in combination with specific co-receptors and/or intracellular signaling adaptor proteins. Furthermore, effects of oxPLs on TLR4 may be selective for certain cell types and cell differentiation/activation states [94].

1.3.2.6 Peroxisome proliferator-activated receptors

PPARs are intracellular ligand-activated transcription factors. Diacyl-oxPLs stimulated a PPAR response element-driven reporter construct in transfected HAECs [115]. The effect of oxPAPC, POVPC and PGPC was mediated by PPARα, according to activation of the ligand binding domain of PPARα but not PPARγ or PPARδ [115]. Long-chain diacyl-oxPCs, such as 1-Palmitoyl-2-(5,6-epoxyisoprostane E2 oyl)-*sn*-glycero-3-phosphocholine (PEIPC) and 1-Palmitoyl-2-(5,6-epoxycyclopentenoneisoprostane)-*sn*-glycero-3-phosphocholine (PECPC), also activate PPARα in transfected HeLa cells [116]. In addition to diacyl-oxPLs, hexadecyl-azelaoyl-PC, 9- and 13-HODE were identified as ligands of PPARγ. PPARγ stimulates expression of CD36 and thereby enhances oxLDL uptake. It also stimulates liver X receptor (LXR) and cyclooxygenase-2 expression [117], [118], [119]. Upregulation of LXR by PPARγ might be important for atherosclerosis, because LXR activates transcription of ABCA1, which is an important transport-protein, mediating cholesterol efflux. The effects of oxLDL on LXR differ in macrophages and endothelial cells. LXR is upregulated in lipid-loaded macrophages due to PPARγ activation [120], but oxLDL appears to decrease LXR activity in endothelial cells [121]. Zhu et al. showed that decreased LXR activity is due to attenuated 27-hydroxycholesterol generation in oxLDL treated endothelial cells, an endogenous ligand and activator of LXR [121].

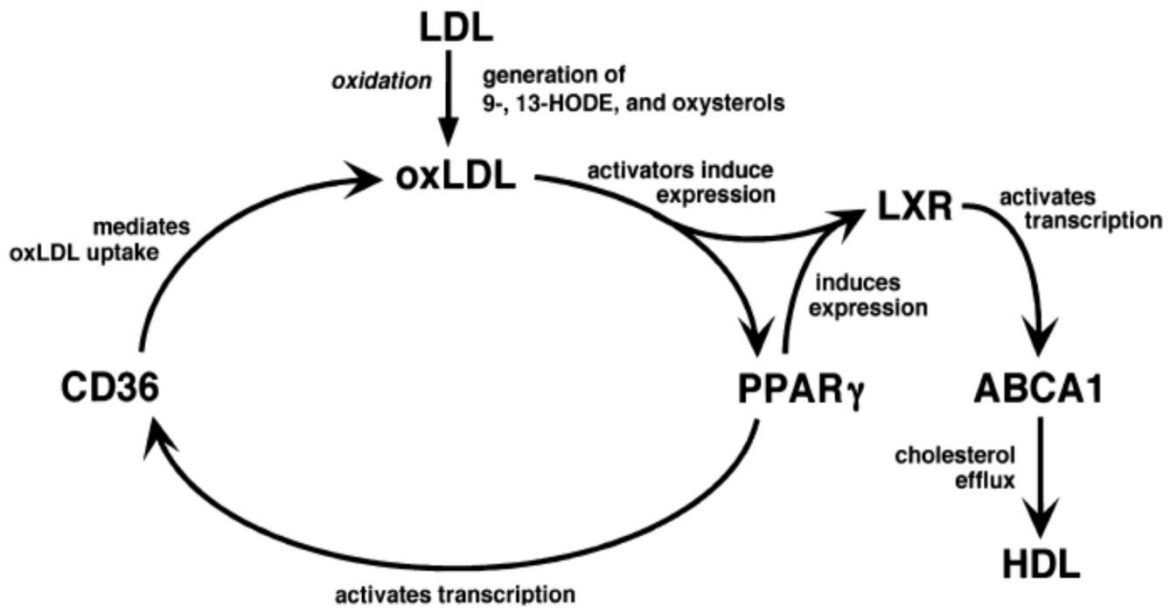

Figure 8: Model of PPARγ activation by oxPLs in macrophages.

OxLDL propagates cholesterol efflux in macrophages through upregulation of transcription of ABCA1 via PPARγ and LXR. In oxLDL treated endothelial cells LXR expression is decreased through attenuated generation of 27-hydroxycholesterol, an endogenous activator of LXR (A. Chawla et. al, [120]).

1.3.2.7 Nonreceptor mechanisms

Certain cellular effects of oxPLs are possibly mediated through various nonreceptor mechanisms. An example for a nonreceptor-mediated oxPL effect is the effect of oxPAPC or POVPC on cholesterol levels in endothelial cells. Yeh and colleagues [122] observed that treatment of endothelial cells with oxPAPC or POVPC for 4 h significantly decreased total cell cholesterol, leading to disruption of caveolae in the plasma membrane. Furthermore, activation of the lipid-sensitive transcription factor sterol regulatory element-binding protein (SREBP) and enhanced expression of IL-8 was demonstrated. These effects were reversible upon addition of exogenous cholesterol, suggesting that cholesterol levels and caveolae in the plasma membrane are correlated to IL-8 expression and activation of SREBP [122]. Furthermore, oxPLs show activation of signaling, characteristic for electrophilic and unfolded

protein stress responses. It is also possible that some oxPLs induce signaling cascades due to their biophysical interactions within the membrane, giving rise to the formation of raft-like membrane domains, which are serving as signaling platforms. The participation of PGPC in formation of such membrane domains was reported by Rhode et al. [89]. Furthermore, actions of oxPLs on membrane-bound enzymes can lead to the formation of second messengers like ceramide.

1.3.3 Second messengers upregulated by oxPLs

Changes in cytosolic Ca^{2+}-levels affect many hormones, growth factors, protein kinases and lipid mediators. MmLDL and oxPAPC induce rapid and reversible Ca^{2+}-responses in endothelial cells [123]. One of the downstream effectors of oxPAPC, related to elevated Ca^{2+} levels, was calcium-sensitive phosphatase calcineurin, that increases expression of tissue factor [124].

An important messenger generated by oxPLs is cyclic adenosine monophosphate (cAMP). OxPLs and mmLDL cause a saturable and dose-dependent increase in cAMP levels in aortic endothelial cells [125], [126], that may result from stimulation of G_s and inhibition of G_i heteromeric G-protein complexes [125]. OxPAPC and its components POVPC and PEIPC also increase intracellular cAMP levels [127]. The amount of PEIPC required for cAMP-dependent cellular processes was 10 fold lower than the required amount of POVPC, suggesting that PEIPC is an important cAMP-elevating oxPL [127]. G_s-coupled prostaglandin E2 receptors and prostaglandin D receptors were identified as mediators of cAMP elevation induced by oxPAPC and PEIPC, but not by POVPC [128]. Another study suggests activation of an adenylate cyclase-coupled membrane receptor by POVPC but not by PGPC [129]. Elevation of cAMP by oxPAPC contributes to inflammation. OxPAPC also induces cAMP-dependent

protein kinase A (PKA), which leads to phosphorylation (activation) of the transcription factor CREB. This results in enhanced expression of heme oxygenase-1, which has antioxidant and anti-inflammatory properties [130]. However, cAMP and PKA play important, mechanistic roles in barrier-protective effects of oxPLs in pulmonary vascular endothelium [131].

A further second messenger activated by OxPAPC, POVPC or PEIPC is the small GTPase R-RAS in endothelial cells, which stimulates expression of fibronectin, thus promoting entry of monocytes into sites of chronic inflammation [127].

OxPLs also induce the formation of the mediator ceramide. Ceramide is a sphingolipid, which is not only involved in modulation of lipid membranes, e. g. contributing to raft and microdomain formation, it can also activate protein kinases.

1.3.4 Intracellular signaling activated by oxPLs.

Small GTPases control various cellular functions including cell proliferation, gene expression, cell motility, regulation of monolayer integrity, and membrane barrier properties [94]. GTPases, whose activity is modulated by oxPLs, are RHO, RAC, CDC42 and RAS family GTPases. These enzymes influence the actin cytoskeleton, cell adhesion, and cell motility [132]. RAS family GTPases mainly control mitogen-activated protein kinase (MAPK) signaling and gene expression [133]. OxPAPC and its bioactive components POVPC and PEIPC induce R-RAS activation by a cAMP-dependent mechanism, leading to enhanced α5β1-integrin-dependent monocyte binding to aortic endothelial cells [127].

Despite the inflammatory effects of oxPLs, oxPAPC and oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphoserine (PAPS) elicit antiinflammatory properties at low concentrations (20 µg/ml) and contribute to the restoration of the endothelial barrier function. This

antiinflammatory effect is mediated through activation of RAC and CDC42 in pulmonary endothelial cells [134], [131], [135]. RAC and CDC42 play a crucial role in enhancement of peripheral endothelial cytoskeleton and endothelial cell barrier properties. CDC42 mediates formation of filopodia [136], [137] and RAC-dependent lamellopodia extension, formation of new cell adhesion sites and enhancement of peripheral endothelial cytoskeleton [138], [139], [137]. PEIPC was identified as the bioactive oxidized species of oxPAPC, whereas PGPC and POVPC did not activate RAC- or CDC42-dependent mechanisms in endothelial cells [134]. A crosstalk between RAC and RHO, leads to significant attenuation of RHO signaling [135]. Both, oxPAPC and oxPAPS attenuate RHO activation by a mechanism, which is not fully elucidated. The current models involves direct PKA-mediated RHO phosphorylation [140] or modulation of RHO-specific guanine-nucleotide exchange factors (GEFs) by PKA, PKC and SRC, known to be activated by oxPAPC [135], [141], [94].

Furthermore, oxPAPC, which is mainly a mixture of POVPC, PGPC and PEIPC, profoundly modulates gene expression [142]. The mechanisms of gene regulation activated by oxPLs often involve different transcription factors than those, activated by normal ligands. For example oxPLs induce VEGF-A expression independent of its major transcription factor HIF-1, but through activation of activating transcription factor 4 (ATF4). IL-8 expression, normally regulated by the NF-κB pathway, is induced by oxPLs independent NF-κB, but likely involve SREBP, STAT3- and PPARα-dependent mechanisms [122], [143], [144]. Expression of tissue factor is also typically induced via NF-κB dependent transcription. Instead, oxPLs induce the expression of this blood clotting initiator via transcription factors early growth response protein 1 (EGR-1) and nuclear factor of activated T-cells (NFAT) [124].

1.4 Metabolism and bioactivity of sphingolipids

1.4.1 Chemical properties of sphingolipids and sphingolipid metabolism

Sphingolipids represent an important class of lipids that are membranes constituents and signaling molecules in eukaryotes [145]. Ceramides are particularly important components of the sphingolipid network. They contain a sphingoid base to which a fatty acyl residue is linked by an amide bond. The fatty acid chain length of ceramide can vary to a considerable extent, with C_{16}- to C_{24}-ceramide being the most abundant species in mammalian cells. These fatty acids are mostly saturated, some are monounsaturated, and sometimes they may contain a hydroxyl group at the C_2-position or the terminal C atom [146], [147]. Biophysically, ceramides are among the least polar, most hydrophobic membrane lipids [148]. For a long time, sphingolipids were mainly considered to play a structural role in membranes formation. More recent research has revealed that many members of the sphingolipid family, including ceramide (Cer), sphingosine (Sph), Sph-1-phosphate (S1P) and Cer-1-phosphate (C1P) as bioactive molecules involved in signal transduction pathways, protein sorting and cell-cell interactions and recognition. Sphingolipids contain two hydrophobic chains and show little spontaneous movement between membranes. Intermembrane transfer is mediated by vesicle transport and/or transfer proteins (e. g. ceramide transfer protein (CERT)). In membranes, sphingolipids may form clusters with or without sterols to form lipid microdomains or rafts, which function as hubs for signal transduction and protein sorting [149].

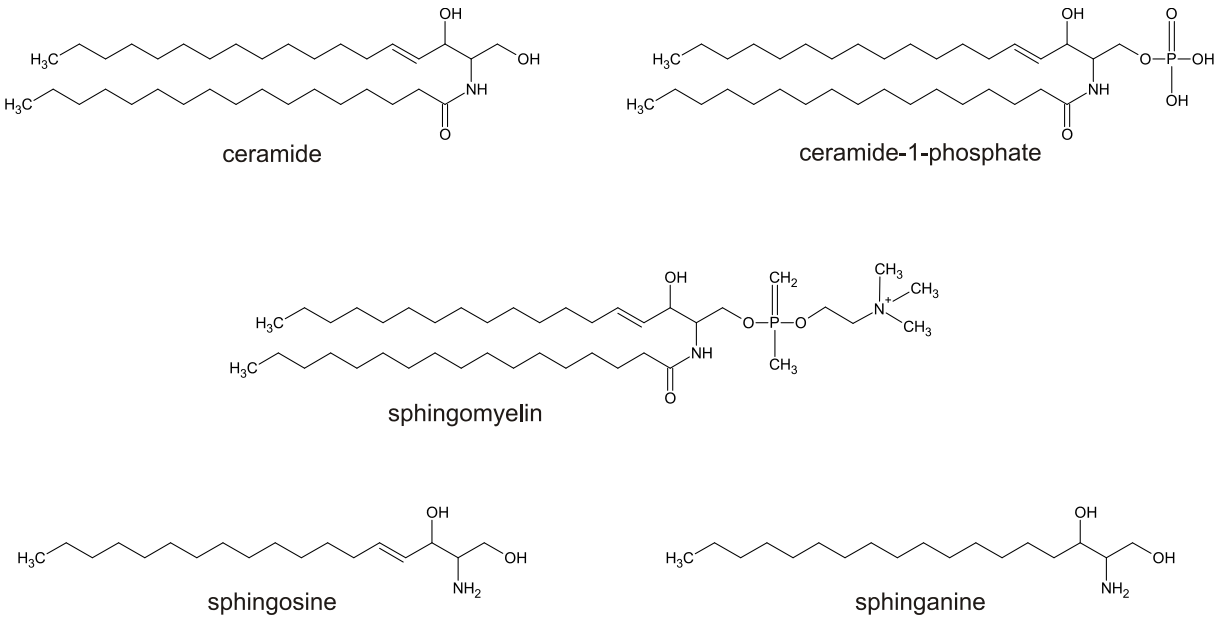

Figure 9: Structures of sphingolipids.

Various exogenous stimuli such as TNF-α, interleukin-1, Fas ligand, ionizing radiation [150], phorbol esters [151], heat stress [152], oxidative stress [153] and chemotherapeutics [154] induce formation of ceramide. Metabolically, ceramide can be formed by at least two distinct pathways. It can be generated through *de novo* synthesis, hydrolysis of complex sphingolipids, especially sphingomyelin (SM) and the salvage pathway, utilizing sphingosine after ceramide hydrolysis. The *de novo* pathway starts with the condensation of serine and palmitoyl-CoA catalyzed by serine palmitoyltransferase. 3-keto-dihydrosphingosine is formed, which is subsequently reduced to form dihydrosphingosine (sphinganine). Sphinganine is further N-acetylated by (dh) Cer synthases (CerS) to produce dihydro-Cer [155]. Six mammalian genes encoding (dh) Cer synthases have been cloned. Six isoforms (CerS 1-6) are known that show substrate preference for specific chain length fatty acyl CoAs, resulting in the generation of specific ceramide species. Dihydro-Cer is further desaturated by dihydro-Cer desaturase, generating ceramide with a 4,5-*trans*-double bond. Once ceramide is formed it is primarily used for the synthesis of SM. In this reaction step a phosphocholine headgroup is transferred by SM synthase from phosphatidylcholine to

ceramide [156]. Cer can be phosphorylated by ceramide kinase (CERK) [157] or glycosylated by glucosyl or galactosyl Cer synthases [158].

Breakdown of complex sphingolipids (glycosphingolipids or sphingomyelin) represents a second important source of Cer formation. Glucosylceramide (GluCer) and galactosylceramide (GalCer) are sequentially hydrolyzed by specific β-glucosidases and galactosidases to release Cer [159]. SM cleavage is catalyzed by one of several sphingomyelinase isoforms (SMases), transferring phosphocholine to diacyglycerol and thereby generating Cer. The SMases are distinguished according to their pH optima and subcellular localization. Several SMases have been characterized: lysosomal acid SMase (aSMase), zinc-dependent secretory SMase, neutral magnesium-dependent SMase (nSMase), and alkaline SMase [160].

Cer can be degraded by ceramidases, cleaving the amide-linked fatty acid to form Sph. Three types of ceramidases have been classified based on their pH optima: acid ceramidase (aCDase), neutral ceramidase (nCDase) and alkaline ceramidase. Sph can be phosphorylated by Sph kinases, SK1 and SK2, to form S1P [161]. In turn, S1P phosphatases dephosphorylate S1P regenerating Sph [162]. Finally, S1P lyase can remove S1P from the sphingolipid network by splitting the lipid into ethanolamine phosphate and hexadecenal [163].

Enzymatic reactions of the sphingolipid metabolism localize to different cellular compartments [164]. The initial steps of SL *de novo* synthesis leading to Cer formation take place on the cytosolic surface of the endoplasmic reticulum (ER) and potentially ER-associated membranes, such as the perinuclear membrane and the mitochondria membranes [165]. Synthesis of SM and GluCer localizes to the Golgi apparatus. Two specific pathways are responsible for the transport of Cer from the ER to the Golgi. The first pathway is catalyzed by CERT, and the second pathway provides Cer for the synthesis of GluCer by

vesicular transport. In this pathway, the transport protein four-phosphate-adaptor protein 2 (FAPP2) is a key mediator, delivering GluCer as precursor for glycosphingolipids (GSL) synthesis [166]. The synthesis of complex GSLs (e. g. gangliosides) occurs in the luminal side of the Golgi. Therefore, GluCer needs to flip from the cytosolic surface to inside the Golgi, which is facilitated by the ABC transporter, P-glycoprotein [167].

SM and complex GSLs are subsequently transported to the plasma membrane by vesicular trafficking. At the plasma membrane, or tightly associated in the lysosomes, SM can be catabolized to Cer, either by aSMase on the outer leaflet of the membrane or in the lysosomes, or by nSMase, which localizes to the inner leaflet of the bilayer. Degradation of complex sphingolipids may serve two purposes. Formation of Cer through SMase activity in the plasma membrane triggers signaling and Cer degradation to Sph in lysosomes makes Sph available as substrate for CerS in the ER (salvage pathway). SM and GluCer are degraded by aSMase and glucosidases to form Cer in the lysosomal compartment, followed by degradation of Cer by aCDase to form Sph. Due to its positive charge, Sph is able to leave the lysosome and moves between membranes, including the ER, where it is recycled [168]. Whereas degradation of SM at the plasma membrane facilitates micro domain formation, serving as a hub for signal transduction, degradation of SM through aSMase in the lysosomes may preferably direct Cer to recycling. Both processes may occur consecutively in some cases, also depending on the activity of ceramide degrading enzymes. The recycling pathway of complex sphingolipids back to the ER represents an important link between sphingolipid catabolism at the plasma membrane (or lysosomes) to sphingolipid metabolism in the ER. Zeidan et al. demonstrated such a link in his study about the sphingolipid response to phorbol 12-myristate 13-acetate (PMA) in MCF-7 mammary carcinoma cells. ASMase was activated followed by an increase in cellular ceramide levels concomitantly with a drop in

sphingomyelin levels. Interestingly, increased ceramide levels were abolished after FumonisinB_1 treatment, which is a potent inhibitor of CerS, but could not be prevented by myriocin treatment, an inhibitor for SPT [169]. These data suggest that the aSMase response to PMA, results in ceramide recycling and transfer of Sph to the ER through the salvage pathway. This Sph supply can bypass SPT and can provide CerS with substrate [169].

A. Loidl et al. showed that oxLDL and its bioactive components POVPC and PGPC trigger activation of aSMase in vascular smooth muscle cells and RAW 264.7 macrophages [58], [59], [170]. Whereas POVPC caused a quick response after 15 minutes of incubation, PGPC revealed no such quick effect at a concentration of 25 µM. In this study it was demonstrated, that both oxPLs, PGPC and POVPC affect aSMase activation at different concentrations, but probably by a different mechanism. The results of this study will be discussed more detailed in chapter 2 of this thesis. Furthermore, Kitatani et al. reported that oxLDL treatment of RAW 264.7 macrophage-like cells for 6 h led to increased ceramide levels. The increase in ceramide concentration was no longer seen after pretreatment of cells with FumonisinB_1, an inhibitor for CerS isoforms, suggesting *de novo* ceramide synthesis being involved in the oxLDL response [171]. L. Hallasidappa found that the oxLDL effect on *de novo* ceramide synthesis is caused by POVPC or PGPC that are bioactive compounds of oxidized lipoprotein particles.

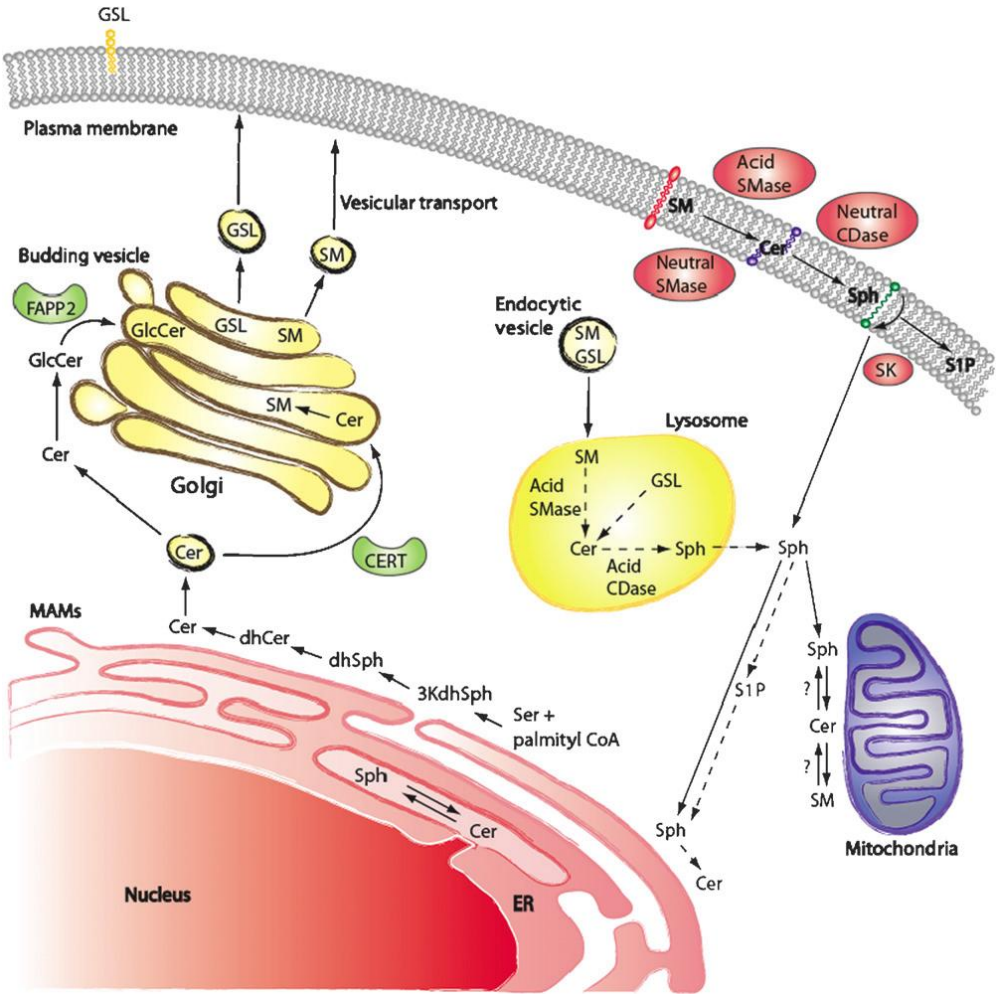

Figure 10: Compartmentalization of metabolites and enzymes of the SL pathways.

Glycoshphingolipid (GSL), sphingomyelin (SM), ceramide (Cer), sphingosine (Sph), sphingosine-1-phosphate (S1P), sphingomyelinase (SMase), ceramidase (CDase), serine (Ser), 3-keto-dihydrosphingosine (3KdhSph), dihydrosphingosine (dhSph), glucosylceramide (GlcCer), four-phosphate-adaptor protein 2 (FAPP2), ceramide transfer protein (CERT), mitochondria associated membranes (MAMs), endoplysmic reticulum (ER), (N. Bartke & Y. Hannun [164]).

1.4.2 Ceramide and ceramide-1-phosphate bioactivity in cell stress

Understanding of sphingolipid bioactivity and how they transmit signals requires elucidation of the mechanisms by which these lipids act. One could envision two general mechanisms for the action of sphingolipids: lipid-lipid interactions, whereby the bioactive lipid affects membrane structure and/or the interaction of membrane proteins with the membrane bilayer; or lipid-protein interactions, whereby changes in lipids modulate the function of target proteins that interact specifically with the bioactive lipid [172].

The physiological levels of bioactive lipids influence their mechanisms of action. Trace lipids such as S1P interact with high-affinity receptors that are capable of sensing their low levels. Lipids, present at intermediate membrane concentrations, such as ceramide, act on targets with intermediate affinity, and changes in local concentrations can alter membrane properties. By contrast, it is difficult to envisage how abundant lipids such as SM can have specific targets, because the affinity of interactions must be low. This does not rule out proteins that may bind to these lipids with high affinity [172].

1.4.2.1 Ceramide

Several candidate proteins have been shown to interact with ceramide *in vitro* and in cells. These include ceramide-activated Ser-Thr phosphatases (CAPPs), such as PP1 and PP2A, which bind ceramide *in vitro* [173]. They are modestly activated by ceramide and show a preference for the natural stereoisomers. Several studies in cells have demonstrated a causal relation between ceramide formation, CAPPs activation and protein dephosphorylation. For example, ceramide-inducing agents, such as TNF-α or cell loading with palmitate, induce dephosphorylation of the retinoblastoma gene product RB [174], PKCα [175], protein kinase B (PKB or AKT) [176] and other proteins in a ceramide-dependent

manner. However, direct cellular activation of phosphatases by ceramide has not been shown on the molecular level [172].

Ceramide has been shown to activate PKCζ [177], [178], the kinase KSR [179] and cathepsin D [180]. The latter enzyme has been proposed as a specific target for lysosomally generated ceramide, and may couple the action of lysosomal aSMase to the mitochondrial pathway of apoptosis. Activation of PKCζ by ceramide has been correlated with the regulation of membrane potential, inhibition of AKT and pro-apoptotic functions [180].

In mammalian cells, *de novo* synthesis of ceramide is enhanced in response to some chemotherapeutic agents, such as etoposide and daunorubicin [154], [181], and other inducers of apoptosis, such as compounds stimulating the B-cell receptor [182]. The pro-apoptotic FAS (death receptor) pathway, which also stimulates *de novo* ceramide synthesis, has also been shown to activate protein phosphatase 1. This process results in dephosphorylation of splicing proteins (SR proteins), which are regulators of alternative splicing of caspase-9b and pro-survival Bcl-x_L. Dephosphorylation of SR proteins directs pre-mRNA processing to the splicing variants Bcl-x_S and caspase-9, which leads to apoptosis [183].

Multiple studies have indicated that sphingoid bases, generated by *de novo* synthesis, mediate specific responses to heat stress [184]. The responding proteins are involved in regulation of nutrient permeases [185], cytoskeletal changes [186], cell-cycle arrest [187] and RNA translation in yeast [188]. Although the significance of this pathway is well established, several key questions remain not understood. An increase in *de novo* sphingolipid synthesis does not necessarily lead to ceramide accumulation if the ceramide consuming pathways are more efficient (e.g. transformation to SM, C1P or glucosylceramide). For instance, in some cases SM synthase and glucosylceramide synthase

(GCS) are suppressed, leading to elevated levels of ceramide from *de novo* synthesis and as a consequence to apoptosis. It is also not clear if the *de novo* pathway acts exclusively in the ER or also in other ER-associated membranes, such as mitochondrial-associated membranes or the nuclear membrane [172].

1.4.2.2 aSMase and the salvage pathway in stress signaling

Multiple stress stimuli, such as ultraviolet and ionizing radiation, ligation of death receptors and chemotherapeuthic agents (including platinum, paclitaxel and histone deacylase inhibitors) have been shown to activate aSMase, usually within minutes of cell stimulation. This quick response is indicative for post-translational modification of aSMase. In a few studies, activation of aSMase was shown to be accompanied by protein translocation to the plasma membrane and the simultaneous generation of ceramide [172]. Downstream targets of aSMase activity were mostly elucidated either through investigating genetic mutants of the enzyme (for example patients with Niemann-Pick disease [189]), knockout mice that lack aSMase [190], small interfering RNA (siRNA) mediated knockdown [191] or pharmacological inhibitors [192]. These studies provided evidence that aSMase mediates apoptotic and stress response to ionizing and UV radiation [190], [169], and to FAS ligands [193].

The mechanisms underlying aSMase activation in response to stress agents are not well understood. Recent results showed that the enzyme is activated by reactive oxygen species (ROS), and perhaps more specifically, by nitrosative stress [183]. Other recent studies provided evidence for stress-induced activation of aSMase by phosphorylation through PKCδ, which was implicated in mediating the effects of UV radiation [191]. In this case, aSMase has been proposed to generate ceramide either in the lysosome or at the plasma membrane. In the lysosome, ceramide has been shown to activate the protease cathepsin D,

which possibly causes cleavage and activation of the pro-apoptotic protein BID [180]. At the plasma membrane, the actions of ceramide are less well defined, but have been suggested to mediate receptor capping and/or microdomain formation [192].

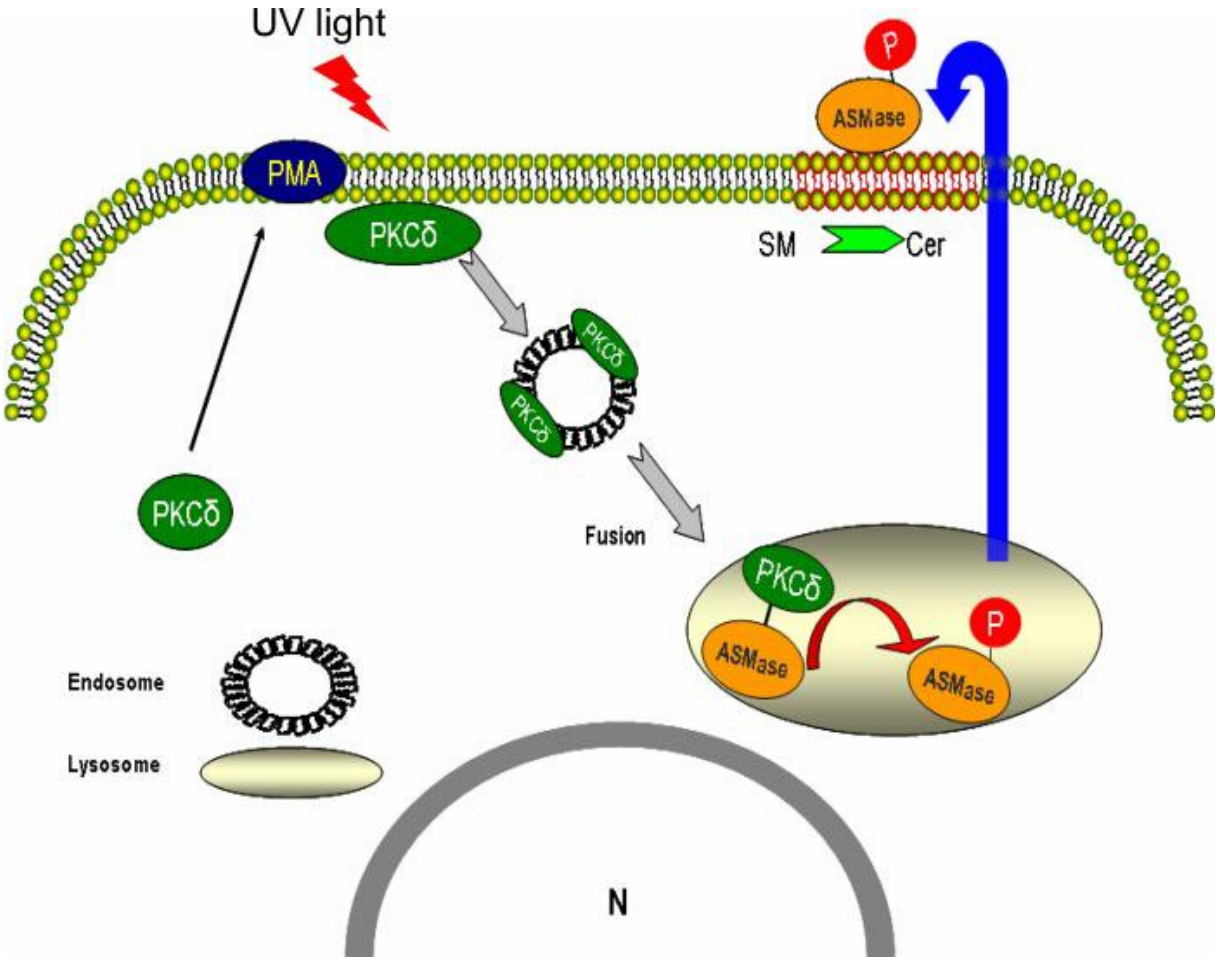

Figure 11: Hypothetical model for aSMase activation by PMA.

The model depicts PKCδ as a kinase upstream of aSMase activation. After translocation to the plasma membrane, PKCδ imigrates to endosomes, causing aSMase phosphorylation at Ser508. The phosphorylated form of aSMase is subsequently targeted to the plasma membrane, where it hydrolyzes membrane SM to ceramide (Y. Zeidan & Y. Hannun [169]).

A more recent a study has shown, that activation of aSMase can lead to increase in ceramide formation through concomitant activation of *de novo* synthesis [194]. As mentioned before, sphingolipid metabolism at the plasma membrane is connected to *de novo* ceramide

synthesis in the ER via the salvage pathway. In this pathway, ceramide generated at the plasma membrane is degraded to sphingosine and recycled to the ER to supply the ceramide synthases. This could explain some of the apparent discrepancies in the literature in which both, aSMase and the *de novo* pathway have been implicated in several cellular responses. Importantly, the commonly used inhibitor FumonisinB_1 inhibits ceramide synthases, but it is not possible to distinguish between the CerS substrates deriving from *de novo* synthesis or from the salvage pathway [172].

1.4.2.3 Neutral SMase2 in stress signaling

Several studies report that nSMase is involved in extracellular cytokine and stress responses [195]. The list of inducers shows much overlap with activators of aSMase. NSMase is acutely activated by the cytokines TNF-α and IL-1. It has been shown to mediate the effects of IL-1 on signal transduction [196], and the effects of TNF-α on gene induction, involving endothelial nitric oxide synthase (eNOS) [197]. The enzyme is also induced during ageing in mouse hepatocytes and mediates the decrease in responsiveness of hepatocytes to IL-1 signaling [198]. nSMase2 has been shown to mediate at least in part the effects of TNF-α on cell adhesion and migration [199].

Several studies suggest a role for nSMase2 in the cytotoxic action of amyloid peptide-β [200], [201]. Persistent activation of the enzyme has been observed upon cell confluency and has been suggested to mediate cell-cycle arrest, which is induced by cell contact [202].

The mechanisms involved in the activation of nSMase2 are poorly known. NSMase 2 is considered to be distributed between Golgi apparatus and the plasma membrane [203]. TNF-α has been shown to induce translocation of the enzyme mainly to the plasma membrane in a p38 MAPK dependent mechanism [199]. In confluent cultured cells nSMase2

resides mostly at the plasma membrane. As mentioned before, it appears to localize to the inner leaflet of the plasma membrane, where only a minor pool of SM may reside [204].

1.4.2.4 Ceramide-1-phosphate

A major metabolite of ceramide is C1P, which can be formed through phosphorylation of ceramide by ceramide kinase. Several studies suggest that C1P is involved in regulation of cell proliferation, suppression of apoptosis [205], [206], inflammatory response [207], [208] and phagocytosis [209], [210]. Recent analyses by Merrill and co-workers have provided information about the amounts of SM, Cer and C1P in RAW 264.7 macrophages (5310, 300 and 1300 pmol/mg protein, respectively (www.lipidmaps.org)). Therefore the C1P amounts of RAW 264.7 cells are several fold higher than the concentration of Cer. One might speculate that this high level of antiapoptotic C1P might be responsible for the relatively high resistance of these cells to enter apoptosis [211].

The first report on C1P bioactivity was on the stimulation of DNA synthesis and cell division in rat fibroblasts [206]. In this study short-chain C1Ps were added to the culture media (C_2-C1P and C_8-C1P). These compounds partially reversed the morphological changes that were induced in rat fibroblasts after prolonged serum deprivation, which is a condition inducing apoptosis. Furthermore short-chain C1Ps decreased the detachment of serum-starved cells from culture dishes [205]. Like short-chain C1Ps, a mixture of long-chain C1Ps also stimulated DNA synthesis. This effect was accompanied by an increase in the levels of proliferating cell nuclear antigen [212], which is a marker of active DNA synthesis [213]. Nevertheless, C_8-C1P has not been found in biological systems and C_8-Cer is a poor substrate for CERK [214]. The involvement of short chain C1P in metabolic or signaling pathways is not well characterized. Induction of extracellular signal-regulated kinase (ERK) phosphorylation

in human osteoblastic cells was stimulated by short chain C1P [215]. Furthermore C_2-C1P or C_8-C1P induced Ca^{2+} mobilization in calf pulmonary artery endothelial cells [216], thyroid FRTL-5 [217], or Jurkat T-cells [218]. In the latter report, Colina and colleagues showed that Ca^{2+} mobilization by C_8-C1P was dependent on inositol 1,4,5-triphosphate accumulation. In contrast, short-chain C1Ps did not induce Ca^{2+} mobilization in fibroblasts [206], [212] or in neutrophils [219]. Long chain C_{16}-C1P failed to influence intracellular Ca^{2+} concentrations in lung adenocarcinoma A549 cells [220]. Whether or not long-chain C1P is able to affect Ca^{2+} homeostasis in any cell type still remains to be investigated.

There is also evidence for stimulation of cell proliferation or inhibition of apoptosis by long chain C1P. The group of Gomez-Munoz reported that C_{16}-C1P blocked cell death in bone marrow-derived macrophages (BMDM) that were incubated in absence of M-CSF [221], a condition known to induce apoptosis in these cells [222], [223]. C1P blocked DNA fragmentation and the stimulation of caspase-9/caspase-3, thereby suggesting that the prosurvival effect of C1P was due to inhibition of apoptosis. Mechanistic studies revealed that C1P exerts its antiapoptotic effect by entirely inhibiting aSMase and ceramide accumulation, which is a crucial step in the apoptotic program of M-CSF deprived BMDM. An important observation in this study was that C1P blocked aSMase activity also in cell homogenates, suggesting that inhibition occurs by direct physical interaction with C1P. It was concluded that C1P is an inhibitor of aSMase, and that inhibition of this enzyme activity is a major mechanism in C1P-mediated cell survival [221]. aSMase activity is also inhibited by S1P in living macrophages, but this inhibitory effect is not mediated by direct lipid-enzyme interaction. As C1P not only affects aSMase activity, but also SPT activity, it is likely to be a sphingolipid regulator of ceramide generation. This important function of C1P will be further discussed below. The physiological relevance of prosurvival C1P effects is emphasized by the

observation that intracellular levels of this lipid are substantially decreased in apoptotic macrophages. It was assumed that the decrease in C1P concentration could prevent inhibition of aSMase, thereby triggering ceramide generation and apoptosis [221].

Another role of C1P as a promoter of cell survival might be based on activation of phosphatidylinositol 3-kinase (PI3-K), which is a target of growth factors. PI3-K in turn activates the transcription factor NF-κB and expression of antiapoptotic genes. A major product of PI3-K is phosphatidylinositol (3,4,5)-triphosphate (PIP3), which directly inhibits aSMase activity [224]. Therefore, PI3-K activation may potentiate the inhibitory effect of C1P on aSMase through generation of PIP3. Whether C1P and PIP3 bind to the same or different domains of aSMase remains to be subject of further investigations.

C1P also stimulates phosphorylation of PKB which is a target of several kinases of different signaling pathways, including PI3-K [225], [226]. Inhibition of PI3-K abolished PKB phosphorylation and blocked the prosurvival effect of C1P, indicating that PKB acts downstream of PI3-K. [227]. Another interesting observation was that C1P caused IκB (inhibitor of NF-κB) phosphorylation and stimulation of NF-κB in cultures of primary mouse macrophages. Pro-survival Bcl-X_L was reported to be a downstream target of NF-κB under these conditions. In summary, these results suggest that C1P to mediates its antiapoptotic effect through activation of the PI3-K/PKB/NF-κB/Bcl-X_L pathway in mammalian cells [227]. Furthermore it should be mentioned that C1P stimulates phosphorylation of cAMP or cAMP-dependent PKA [228], [229] and PKC-ζ [230].

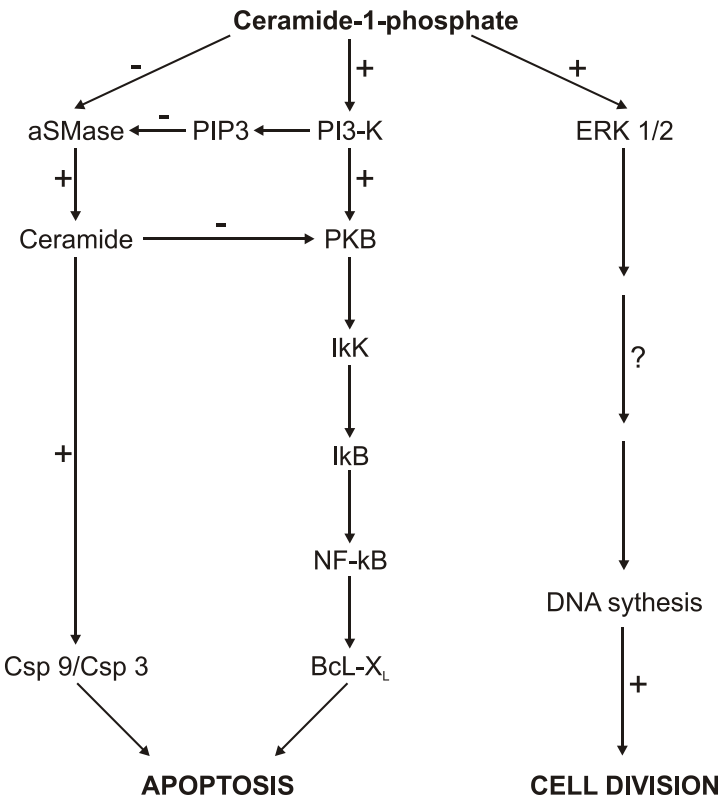

Figure 12: Mechanistic model for stimulation of cell survival and of cell growth by ceramide-1-phosphate.

C1P prevents apoptosis by inhibition of aSMase through direct interaction with the enzyme and by stimulating the formation of PIP3. In addition, C1P activates ERK1/2, which stimulates cell proliferation (A. Gomez-Munoz [211]).

Since C1P inhibits SPT activity, it also regulates *de novo* ceramide synthesis. In this study [231], apoptosis of NR8383 alveolar macrophages was investigated under conditions of serum deprivation. After preincubation with [^{3}H]palmitate for 24 h, labeled SM was formed in these cells. Serum deprivation did not induce formation of labeled Cer from labeled SM hydrolysis, suggesting that neither aSMase nor nSMase play an important role under investigated conditions. However, a sharp increase in labeled ceramide from *de novo* sphingolipid synthesis was observed. Myriocin, a specific inhibitor of SPT, abolished serum deprivation induced ceramide formation. Synthesis of ceramide was also blocked after addition of exogenous C1P. This lipid inhibited SPT in lysates of macrophages too, which had

been incubated in serum free medium for 48 h, to increase the activity of this enzyme. Overall, these results suggest a direct physical interaction of the sphingolipid with SPT [231], similar to the inhibitory effect of C1P on aSMase in BMDM [221]. This hypothesis is further supported by the finding, that SPT expression levels was not changed under these conditions [231].

The inhibitory effects of C1P on aSMase and SPT support the assumption that this sphingolipid represents a key regulator of ceramide synthesis and functions as an antagonist of ceramide in terms of biological activity. For all these reasons, the ratio of ceramide to C1P must be strictly controlled in resting cells since a change of this value decides between life or death. It is important to note that different cell types contain different levels and ratios of these sphingolipids. Any change of this equilibrium could potentially result in severe disease, atypical cell proliferation or death [231].

1.5 Sphingolipids and ceramides as raft components

The understanding of cell membranes began with the Singer-Nicholson model. It views the membrane as a “fluid mosaic” in which a homogenous phospholipid bilayer acts mainly as a solvent for integral proteins and as a permeability barrier for the cell. Observations such as the resistance of sphingolipids, cholesterol and a subclass of membrane proteins to cold detergent extraction [232], or mechanical disruption more and more questioned the concept of homogenous cell membranes. Nevertheless the question remained if these observations were based on methodical artifacts. To date, however, the advancement of technology has enabled data, which supported that self-organization of lipids and proteins can induce subcompartmentalization and shifted the understanding of cell membranes to a concept which includes lateral heterogeneity, lipid microdomains and rafts. The plasma membrane is

now viewed as a heterogeneous dynamic entity, containing various lipid domains that can assume different functional states [148], [233].

Important techniques that provided the data for the base for this concept were fluorescence correlation spectroscopy, single-particle tracking, high spatial and temporal resolution fluorescence resonance energy transfer (FRET), fluorescence photoactivation localization microscopy (FPALM), stimulated emission depletion microscopy (STED) and electron paramagnetic resonance (EPR) spectroscopy. Single-particle tracking of colloidal gold-labeled glycophosphatidylinositol (GPI)-anchored receptors reveals “stimulation-induced temporary arrest of lateral diffusion” (STALL) in short-lived (~0.5 s) 50 nm areas as a bioactive feature of receptor function [234]. Investigation of GPI-anchored proteins with fluorescence correlation spectroscopy showed < 120 nm assemblages that fluctuate on a sub-second time scale [235]. Data from high spatial and temporal resolution FRET measurement suggest GPI-anchored receptors residing in more temporally stable clusters of ~10 nm. FPALM has revealed a dynamically clustered nanoscale distribution of hemagglutinin [236], a transmembrane protein described as raft-associated [237]. The role of the association between cholesterol and sphingolipids in assembly formation has been analyzed by STED. It was shown that unlike glycerophospholipids, plasma-membrane sphingolipids display transient cholesterol dependent confinement in areas of < 20 nm [238]. The differences in diffusion of glycerophospholipids compared to sphingolipids were attributed to differential hydrogen-bonding capacities. Numerous studies with these techniques led to the current view on cell membranes as a heterogeneous, self-organized, dynamic assembly containing lipid rafts, which are enriched in sphingolipids, cholesterol, GPI-anchored proteins and other raft-associated proteins [239], [233].

1.5.1 Biophysical properties of sphingolipids

Data from numerous studies suggest that the key role of sphingolipids in raft assembly is based on their unique biophysical properties. Two biophysical properties of sphingolipids are considered most important: The ability to match hydrophobically with cholesterol and the ability to act as donors and acceptors of hydrogen bonds.

Hydrogen bonds in lipid segregation. The polar headgroup of ceramide including the amide linkage and the hydroxyl groups of sphingosine and in some instances the fatty acid chain, are responsible for extensive hydrogen bond formation in the interface of the phospholipid bilayer. The tight interactions between ceramide molecules give rise to in-plane phase separation of ceramide-rich and –poor microdomains [240]. This propensity to undergo extensive hydrogen-bonding is what distinguishes sphingolipids from glycerophospholipids. While the latter lipids can only act as acceptors of hydrogen bonds in the interface, sphingolipids, such as ceramide and sphingomyelin, can act as both, acceptors and donors through their hydroxyl and amide groups. However, ceramides and sphingomyelin differ in their affinity for other lipids in the membrane, namely cholesterol. Sphingomyelin interacts very tightly with cholesterol, through hydrogen-bonding between the C_3-hydroxyl group of cholesterol and the sphingosine moiety of sphingomyelin. Ceramides, on the other hand, have poor affinity for cholesterol and tend to separate into microdomains exclusively enriched in ceramide [240], [148].

Hydrophobic match in lipid segregation. Rafts formation is a cholesterol-dependent lateral phase segregation, in part driven by the preference of cholesterol for specific lipid interaction partners. The planar, rigid sterol ring favors interaction with the straight and stiff

hydrocarbon chains of saturated lipids and it disfavors interaction with the more bulky unsaturated species [241]. Interaction with cholesterol also forces neighboring hydrocarbon chains into more extended conformations, increasing membrane thickness [242]. Sphingolipids tend to display longer and more saturated hydrocarbon chains, mediating interdigitation between membrane leaflets [243] and favoring interaction with cholesterol. This hydrophobic match/mismatch not only affects the interaction of sphingolipids with cholesterol, it also influences the affinity of raft-associated proteins for sphingolipid-enriched domains. Generally, it is believed that the hydrophobic membrane-spanning part of membrane proteins is stiff with no appreciable flexibility. This proteins preferably associate with lipids that match their steric requirement especially close to the protein-lipid interface [244]. The stabilization of a sterically favored lipid environment is defined as “wetting” [245]. Some membrane proteins retain tightly bound lipids even in detergent solution [246]. In such cases, lipids may be bound at defined binding sites, and specifically intercalated into the protein structure [247]. Lipid incorporation is a function of specific polar-head group interactions, hydrophobic interactions and hydrocarbon-chain space-filling functions [247]. Many of these interactions can be very strong, being even resistant to detergents. Binding of cholesterol to oligomers of caveolin-1 is such an example [248]. Formation of lipid rafts is driven by both, lipid-lipid and protein-lipid interactions. On top of this, the recruitment of proteins into rafts may be accompanied by conformational changes that modify protein activity [233].

Biophysical properties of ceramides. Ceramides mix poorly with phospholipids in bilayers, segregate into distinct high-temperature melting ceramide-enriched microdomains and facilitate the lamellar-hexagonal transition of lipids [249]. These properties affect the

ordering of lipids in the membrane, tending to destabilize them and cause membrane leckage, fusion, or budding of vesicles. Membrane fusion seems to be critical for many cellular processes, e. g. membrane biogenesis and viral infections [240]. In model membranes ceramide induces membrane fusion or fission. Ruiz-Arguello et al. reported that large unilamellar vesicles, containing sphingomyelin, phosphatidylethanolamine and cholesterol, show aggregation or efflux of intravesicular components after generation of ceramide by exogenous aSMase [250], [251]. Moreover, addition of 5-10 mol % ceramide to PC-containing vesicles reduced the time required for fusion of small vesicles activated by phospholipase C. The effect of sphingomyelinase was due to non-lamellar phase transition and induced fusion. Montes et al. showed that in large unilamellar vesicles composed of different phospholipids and cholesterol, both externally added and enzymatically produced ceramide (10 mol % of total lipid), induced release of vesicle contents [252]. Furthermore, Holopainen et al. used microscopy to detect formation of ceramide-enriched microdomains followed by vesicular budding after addition of sphingomyelinase in PC/sphingomyelin vesicles [253]. These biophysical findings in artificial membranes could have a biologic correlate. Zhang et al. observed that endocytic vesicles, 400 nm in diameter, are formed in ATP-depleted macrophages when treated with exogenous sphingomyelinase or ceramide. The respective vesicles pinch off the plasma membrane to become internalized and are free of caveolin or clathrin [254]. This is in agreement with the assumption that sphingomyelin promotes stability of lipid bilayers, whereas ceramide due to its relatively small polar headgroup (conical shape), induces a negative curvature, thus favoring vesicle formation [148].

Ceramide also induces pore formation. Siskind and Colombini showed that C_2- and C_{16}-ceramide have the capacity to form pores in model phospholipid bilayers whereas the

channel-forming capacity of dihydroceramide is limited [255]. The authors argue that the pore-forming ability is due to the extensive hydrogen-bonding capacity of ceramide, which is greatly reduced in the dihydroceramides. This could explain some of the biological effects of ceramide: Ceramide generation at the plasma permeability barrier could result in abnormal ion fluxes. Release of ions, such as calcium, would in turn affect the activity of local enzymes, activating specific local signaling cascades [148].

Ceramide, when added to model membranes, is able to induce lateral segregation followed by microdomain formation. Huang et al. examined the structure of bilayers composed of deuterated dipalmitoyl PC and bovine brain ceramide using nuclear magnetic resonance spectroscopy [256]. They found that the addition of ceramide induced lateral phase separation of fluid phospholipid bilayers into regions of gel and liquid crystalline phases, where ceramide partitioned mostly into the gel phases. Similarly, Veiga et al. showed that in the lamellar phase, ceramides do not mix ideally with phospholipids, leading to the co-existence of domains that undergo gel-fluid transition at different temperatures [249]. Ceramide-enriched microdomains were also detected in PC/phosphytidylserine mixtures using pyrene-labeled fluorescent phospholipids to probe lateral mobility in the membrane [257]. In these studies, atomic force microscopy revealed that long chain ceramides mixed very poorly with cholesterol and lateral phase separation ensues, indicating that ceramide possesses the ability to spontaneously form microdomains in a glycerophospholipids bilayer [257] [148].

The lipid properties described above contribute to the activities of ceramides in membranes. Generation of ceramide in response to stress signals or other agonist induce local changes in membranes. This effect could influence the permeability and fluidity of the membrane, induce ion fluxes, increase movement of proteins into or from rafts, cause conformational

changes in membrane-associated enzymes or receptors, or alter the transbilayer movement of lipids. The poor miscibility of ceramides and phospholipids and the subsequent formation of ceramide-rich membrane domains might also cause packing defects, allowing ceramide to modulate activities of membrane-bound enzymes in this way. Huang et al. suggested that this could be the mechanism for activation of phospholipase A_2 [256]. Ceramide generation might also change membrane curvature, activating or inhibiting the three-dimensional structure of membrane-bound enzymes.

Furthermore, direct binding of ceramide by specific proteins is possible. Ceramide can recruit proteins such as kinase suppressor of ras (KSR) [179] or PKCζ. Fox et al. found that treatment of vascular smooth muscle cells with C_6-ceramide results in increased localization and phosphorylation of PKCζ within caveolin-enriched lipid microdomains [258].

1.5.2 Membrane rafts

It is well established that lipids are distributed nonrandomly in the plasma membrane leading to the formation of sub-microscopic microdomains, some of them termed rafts. Rafts are composed mainly of sphingomyelin and cholesterol [259], and represent regions of tightly packed sphingolipids [148]. Lipid analysis has revealed that up to 70 % of total cellular sphingomyelin is found in rafts [260]. Treatment of cells with cholesterol-depleting agents leads to disruption of rafts by sequestering cholesterol and removing it from the membrane. This technique can also be used to study the function of these rafts [148].

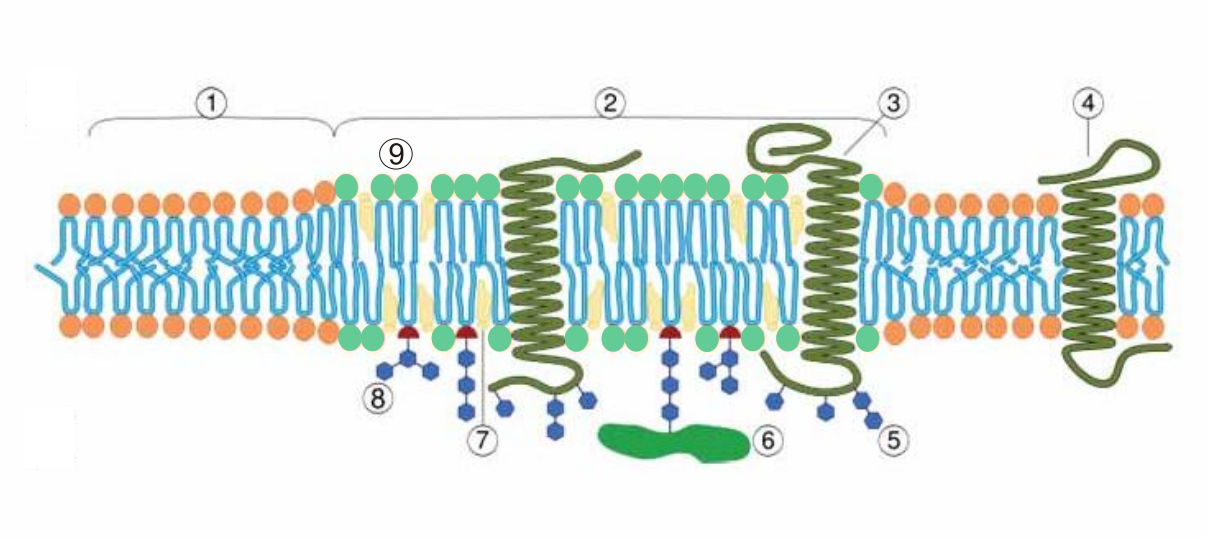

Figure 13: Lipid raft organisation scheme.

1. Non-raft membrane, 2. Lipid raft, 3. Lipid raft associated transmembrane proteins, 4. Non-raft membrane protein, 5. Glycosilation modifications on glycosphingolipids and glycoproteins, 6. GPI-anchored protein, 7. Cholesterol, 8. Glycosphingolipid, 9. Sphingomyelin (K. Simons & E. Ikonen [149]).

There are two types of rafts: those containing the structural protein caveolin-1, and those lacking this protein. The latter contain two other raft-specific proteins, called flotillin-1 and -2 [261]. Since caveolar and non-caveolar rafts are highly enriched in sphingolipids and glycosphingolipids, they are also known as glycolipid-enriched microdomains. These rafts are highly enriched in gangliosides, especially monosialotetrahexosylganglioside (GM1), which almost exclusively localizes to them. Parton et al. found that GM1 is enriched in caveolae using immune-electron microscopy [262]. The presence of GM1 in detergent-resistant membranes has also been taken as evidence that it resides in microdomains or rafts [263]. GM1 binds cholera toxin, and the toxin`s affinity for it is often used as a marker for these microdomains. Rafts are resistant to solubilization with cold non-ionic detergents, whereas the residual of the membrane lipids are soluble [264]. For this reason, rafts have also been termed detergent-insoluble-glycolipid-enriched microdomains.

Non-caveolar microdomains can be 10-300 nm in size and can adopt different shapes. They are flat rather than invaginated [265]. When the structural protein caveolin-1 is inserted into rafts of model membranes, they form the invaginated 50-100 nm flask-shaped structures termed caveolae. Caveolae were first discovered in the late 1950s by Palade and Yamada [266], who observed membrane structures that looked like “little caves”, and hence gave them the name caveolae [260]. Since Palade observed these structures in endothelial cells, he proposed that they are involved in the uptake of material from the blood stream and their transport into cells, a process referred to as transcytosis. The function of caveolae remained obscure. However, the discovery of the integral membrane protein caveolin-1, made isolation, analysis of composition and function of caveolae possible. Since then caveolae have been found in several cell types, including fibroblasts, adipocytes, endothelial and epithelial cells, smooth muscle and striated muscle cells, whereas non-caveolar rafts are believed to be ubiquitously present in cells. Caveolins are palmitoylated hairpin-like proteins and include caveolin-1 and 2, which are usually co-expressed in a variety of cells, and caveolin-3 which is only found in muscle cells [148].

1.5.3 Functions of rafts

It is now known that the composition and function of membrane rafts can be modulated in response to a variety of factors and stress conditions. Thus, a correlation between the physical state of the membrane and the physiological state of the cell can be assumed.

Many signaling proteins have been shown to reside in rafts during the process of signal transmission. These proteins include the epidermal growth factor (EGF) receptor, insulin receptor, non-receptor tyrosine kinases (fyn and src), G proteins, Ras, nitric oxide synthase,

adenylate cyclase, PI3-K, several PKC isoforms, Fas and the TNF-L receptor [260]. The list of raft-associated proteins is increasing rapidly.

Caveolin-1 can directly interact with many of these signaling molecules through a conserved 20 amino acid domain termed the caveolin scaffolding domain, which appears to hold molecular targets in an inactive conformation. The best characterized example of this interaction is the association of caveolin-1 with eNOS [260], [267]. eNOS is found in endothelial cells and produces NO in response to various stimuli including hormones and neurotransmitters. NO helps dilate and relax the blood vessels. Several groups have reported that eNOS is bound to caveolin-1, and that this interaction keeps the enzyme inactive [268]. In contrast, signals that activate eNOS induce its dissociation from caveolin-1 [269]. Similarly, the EGF receptor interacts with caveolin-1 within caveolae of quiescent fibroblasts, and is rapidly released in response to EGF stimulation [270]. Migration of EGF out of caveolae appears to be important for normal cellular function since mutant EGF receptors, which are incapable of moving out of caveolae, induce an oncogenic phenotype [270].

Alternatively, some proteins migrate into caveolae, which in some instances may be a ceramide-dependent event. Giaccia and co-workers showed that radiation-induced ceramide formation through aSMase modulates caveolin-1 function, leading to inhibition of PI3-K, which translocates into caveolae. This effect was mimicked by the addition of exogenous C_2-ceramide [271]. Migration of CD40 and the Fas receptor into rafts has also been observed. Ceramide plays a role in signal transmission by this protein [272], [273].

1.5.4 Capping: definition and the role of rafts

Capping is a process during which cell surface receptors or proteins aggregate on one pole of the cell after binding their cognate ligands or agonistic antibodies [274]. It is supposed to be a prerequisite for signaling by many receptors such as receptors for insulin, L-selectin, EGF, Fas and immunoglobulins, to list a few [274]. For instance, the release of histamine from mast cells results from the rapid clustering of Fc receptors on the cell surface.

The exact mechanism of the capping process is not well understood. The cytoskeleton is likely to be involved directly or indirectly in the lateral redistribution of surface molecules into a cap structure. The contribution of contractile microtubules was reported by Wessel et al. [275]. Wessel and colleagues observed that cytochalasin B (mycotoxin that inhibits network formation by actin filaments) partially inhibited capping in mouse splenic lymphocytes. However, in other cell lines, cytochalasin B induced capping. This suggests that differences exist in the way how microfilaments are involved in capping depending on cell type and receptor. At least two mechanisms are suggested for capping: one involves association between surface receptors and the sub-membrane microfilament and cytoskeletal network [274]. Another one emphasizes the influence of lipid properties that direct the receptors to membrane microdomains [272], [276].

Hoover et al. provided evidence that lipids modulate capping processes [277]. Their studies demonstrate that lowering of cellular cholesterol is associated with capping of surface immunoglobulins in murine lymphocytes. The authors proposed that the capped proteins were found in a gel-like domain of the membrane. In addition, removal of cholesterol reduced the gel state, increased fluidity and thus interfered with capping [277]. It is now well established that membrane rafts are also in a gel-like state. The importance of rafts enriched in sphingolipids for receptor capping was also supported by this study [278]. These authors

demonstrated that prolonged exposure to C_2-ceramide resulted in trkA receptor activation by mediating formation of trkA receptor dimers. The authors propose that these effects are caused by changes in the lipid composition and properties of rafts. CD28 activation on the surface of T lymphocytes also leads to clustering of rafts at the site of T cell receptor (TCR) activity, and the formation of a dense cap [279], [280]. It was proposed that this process leads to amplification of TCR-induced signaling, resulting in increased recruitment of kinases [281]. Harder et al. found that direct cross-linking of raft-associated gangliosides GM1 by cholera toxin results in co-capping of GM1, Thy1, the TCR complex and the src tyrosine kinase fyn in a signaling cluster [282]. Furthermore, capping can occur in non-caveolar rafts. Stuermer et al. demonstrated that flotillin-1 and 2 associate with the activated TCR complex during capping [283] in neurons and Jurkat cells that do not express caveolin-1 and lack caveolae. The importance of sphingolipid rafts in capping is further emphasized by the studies of Drezewieska et al. and Hoover et al., who showed that disruption of rafts by cholesterol-depleting agents, such as cyclodextrin or nystatin, abrogate capping and signaling through the Fc receptor and immunoglobulin receptors, respectively [277], [284].

In summary, these studies suggest that rafts play a critical role in capping and activation of distinct receptors, resulting in regulated signaling of diverse biologic processes, including apoptosis, mitogenesis and immune signaling [148].

1.5.5 Ceramide formation, sphingomyelinase and rafts

Several lines of evidence suggest that membrane rafts are the specific sites for ceramide generation in response to various agonists and stress signals [271], [272], [285], [286], [287]. Liu and Anderson first reported that ceramide levels were elevated and sphingomyelin levels decreased in caveolae of human fibroblasts in response to IL-1 treatment [286].

Furthermore, this increase in ceramide was due to the activity of a zinc-dependent acid sphingomyelinase, which was found to be enriched in these fractions. It was observed that the quick increase in ceramide concentration in response to IL-1 treatment correlated with decreased thymidine uptake.

It has also been shown that rafts are the sites of ceramide generation in response to heat shock and ionizing radiation. Zundel et al. found that radiation-induced ceramide formation by aSMase within caveolae changes caveolin-1 activity, which results in PI3-K inhibition. The role of different sphingomyelinases in ceramide generation within rafts is the subject of ongoing research. Activation of aSMase and nSMase occurs in part differently in response to some stimuli [271], [146]. aSMase and nSMase have both been detected in rafts [271], [288]. Levade et al. reported a correlation between TNF, caveolae and SMases. Binding of TNF leads to movement of the receptor into caveolae. Concomitantly nSMase segregates from caveolae, followed by an increase in ceramide and a decrease in sphingomyelin in this compartment. This observation suggests that aSMase might be responsible for sphingomyelin hydrolysis in this case [288].

Cremesti et al. have found that aSMase has the capacity to localize rapidly from an intracellular compartment (lysosomes) to the plasma membrane upon Fas stimulation [272]. Concomitantly ceramide is formed seconds after Fas receptor activation. The resulting ceramide patches rapidly merge to form larger platforms which contain the activated Fas receptors, aSMase and other raft constituents [289]. Since the addition of long-chain ceramides to the surface of Jurkat cells or generation of endogenous ceramide directly resulted in patch formation and coalescence into larger platforms, it appears that the driving force for capping may be ceramide itself [148].

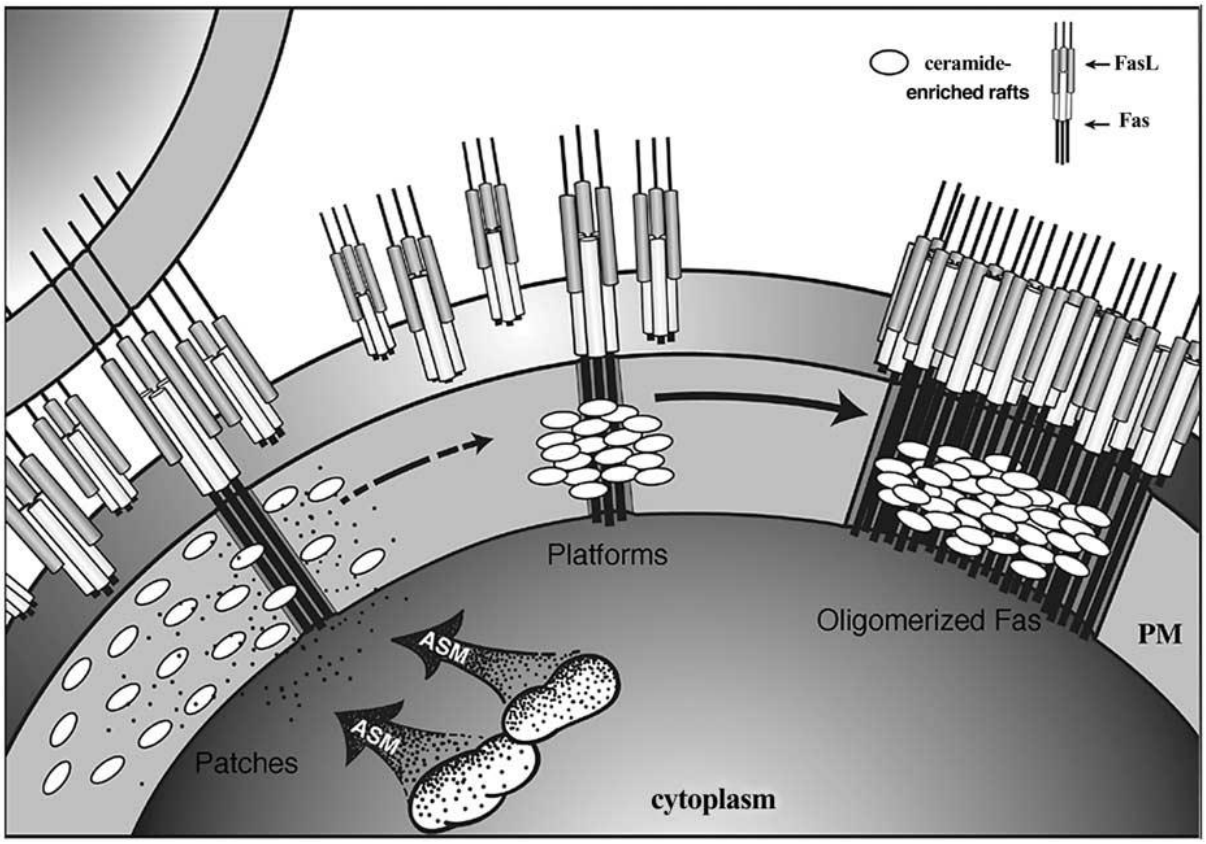

Figure 14: Scheme of Fas receptor capping through ceramide induced raft formation.
Seconds after Fas activation, aSMase translocates to the membrane and hydrolyzes SM to Cer, thereby mediating the formation and coalescence of ceramide-enriched rafts and Fas receptor multimerization. Only ligated Fas receptors are able to enter these rafts (A. E. Cremesti et al. [148]).

The signaling mechanism described above is not restricted to Fas. ASMase is also essential for the clustering of another TNF superfamily member, namely CD40 [273]. Stimulation of lymphocytes through CD40 binding propagates translocation of aSMase to the plasma membrane, followed by formation of ceramide, which in turn mediates CD40 clustering in sphingolipid-rich membrane domains. Deficiency of aSMase or destruction of sphingolipid-rich rafts prevents CD40-clustering and CD40-induced cell signaling. This clustering of CD40 in rafts is required for its signaling [273]. Furthermore, the p55 TNF receptor, which activates nSMase, also localizes to rafts. Raft disruption prevented TNF-induced cell death in Jurkat cells [290]. These results can also be taken as evidence that rafts are specific sites of

ceramide formation, which appears to be essential for capping and signaling by several TNF receptor superfamily members [148].

2 Effects of the oxidized phospholipids PGPC and POVPC on enzymes of ceramide metabolism

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2.1 Abstract

The truncated oxidized phospholipids PGPC and POVPC are biologically active components of oxLDL which is causally involved in the development of atherosclerosis. Lipoprotein and phospholipid toxicity in vascular macrophages is associated with the formation of ceramide which is considered to be a mediator of apoptosis. It has already been shown that oxLDL and the oxidized phospholipids PGPC and POVPC stimulate acid sphingomyelinase (aSMase) in cultured macrophages and vascular smooth muscle cells. This activation was causally related to the activation of stress-associated kinases and induction of caspase-3 mediated apoptosis. In this study, we investigated the role of enzymes of the sphingolipid “rheostat” under the conditions of oxidative phospholipid stress. PGPC and POVPC stimulated the activities of aSMase and nSMase. The effects of both oxPLs were concentration- and time-dependent.

Synthesis of ceramide, catalyzed be enzymes of the de novo pathway was also stimulated by PGPC and POVPC although in a different fashion. These effects correlated with results from sphingolipidome analysis showing that POVPC induces a faster ceramide response, whereas PGPC elicits a slower but persistent effect on ceramide formation. Both oxPLs did not affect the activities of ceramidases. Thus, influences of PGPC and POVPC on ceramide degrading pathways are unlikely. The effects of oxPLs on ceramide modification, e. g. phosphorylation and glycosylation are still unknown and will be subject to separate studies.

2.2 Introduction

Oxidized phospholipids are biologically active components of oxLDL which is causally involved in the development of atherosclerosis. Cellular uptake of this particle by the macrophages of the arterial wall leads to foam cell formation and sustained exposure of the cells to the modified lipoproteins induces cell death by apoptosis and eventually by necrosis. Typically, large amounts of oxLDL and its oxidized (phosphor)lipid components are found in atherosclerotic plaques [1], [2], [3]. It is important to note that the cellular effects of oxLDL are time- and concentration-dependent. Whereas small amounts of oxLDL in the micromolar range are proliferative on cultured macrophages, higher amounts, typically in the range above 20 µM elicit the above mentioned toxic effects [291], [292], [293], [294].

Lipoprotein toxicity in vascular macrophages is associated with the formation of ceramide which is considered a mediator of apoptosis [295]. It may function in two ways. On the one hand it propagates an apoptotic signal from the plasma membrane to downstream (kinase) cascades. On the other hand it may be involved in the execution program. This sphingolipid gives rises to alterations in biophysical properties. It may induce phase separations making (mitochondrial) membranes leaky or increase membrane curvature which facilitates

(apoptotic) membrane blebbing [249]. The steady-state concentrations of ceramide under given conditions are subject to a complex network of modifications that are catalysed by specific enzymes. Basically, three pathways participate in ceramide formation. *De novo* ceramide synthesis starts from serine and acyl CoA which is catalysed by SPT. A key enzyme of this pathway is ceramide synthase which catalyses the attachment of fatty acyl chains to the sphingolipid backbone leading to the formation of distinct ceramide species. A second mechanism is based on the action of sphingomyelinases which catalyse ceramide formation by degradation of sphingomyelin. Whereas *de novo* ceramide synthesis localizes to the ER, sphingomyelinase triggers ceramide formation in the plasma membrane under the influence of external stimuli. Finally, ceramide can be provided by the salvage pathway which reutilizes sphingosine generated from ceramide after sphingomyelin cleavage. On top of these synthetic pathways, ceramide concentration depends on a variety of enzymes catalysing the modification, e. g. phosphorylation, glycosylation or degradation of this sphingolipid [296].

It has already been shown that oxLDL and the truncated oxidized phospholipids PGPC and POVPC stimulate acid sphingomyelinase in cultured macrophages and vascular smooth muscle cells [58], [59], [68], [170]. This activation was causally related to the activation of stress-associated kinases JNK and p38 and induction of caspase-3 mediated apoptosis. Under the influence of oxLDL or oxPLs, ceramide is also formed in macrophages via the *de novo* pathway. CerS was identified as a key enzyme which was activated by the oxidized lipoprotein and phospholipids (L. M. Halassiddappa, T. Futerman, A. Hermetter, unpublished). Whereas activation of aSMase was very fast, ceramide production catalysed by ceramide synthases was much slower (typically several hours).

It was the aim of this study to identify the role of other enzymes of the sphingolipid “rheostat” in ceramide formation under the condition of oxidative phospholipid stress. For

this purpose, we determined the effects of PGPC and POVPC on the activities of SPT, ceramidases, aSMase and nSMase. The emphasis of the SMase studies was on the concentration dependence of oxPL effects in order to find out whether the concentration-dependent toxicities of oxLDL are related to the cellular effects of its toxic phospholipid components. In addition, the effects of PGPC and POVPC on the ceramide patterns were analysed. We found that ceramide formation correlated with the influence of the oxPLs on enzyme activities. POVPC preferentially stimulated ceramide formation within short incubation times, whereas PGPC led to a slow but persistent increase in ceramide formation.

2.3 Experimental procedures

Materials

The oxidized phospholipids PGPC and POVPC were synthesized in our laboratory as described by A. Moumtzi et al. [97]. Organic solvents and other chemicals were purchased from Carl Roth (Karlsruhe, Germany), Sigma-Aldrich (Steinheim, Germany) or Merck (Darmstadt, Germany). Tissue culture materials were purchased from Sarstedt (Nürmbrecht, Germany). Dulbecco`s modified Eagle medium (DMEM, 4,5 g/l Glucose) with and without phenol red and heat-inactivated fetal bovine serum were obtained from Invitrogen (Leek, Netherlands). PBS and cell culture supplements were purchased from PAA (Linz, Austria). Fluids for flow cytometry and FACS tubes were from BD bioscience (Heidelberg, Germany). The Alexa Fluor® 488 Annexin V/ Dead Cell Apoptosis Kit was purchased from Invitrogen.

Cell culture and incubation with oxidized phospholipids

The RAW 264.7 macrophage-like cell line (ATCC No. TIB-71, American Type Culture collection, Rockville, MD, USA) was routinely grown in DMEM (glucose 4.5 g/l, HEPES 25 mM, L-glutamine 4 mM, without sodium pyruvate) supplemented with 10 % (v/v) heat-inactivated fetal calf serum (FCS) and penicillin/streptomycin 100 U/ml at 37 °C in humidified CO_2 (5 %) atmosphere.

Cells were routinely splitted at a confluence of 80 % in DMEM-medium supplemented with heat-inactivated FCS 10 % (v/v). Incubation media containing oxPLs were prepared in DMEM media without heat-inactivated FCS using the ethanol injection method [297]. Briefly, oxPLs were dissolved in Ethanol and injected in the media with a hamilton syringe. The final concentrations of PGPC or POVPC in the media were 50 µM, unless otherwise indicated. The final EtOH concentration in the incubation media did not exceed 1 % (v/v).

Serine palmitoyltransferase assay

To determine serine palmitoyltransferase activity, a metabolic method based on lipid labelling with D^3-serine was used. SPT activity was calculated from the amount of D^3-sphingosine and D^3-sphinganine in lipid extracts isolated from oxPL-treated cells and compared to untreated control cells. Briefly, 4 millions of cells were seeded in a 21 cm^2 cell culture dishes and left in DMEM-media supplemented with 10 % heat inactivated FCS (v/v) for 36 h. DMEM-media with heat-inactivated FCS was removed 1 h before the experiment and cells were incubated with DMEM-media without FCS. Cells were further preincubated with DMEM-media without FCS, containing 1 mM D^3-serine, 1 h before addition of oxPLs. In some cases, the medium containing 2.5 µM myriocin at this point. The preincubation medium was removed, cells were washed with warm DMEM-medium without FCS and

DMEM-medium without FCS containing 1 mM D^3-serine and 50 µM oxPL (had been added to the medium using the ethanol injection method) was added to the cells. Cells were harvested after different incubation times (see Results) by scraping and transferred to a falcon tube on ice and centrifuged at 453 g at 4 °C for 7 minutes. Pellets were resuspended in 3 ml ice cold PBS. 1.5 ml of the cell suspensions were used for quantitative determination of protein using a plate assay according to the method of Bradford [298]. 1.5 ml of the cell suspension was transferred to reaction tubes for SPT-activity measurement. Cells were centrifuged in an Eppendorf centrifuge at 14000 rpm and 4 °C for 10 minutes. The supernatant was discarded and pellets were stored at – 20 °C. The D^3-sphingolipids in the cell pellets were analysed by HPLC-MS after alkaline hydrolysis in the laboratory of T. Hornemann. Results were normalized to protein content and relative values of test samples to control samples were calculated.

Lipid extraction and HPLC-MS analysis of ceramide concentration

9 Millions of cells were seeded in 58 cm^2 cell culture dishes and incubated with 11 ml of DMEM-medium with 10 % heat-inactivated FCS for 16 h. The medium was removed, cells were washed with DMEM-medium without heat-inactivated FCS and incubated with oxPL incubation media as described above. Control samples were incubated with FCS free DMEM-medium and 1 % EtOH (v/v) under the same conditions. Cells were harvested after 2 h, 4 h or 8 h by scraping, cell suspensions were transferred into 15 ml Falcon tubes on ice, followed by centrifugation at 453 g at 4 °C for 7 minutes. After cell harvest, all working steps were carried out on ice. The supernatant was discarded, cells were washed with 5 ml ice-cold PBS and a 0.5 ml aliquot of the suspension was used to determine the protein concentration. For this purpose, cells were spinned down, resuspended in 1 ml lysis buffer (50 µM Tris/HCl; pH

7.4) and lysed by ultrasonication. Protein concentration was determined using a plate assay according to the method of Bradford [298]. Samples for lipid extraction were transferred in a glass vial and centrifuged again at 453 g for 7 minutes. The supernatant was discarded, 3 ml $CHCl_3$/MeOH (2:1 v/v) were added and the mixture was shaken at 1500 rpm at 4 °C for 1 h. 700 µl $MgCl_2$-solution (0,036 %) were added to improve phase separation and the mixture was shaken for additional 10 minutes. The samples were centrifuged at 300 g for 5 minutes and the lower organic phase was transferred with a syringe to a fresh glass vial and the organic solvent was removed under a stream of nitrogen. For mild alkaline hydrolysis, 400 µl of $CHCl_3$/MeOH/H_2O (16/16/5 per vol.) were added to the solvent-free lipids and the solution was shaken vigorously. After addition of 400 µl 0.2 M NaOH in MeOH, the samples were incubated at RT for 45 minutes. Following addition of 400 µl 0.5 M EDTA and 150 µl CH_3COOH and vigorous shaking, 1 ml $CHCl_3$ was added to extract the lipids. Extracts were shaken for 5 minutes and centrifuged for 3 minutes at 300 g to facilitate phase separation. The chloroform phase was transferred to a new vial and the solvent was removed under a nitrogen stream. Solvent-free lipid extracts were dissolved in 100 µl $CHCl_3$/MeOH (1:1 v/v) containing 100 pmol CER 12:0, CER 25:0 and SM 12:0 each as internal standards. Sphingolipid species were determined by reversed phase HPLC coupled to a TSQ Quantum Ultra (Thermo Scientific) triple quadrupole mass spectrometer as described by Fauland et al. [299] and Radner et al. [300]. Cer and SM peak areas were calculated by QuanBrowser software for all lipid species and quantification was performed by correlation to internal standards. Results were normalized to protein content and relative values for lipid concentrations in test samples compared to control samples were calculated.

SMase assay

The neutral and acid SMase assays are based on the method published by A. Loidl et al. [301]. Adaptions due to different SMase activity levels in RAW 264.7 macrophages are listed below. Briefly, 4 millions of cells were seeded in 21 cm^2 cell culture dishes and left in DMEM-medium supplemented with 10 % heat-inactivated FCS (v/v) for 36 h. Before the experiment cells were washed with warm DMEM-medium without FCS and incubated with incubation medium containing oxPLs and control medium without oxPLs, which had been prepared as described above. Cells were harvested after different incubation times (see Results) by scraping and transferred to Falcon tubes on ice, centrifuged at 453 g at 4 °C for 7 minutes and washed with 3 ml ice-cold PBS. After washing the cell pellets were resuspended in 100 µl lysis buffer and enzyme activities were determined according to the method published by A. Loidl et al. [301], with exceptions listed in Table 1. Briefly, protein concentrations of the cell lysates were determined using a plate assay according to the method of Bradford [298]. 30 µl aliquots containing defined amounts of total cell protein were incubated with 200 µl reaction buffer containing 10 µl NBD-sphingomyelin substrate, as described [301]. Lipids were extracted with 300 µl $CHCl_3$/MeOH (2:1 v/v) followed by separation of substrate and product by thin-layer chromatography using $CHCl_3$-MeOH-H_2O, 65:25:4 (v/v/v) as a solvent. The fluorescent lipids were quantified with a CCD camera (Herolab, Vienna; excitation wavelength 365 nm) using EasyWin software. SMase activities were expressed as ratios of sample to control values.

Table 1: Assay conditions and buffer compositions for neutral and acid SMase-assays.

Inhibitors used by A. Loidl et al. [301] were added to the lysis buffers directly before use.

	aSMase-assay	nSMase-assay
Reaction time [h]	2.5	1.0
Sample protein amount [µg]	20	40
Lysis buffer	250 mM Sodium-acetate, 0.2 % Triton X-100 (v/v); pH 5.0	20 mM HEPES, 10 mM $MgCl_2$, 2 mM EDTA, 0.1 mM Na_2MoO_4, 1 mM NaF, 0.2 % Triton X-100 (v/v); pH 7.5
Reaction buffer	250 mM Sodium-acetate, 1 mM EDTA; pH 5.0	20 mM HEPES, 1 mM $MgCl_2$; pH 7.4

Flow cytometric assay for apoptosis

Briefly, $3.5*10^5$ RAW 264.7 macrophages were seeded in a 24 well plate (volume 3 ml) 40 h before the experiment. In some cases, samples were preincubated with enzyme inhibitors 1 h before incubation with oxPLs. Inhibitors were NB19 (10 µM) for aSMase, FumonisinB_1 (13.85 µM) for CerS and for K1 (50 µM) for CERK, suspended in DMEM-medium without phenol red. After preincubation, the medium was removed, cells were washed with PBS, and corresponding incubation medium containing oxPL was added. PGPC or POVPC were suspended in DMEM-medium without phenol red and without heat-inactivated FCS. DMEM-media for control samples contained 1 % EtOH (v/v) but no phenol red nor heat-inactivated FCS. After 4 h, cells were harvested by scraping and washed with cold PBS containing 2 % glucose (m/m). Cells were suspended in Annexin V binding buffer and 100 µl of the cell suspension were transferred to a FACS tube. 5 µl Alexa Fluor® 488 Annexin V and 5,5 µl Propidium iodide (PI; 1 mg/ml) were added and the mixture was incubated on ice in the dark for 15 minutes. The samples were diluted in 400 µl PBS containing 2 % glucose (m/m) and kept on ice until measurement start. The samples were analysed with a FACS Calibur flow cytometer (BD Bioscience, Heidelberg, Germany). The green fluorescence was measured at

530 nm and the red fluorescence at 575 nm, respectively. Cell populations were separated into four groups. Apoptotic cells were stained by green fluorescent Alexa Fluor® 488 Annexin V which binds to phosphatidylserine on the cell surface; necrotic cells are permeable for PI which stains DNA fragments; living cells remained unstained. Double-stained cells were considered as late apoptotic or early necrotic and were excluded from analysis. The percentage of apoptotic cells was calculated using the WinMDI 2.8 software.

Ceramidase assay

We developed a quick fluorescence method for the determination of relative changes in acid and neutral ceramidase activity using NBD-labelled C_{12}-ceramide.

The procedure was analogue to the SMase assays published by A. Loidl et al. [301], only the buffers and the substrate were different. Culturing and incubation of cells with oxPLs, as well as cell harvest and lysis were the same as described for the SMase assays, except for the lysis buffers (for buffer and reaction conditions see Table 2). We used the reaction buffer published by He et al. [302] for the acid ceramidase assay, which also served as acid lysis buffer. Sample aliquots containing 40 µg protein were diluted to a volume of 30 µl. 200 µl Reaction buffer containing 10 µM NBD-C_{12}-ceramide was added as described [301]. After different incubation times (see Results), lipids were extracted with 300 µl $CHCl_3$/MeOH (2:1 v/v) followed by separation of substrate and product by thin-layer chromatography using $CHCl_3$-MeOH-25 % NH_3, 90:20:0.5 (v/v/v) as a solvent. The fluorescent lipids were quantified with a CCD camera (Herolab, Vienna; excitation wavelength 365 nm) using EasyWin software. Ceramidase activities were expressed as ratios of test to control values. Commercial ASAH2, a recombinant neutral ceramidase from R&D Systems, was used for

assay development (see supplemental information Figure 28, Figure 29a, Figure 29b, Figure 30).

Table 2: Assay conditions and buffer composition of acid ceramidase and neutral ceramidase assay.

Protease inhibitors published by A. Loidl et al. [301] were added to the lysis buffers directly before use.

	acid ceramidase assay	**neutral ceramidase assay**
Reaction time [h]	1	1
Applied protein amount [µg]	40	40
Lysis buffer	See reaction buffer	20 mM HEPES, 10 mM $MgCl_2$, 2 mM EDTA, 0.1 mM Na_2MoO_4, 1 mM NaF, 0.2 % (v/v) Triton X-100; pH 7.5
Reaction buffer (see [302])	200 mM Citric acid. monohyd., 200 mM Na_2HPO_4 * $12H_2O$, 0.5 % (w/w) Sodium-Taurocholate, 0.5 % (v/v) Triton X-100; pH 4.5	50 mM Tris/HCl, 20 mM Sodium-Taurocholate; $pH_{25\,°C}$ 7.37 ($pH_{37\,°C}$ 7.0)

2.4 Results

Here we report on the effects of PGPC and POVPC on enzymes of ceramide homeostasis in the RAW 264.7 macrophage like cell line. Specifically, we analysed the effects of PGPC and POVPC on ceramide levels, as well as the activities of SPT, aSMase, nSMase, aCDase and nCDase. To gain information on the relevance of the enzyme effects for oxPL cytotoxicity, we performed FACS experiments to determine the extent of induced cell death under the influence of enzyme inhibitors. The respective compounds were NB19 for aSMase, K1 for Ceramide kinase, Myriocin for SPT and FumonisinB_1 for Ceramide Synthases.

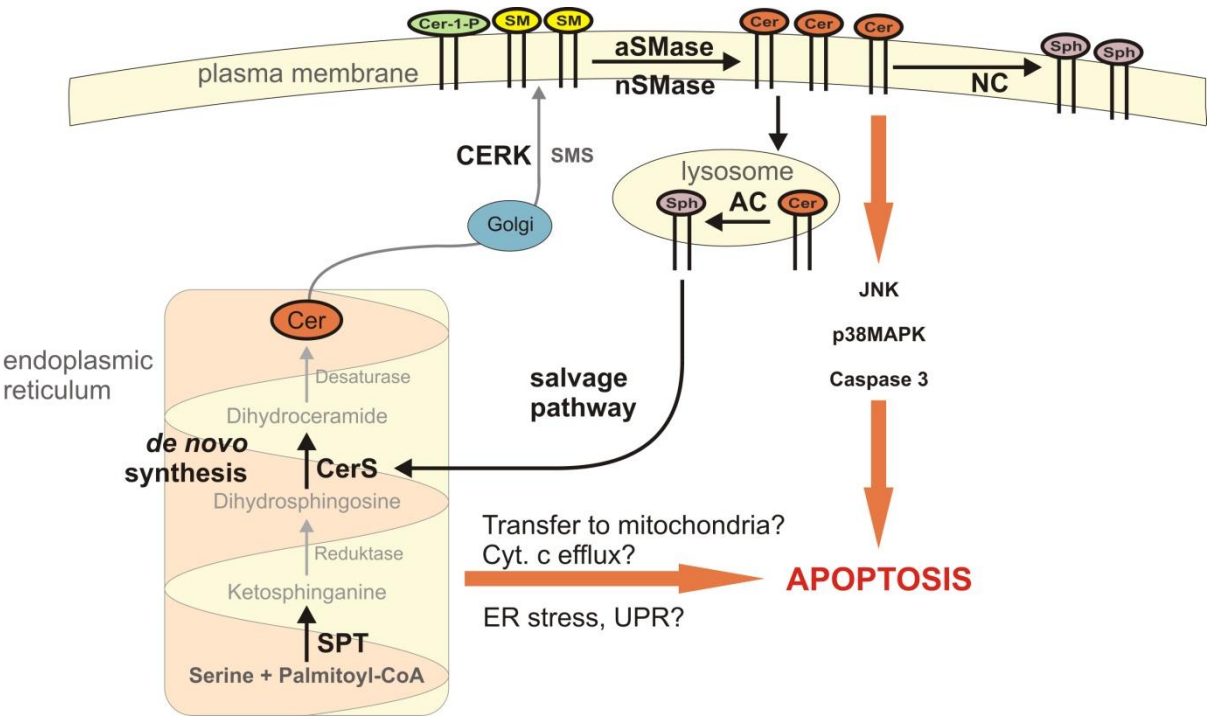

Figure 15: Pathways of ceramide formation and modification.

Cer: ceramide, SM: sphingomyelin, Cer-1-P: ceramide-1-phosphate, Sph: sphingosine, aSMase: acid sphingomyelinase, nSMase: neutral sphingomyelinase, NC: neutral ceramidase, AC: acid ceramidase, CerS: ceramide synthase, SPT: serine palmitoyltransferase, CERK: ceramide kinase, ER: endoplasmic reticulum, Cyt. c: Cytochrome c, UPR: unfolded protein response

Ceramide content. To study the effects of oxPL on the levels of distinct ceramide species in RAW 264.7 macrophages, cells were incubated with dispersion of 50 µM PGPC and POVPC in DMEM-media, followed by lipid extraction and MS-analysis of ceramides. Both oxPLs elicited an increase in cellular ceramide levels, but within different time frames (see Figure 16). POVPC induces a faster response, with ceramide concentration at maximum at 2 h incubation time and returned back to control level at longer incubation times. The PGPC effect is slower but persistent for several hours. Basically, three possible pathways are available for ceramide formation including lipid degradation by SMase at the plasma

membrane, *de novo* ceramide synthesis in the ER, and the salvage pathway utilizing sphingosine. In order to identify the source of ceramide levels under lipid stress, we investigated the effects of the oxPLs on the activities on SPT, CerS, SMase and CDase.

SPT. Preliminary data suggest that activation of SPT by POVPC is faster than by PGPC (see Figure 17). However, the activation by PGPC is longer lasting. In this context it has to be noted, that expression of SPT on the protein level is minimally affected by oxPL treatment. Recently, a model has been suggested for the regulation of SPT activity by activator proteins [303]. To date it is unknown whether oxPLs interfere with these interactors. Inhibition of SPT with Myriocin surprisingly led to an increase of apoptotic cells. Obviously *de novo* sphingolipid synthesis is an absolute requirement for cell survival under the influence of oxPLs and cannot be compensated by the SMase and salvage pathways. In fact, the SPT activation nicely matches the change in ceramide levels induced by either oxPL, suggesting relationship between Cer formation and SPT activity under the influence of oxPLs.

CerS. L. M. Hallasidappa could demonstrate an important role for CerS in ceramide formation under the influence of oxPLs (L. M. Hallasidappa, PhD thesis, TU-Graz, 2012). Therefore, we made an attempt to relate the activation of these enzymes to lipid induced cell death. For this purpose cells were pretreated with the CerS inhibitor FumonisinB_1 for 1 h followed by incubation with oxPLs for 4 hours. Interestingly, inhibition of CerS did not decrease apoptosis, showing again that *de novo* synthesized ceramide is important for cell viability (see Figure 18).

aSMase. Ute Stemmer et. al. [170], found that aSMase was activated in RAW 264.7 macrophages by POVPC within 15 minutes, whereas PGPC did not show any effect. Since it was known that aSMase activation by oxLDL depends on lipid concentration, we studied the effects of various amounts of POVPC and PGPC on the cells, since these lipids are components of the lipoprotein particle. In agreement with results obtained by Ute Stemmer et. al., 25 µM PGPC did not activate aSMase within 15 minutes, whereas 50 µM lipid stimulated this enzyme und the same conditions. In contrast to PGPC, 25 µM POVPC activated aSMase after 15 minutes, whereas 50 µM POVPC stimulated aSMase only after 2 hours (see Figure 19). Obviously, different mechanisms of enzyme activation are involved in oxPL effects on aSMase, depending on concentration and time. These mechanisms are currently unknown. To determine the biological relevance of the aSMase response to PGPC and POVPC, we determined the fraction of apoptotic macrophages at treatment with 50 µM oxPL and aSMase inhibitor NB19 for 4 hours. Specifically we preincubated the cells with NB19 for 1 h, followed by incubation with oxPL for 4 hours and FACS analysis of cell death. Figure 20 shows that the apoptotic effects of both oxPLs were significantly decreased by NB19, demonstrating a causal relationship between apoptosis and aSMase activity. OxPL toxicity seems to be based entirely on the enzyme activity level since aSMase expression was not affected by oxPLs in RAW macrophages (data not shown).

nSMase. POVPC and PGPC also stimulated the activity of nSMase. The concentration and time dependence of this effect is different compared to aSMase (see Figure 21). 50 µM POVPC quickly increased nSMase activity after 15 minutes, whereas 50 µM PGPC activated this enzyme after 4 h incubation. However, it has to be noted, that aSMase activity contributes a lot more to ceramide formation than nSMase. Basal aSMase activity was

approximately five times higher than the basal nSMase activity in lysates of control RAW 264.7 macrophages, and this difference is not compensated by the extent of oxPL induced activation of both isoforms.

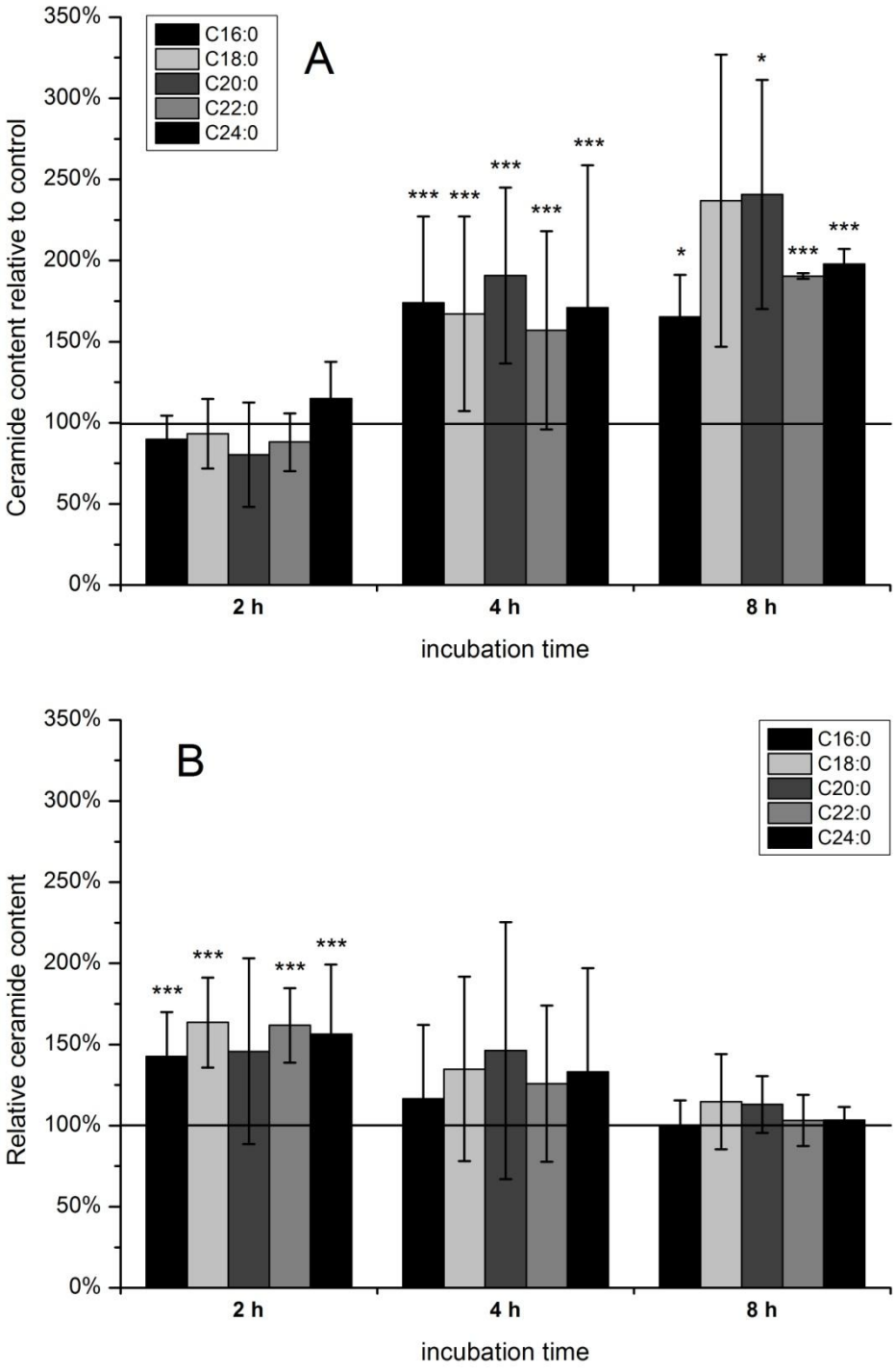

Figure 16: Effects of oxPL on ceramide levels in RAW 264.7 macrophages.

RAW 264.7 macrophages were incubated with 50 µM PGPC (panel A) or 50 µM POVPC (panel B) in DMEM media for various times. Lipid extracts were prepared and ceramide species were determined by MS as described in Experimental procedures. N-acyl chains of the individual ceramide species are specified in the insert. Data are expressed as means ± SD (n = 5).

* $p \leq 0.05$, *** $p \leq 0.005$

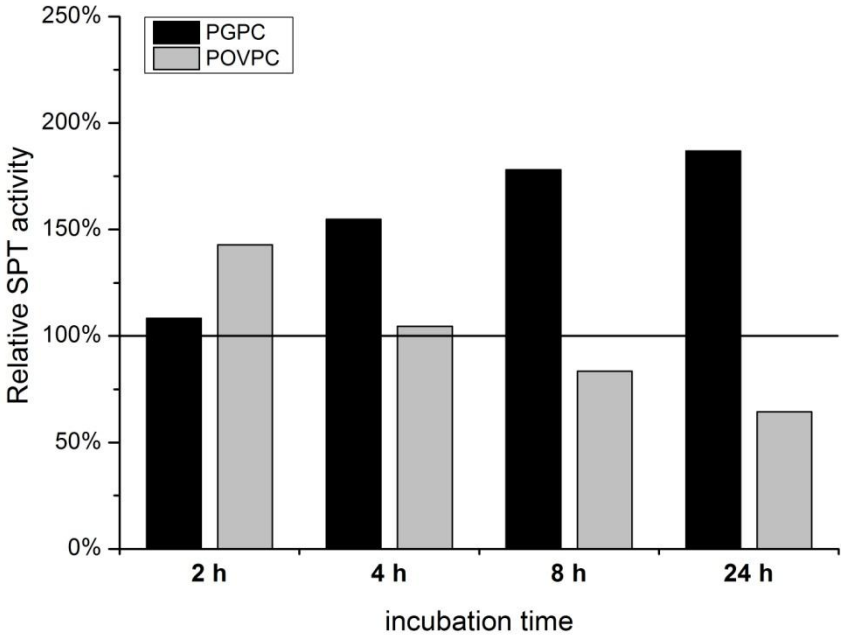

Figure 17: OxPL effects on SPT activity in RAW 264.7 MCs.

Cells were preincubated with deuterated serine, followed by incubation with 50 µM PGPC or POVPC in DMEM media for various times. Lipid extracts were prepared, hydrolyzed and relative SPT activities were obtained from MS analysis of deuterated sphingosine and sphinganine amounts.

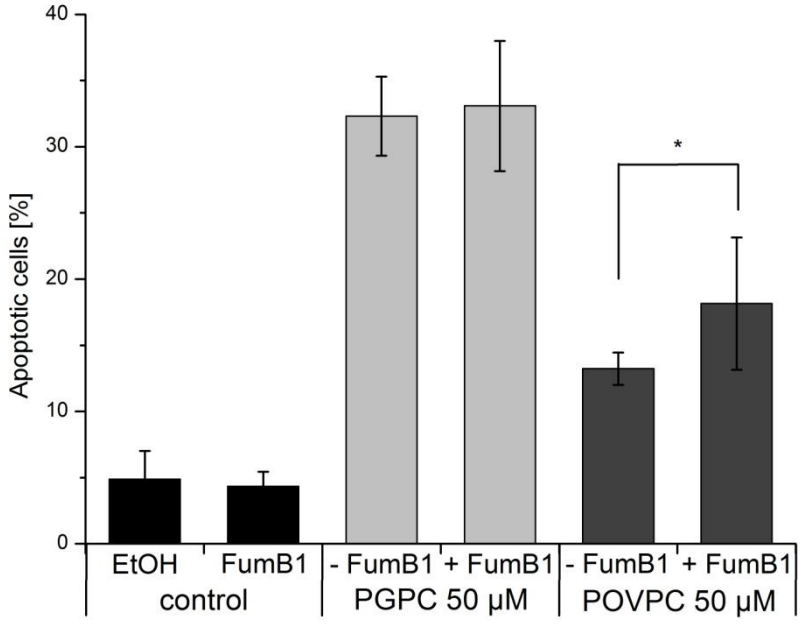

Figure 18: Effect of CerS inhibition on oxPLs induced apoptosis in RAW 264.7 macrophages.

RAW 264.7 macrophages were preincubated with the CerS inhibitor FumonisinB_1, followed by incubation with 50 µM PGPC or POVPC in DMEM media for 4 hours. The fraction of apoptotic cells was determined by FACS. Data are expressed as means ± SD (n = 6). * $p \leq 0.05$

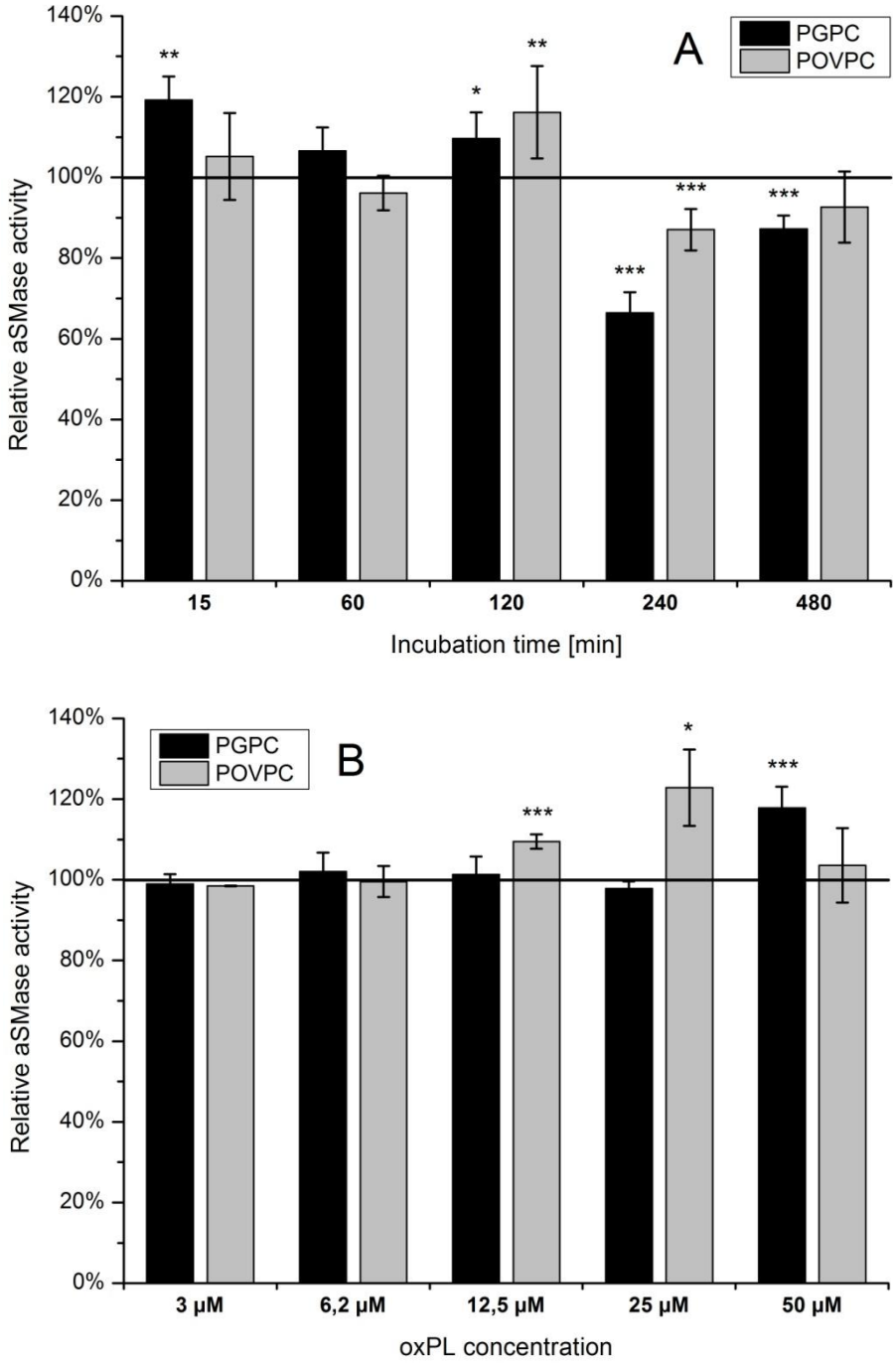

Figure 19: Effects of oxPL on aSMase activity in RAW 264.7 macrophages.

Panel A: RAW 264.7 macrophages were incubated with 50 µM PGPC or 50 µM POVPC in DMEM media for various times and aSMase activities relative to controls were determined using the described fluorescence assay [301]. Data are expressed as means ± SD (n = 5).

Panel B: Cells were incubated with various concentrations of PGPC or POVPC in DMEM media for 15 min and aSMase activities relative to controls were determined. Data are expressed as means ± SD (n = 4). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$

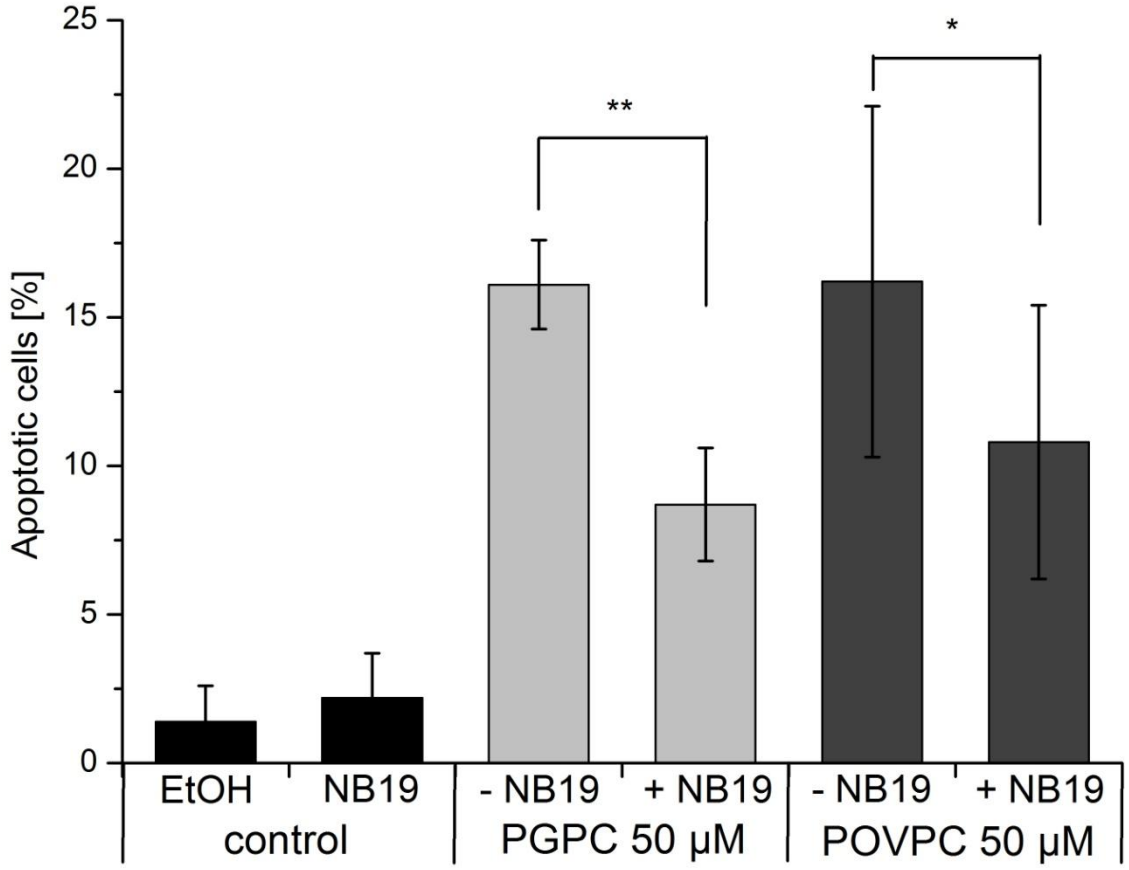

Figure 20: Effect of aSMase inhibition on oxPLs induced apoptosis in RAW 264.7 macrophages.

RAW 264.7 macrophages were preincubated with the aSMase inhibitor NB19, followed by incubation with 50 µM PGPC or POVPC in DMEM media for 4 hours. The fraction of apoptotic cells was determined by FACS. Data are expressed as means ± SD ($n_{PGPC/NB19}$ = 11; $n_{POVPC/NB19}$ = 6). * $p \leq 0.05$, ** $p \leq 0.01$

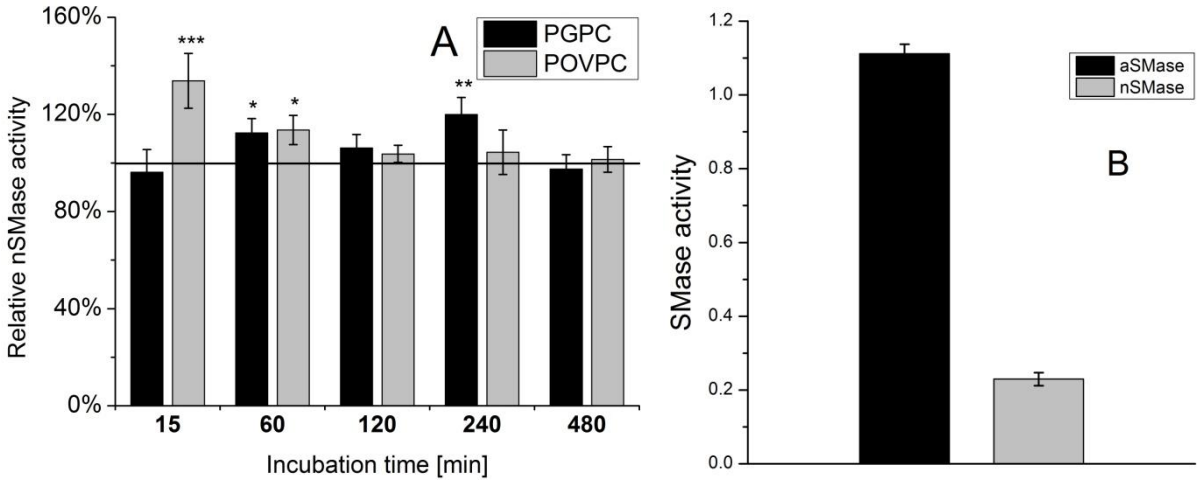

Figure 21: Effects of oxPL on nSMase activity in RAW 264.7 macrophages.

Panel A: RAW 264.7 macrophages were incubated with 50 µM PGPC or 50 µM POVPC in DMEM media for various times and nSMase activities relative to controls were determined using the described fluorescence assay [301]. Data are expressed as means ± SD (n = 5).

Panel B: Basal aSMase and nSMase activities were determined in cell lysates of control RAW 264.7 MCs. Activities are expressed as ratios of moles ceramide / moles sphingomyelin ± SD (n = 4). * $p \leq 0{,}05$, *** $p \leq 0{,}005$

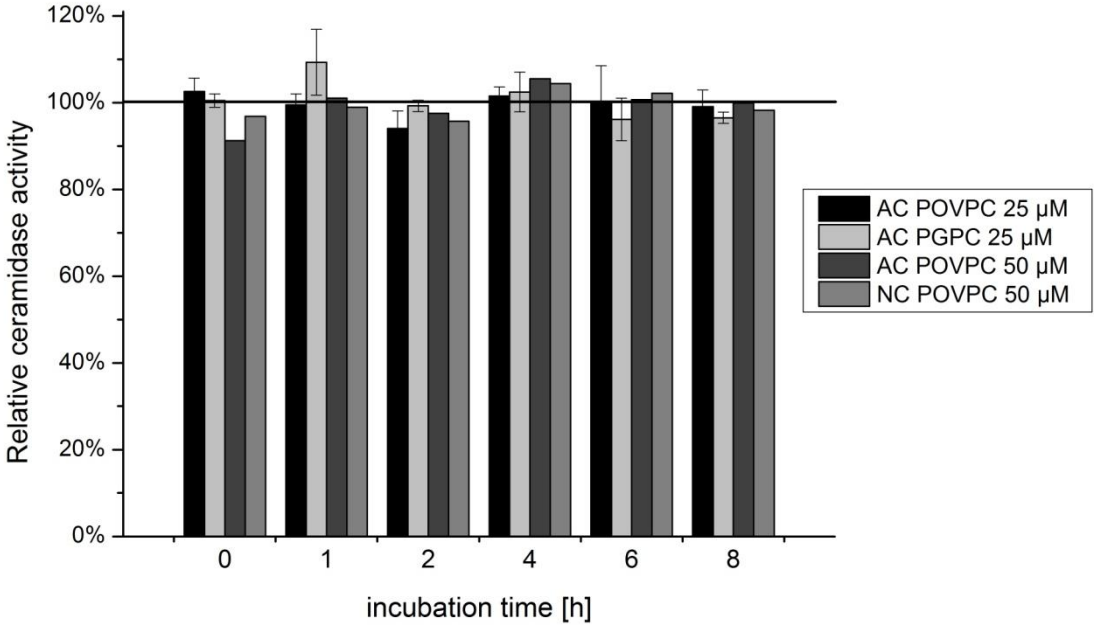

Figure 22: Effects of oxPLs on NC and AC activities in RAW 264.7 macrophages.

Cells were incubated with PGPC or POVPC in DMEM media for various times and relative changes in aCDase or nCDase activities were determined using fluorescent ceramide as substrate (see Experimental procedures). Data are expressed as means ± SD ($n_{AC/25\mu M}$ = 4; $n_{AC/50\mu M}$ = 1; $n_{NC/50\mu M}$ = 1).

2.5 Discussion

This work aimed at identifying the effects of PGPC and POVPC on enzymes of the ceramide rheostat in order to obtain information about the mechanisms of ceramide formation and modification in RAW 264.7 macrophages under the influence of oxidized phospholipid stress. Short incubation of the cells with both oxPLs leads to the activation of aSMase in a concentration- and time-dependent manner. After 15 min, concentrations around and below 25 µM POVPC activate the enzyme, whereas 50 µM lipid does not show any effect. Enzyme activation by PGPC was observed at 50 µM lipid concentration. Obviously, the response to the aldehydolipid is more sensitive. This difference can be explained at least in part by the capacity of POVPC of covalently modifying proteins by Schiff base formation. Kinnunen and colleagues have already shown, that the activity of phospholipase A_2 in model membranes is significantly enhanced, very likely due to the same effect [95]. Our data would also be in line with the assumption that aSMase is lipidated by POVPC. As a consequence, the enzyme is more firmly attached to its membrane substrates and can work more efficiently. PGPC can only physically interact with its molecular neighbors and affect the activity of membrane-associated proteins either by direct interactions or more general supramolecular effects. So far, we do not have evidence for bimolecular aSMase interactions with PGPC, but we know that PGPC preferentially localizes to and accumulates in membrane regions of high curvature, e. g. caveolae, and promote vesiculation [89]. If according to reports from the literature aSMase preferentially localizes to caveolae [290], [271], [286], it seems plausible that PGPC affects enzyme activity in these membrane subdomains, both, on the level of the plasma membrane and endosomes released inside the cells. In contrast, POVPC is likely to modify aSMase in the plasma membrane. This hypothesis is supported by fluorescence microscopy of labeled oxPL analogs in RAW cells. POVPC stays on the cell surface for quite

some time whereas PGPC is quickly internalized ending up in the endoplasmic reticulum after very long incubation times [88], [85]. Furthermore lipidome studies will be needed to validate this model showing that ceramide levels respond to oxPLs in the relevant subcellular domains in the same time frame. Irrespective of the mechanisms underlying the activation of aSMase, the stimulation of this enzyme is mandatory for the cytotoxic properties of PGPC and POVPC. Inhibition of aSMase by a chemical inhibitor significantly reduced the fraction of apoptotic cells after treatment with oxPLs.

Whereas 50 µM PGPC stimulated aSMase, POVPC did not show any effect on enzyme function at the same concentrations. We can only speculate that the high aldehydolipid concentration which is confined to the plasma membrane at short incubation times also affects membrane organization physically in a way that is not favorable for the enzyme.

If the cells were exposed to the oxPLs for 2 h, a second rise of aSMase activity was observed. From microarray analysis, we know that oxPLs stimulate TNFα expression under the same conditions. Thus, we conclude that TNFα formation in turn stimulates activation of aSMase, as reported from several studies [160], [304], [305]. This result would be in line with the observation that oxLDL shows the same delayed effect on aSMase as the oxPLs which are components of this lipoprotein particle. In addition, oxLDL also activates TNFα expression and very likely increases aSMase activity via this cytokine [306].

So far, we have information from sphingolipidome analysis that oxPLs give rise to elevation of distinct ceramide isoforms under conditions of prolonged incubation. According to the activity of profiles of aSMase, this enzyme cannot solely be responsible for this effect. Here we provide evidence that *de novo* sphingolipid synthesis largely contributes to the increase of cellular ceramide. According to preliminary data, the activity profile of SPT under the influence of oxPLs matches the time-dependent ceramide profiles. PGPC slowly but

persistently increases ceramide levels and SPT activity. In contrast, POVPC increased lipid and enzyme activity levels much faster, but these effects abate after some time (4 h). We have already sound information that certain ceramide synthase isoforms also play a key role in the long term effects of oxPLs on ceramide formation. PGPC and POVPC stimulate a subset of CerS isoforms catalyzing the formation of distinct ceramide species. In order to search for a causative role of CerS in oxPL toxicity in RAW macrophages, we studied the effects of PGPC and POVPC in these cells under the influence of the enzyme inhibitor FumonisinB_1. Surprisingly, FumonisinB_1 treatment enhanced the susceptibility of the cells towards cell death at incubation with POVPC. We can only speculate that macrophages need a certain level of ceramide for survival [221], [227]. These cells display very high ceramide kinase activities, which leads to a ceramide-1-phosphate level that is four times higher than the ceramide level (www.lipidmaps.org). Cutting the ceramide supply to this enzyme could result in the observed higher tendency of the cells to undergo apoptosis. To definitely answer these questions, more detailed studies on the functional role of ceramide kinase and other ceramide modifying enzymes (e. g. glucosylceramide transferase) will be needed.

Finally, we analyzed the effects of oxPLs on the ceramide degrading enzyme ceramidase. These biocatalysts play an important role in cancer cells, because they help the malignant cell to survive under the influence of cytotoxic agents that would otherwise increase the levels of apoptotic ceramide [307]. In RAW 264.7 macrophages, PGPC and POVPC did not influence ceramidase activities (see Figure 22). This insensitivity of the ceramide hydrolase may have at least two consequences. First of all, ceramide which is formed by sphingomyelinase cannot be reutilized for the formation of other ceramide species by the salvage pathway. Secondly, the rise of ceramide under oxPL stress conditions must be compensated by ceramide modification in these cells (phosphorylation, glycosylation,

sphingomyelin synthesis). The role of all these components in the ceramide rheostat will be subject to future studies.

3 Effects of PGPC and POVPC on gene expression in RAW 264.7 macrophages

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3.1 Abstract

Oxidized phospholipids are compounds of oxLDL. It is known that oxLDL activates expression of a series of atherogenic genes.

In this study we present the effects of PGPC and POVPC on total gene expression of RAW 264.7 macrophages. These two oxPLs represent major bioactive compounds of oxLDL and oxPAPC and largely contribute to their bioactivity. Our microarray analysis shows that PGPC has vast gene regulatory activity, whereas POVPC showed only very minor effects. PGPC altered especially gene expression of genes related to cell death, angiogenesis, cholesterol efflux, procoagulant mechanisms, atherogenesis, inflammation, and cell cycle. Many of these effects are reported from studies with oxLDL or oxPAPC, suggesting that PGPC is at least partially responsible for these effects of the oxidized lipid mixtures. From previous studies it

is known that POVPC efficiently reacts with proteins by Schiff base formation, whereas PGPC is not chemical reactive. Thus, we conclude that POVPC affects cell physiology on the protein level at the plasma membrane, whereas PGPC could undergo a great variety of physical interactions with proteins and other biomolecules and is modulating gene expression directly or indirectly via transcription factors.

3.2 Introduction

Oxidized phospholipids are components of oxidized LDL which plays an important role in atherogenesis. This lipoprotein particle interacts with the cells of the arterial wall leading to cell-specific pathophysiological consequences. The uptake of oxLDL by macrophages is a hallmark of atherosclerosis. Accumulation of the particle contents inside the cells gives rise to the formation of foam cells and eventually leads to cell death by apoptosis and necrosis. It has already been established that the truncated oxidized phospholipids PGPC and POVPC are toxic components of oxLDL and induce apoptosis in cultured macrophages and vascular smooth muscle cells [1], [2], [3]. Both compounds are generated from PAPC under oxidative stress and are structurally very similar. They contain a long fatty acyl chain and a short carboxylic acid residue in positions *sn*-1 and -2, respectively. They only differ by the functional group at the ω-position of the *sn*-2 substituent which is an aldehyde residue in POVPC and a carboxy group in PGPC [71]. The aldehydic function makes POVPC chemically reactive towards the aminogroups of phospholipids and proteins and for that reason specifically affects the interaction of this oxPL with the cells. While PGPC is rapidly and efficiently internalized by cultured vascular cells, POVPC is retained in the plasma membrane for some time [170], [85], [88]. Therefore it has been speculated that POVPC might preferentially interact with the cellular components on the protein level, whereas PGPC

could undergo a great variety of physical interactions with proteins and other biomolecules, e.g. by modulating gene expression directly or indirectly via transcription factors.

A series of investigations has been performed to identify the effects of oxLDL and oxPAPC (a mixture of oxidized phospholipids) on the expression of selected genes. Hägg et al. found that oxLDL coordinately upregulated gene expression of the glutathione and thioredoxin system in human macrophages [308]. Hirose et al. detected different responses of human polarized macrophages to oxLDL depending on the activation state (M0, M1, M2) [309]. Almost 93 % of the top 30 upregulated genes in M2 macrophages were also upregulated in M0. However, all subsets of macrophages share upregulated genes. M0 and M2 cells shared high similarities in top 10 functional ontology categories and gene expression in M1 cells differed to some extent as compared to the other activation states. According to the study by Groeneweg et al., oxLDL increases the transcriptional response of murine macrophages to lipopolysaccharide-induced gene expression [310]. The genes induced by the oxidized phospholipid mixture oxPAPC in human aortic endothelial cells (HAECs) were identified by Reddy et al. [311]. Bochkov and Gargalovic et al. specifically studied the effects of oxPAPC on the genes of angiogenesis [102], [312]. Leitinger et al. used the chemically defined oxPLs PGPC and POVPC to study their effects on the specific expression of inflammatory genes in HUVECS.

Most of the studies listed above have in common that they explored subsets of the human or murine genome. In order to obtain more global information on oxPL effects on gene expression, we screened the murine transcriptome under the influence of PGPC and POVPC using microarrays displaying probes for virtually the entire mouse genome. In cultured RAW 264.7 macrophages we found that PGPC upregulated a large number of genes relevant to atherosclerosis, whereas POVPC showed only minor effects on the transcriptional level. This

result further supports the assumption that toxicity of oxPLs in vascular cells is dramatically altered by small functional differences in the oxidized acyl chain. The chemically reactive POVPC is efficiently scavenged by proteins and modifies their functions by direct contact whereas PGPC is free to interact with other molecules including those, modulating gene expression.

3.3 Experimental procedures

Materials

The oxidized phospholipids PGPC and POVPC were synthesized in our laboratory as described by A. Moumtzi et al. [97]. Organic solvents and other chemicals were purchased from Carl Roth (Karlsruhe, Germany), Sigma-Aldrich (Steinheim, Germany) or Merck (Darmstadt, Germany). Tissue culture materials were purchased from Sarstedt (Nürmbrecht, Germany). Dulbecco`s modified Eagle medium (DMEM, 4,5 g/l Glucose) with phenol red and heat-inactivated FCS were obtained from Invitrogen (Leek, Netherlands). PBS and cell culture supplements were purchased from PAA (Linz, Austria).

Cell culture and RNA preparation

RAW 264.7 macrophage-like cells (ATCC No. TIB-71, American Type Culture collection, Rockville, MD, USA) were routinely grown in DMEM (4,5 g/l glucose, 25 mM HEPES, 4 mM L-glutamine, without sodium pyruvate) supplemented with 10 % (v/v) heat-inactivated FCS and 100 U/ml penicillin/streptomycin at 37 °C in humidified atmosphere containing CO_2 (5 %).

Cells were grown in DMEM supplemented with 10 % (v/v) heat-inactivated FCS to 80 % confluency. Aqueous lipid dispersions were prepared using the ethanol injection method [297] in DMEM without heat-inactivated FCS. The final EtOH concentration of the incubation

media did not exceed 1 % (v/v). Cells were incubated with lipid dispersions for 1 h, 2 h, or 4 h, with DMEM containing 1 % (v/v) EtOH as controls. Cells were harvested after 1 h, 2 h, or 4 h of incubation, washed with PBS. mRNA Isolation was performed using the RNeasy® Mini Kit from QIAGEN (Venlo, Netherlands) according to the procedure described for animal cells in the RNeasy® Mini Handbook (Fourth Edition). The RNA of cells grown in a 58 cm^2 culture dish was isolated using two columns and pooled afterwards. mRNA quantity and quality was checked using the Agilent 2100 Bioanalyzer RNA assay. Results are presented as means of three independent experiments (biological replicates) and are refered to control samples (incubation of cells with oxPL-free medium).

Microarray analysis

The used mouse cDNA microarrays (>27k elements), labelling and hybridization procedures were described previously (Pinent et al. [313], Hackl et al. [314]). Briefly, cDNA was prepared from 25 µg total RNA with Random Hexamers and Superscript Reverse Transcriptase II, in the presence of amino allyl dUTP. cDNA samples were purified with QIAquick kit from QIAGEN, according to the manufacturer´s instructions, but using potassium phosphate wash and elution buffer instead of supplied buffers. N-Hydroxysuccinimide esters of Cy3 and Cy5 were coupled to the aadUTP incorporated in the cDNA. Coupling reactions were quenched by 0.1 M sodium acetate (pH = 5.2) and free dyes were removed using QIAquick columns (QIAGEN). Fluorescent samples were dried, resuspended in hybridization buffer (50 % formamide, 5 * SSC, 0.1 % SDS) and combined. In all Cot1 DNA and 20 µg poly(A) DNA were added and denaturated at 95 °C for 3 min. The samples were applied to the prehybridized slide (incubation at 42 °C for 45 min in 5 * SSC, 0.1 % SDS) and hybridized in a humidified chamber overnight at 42 °C in the dark. The slides were washed at room temperature twice

for 2 min in a 1 * SSC, 0.2 % SDS solution, for 4 min in 0.1 * SSC, 0.2 % SDS, for 4 min in 0.1 * SSC and for further 2 min in 0.1 * SSC and dipped twice in MQ water. The slides were dried and scanned with a GenePix 4000B microarray scanner (Axon Instruments) and the resulting TIFF images were analysed with GenePix Pro 4.1 (Axon instruments). Microarray analyses were performed in triplicates with dye swap, corresponding to three independent experiments (biological replicates). Features were filtered for low-quality spots and arrays were global mean and dye-swap normalized, and log2-transformed using ArrayNorm [313]. Genes with >1.5 fold change and adjusted p-value (FDR) < 0.05 were considered significantly differentially expressed using the R/Bioconductor package *limma*.

Bioinformatic analysis

Significantly up or down regulated genes were analysed using DAVID [315], [316], which systematically maps these genes to associated biological terms (i.e. gene ontology) and analyses if annotation terms are significantly enriched (over represented) compared to chance (background population of all known mouse genes from the Gene database). As POVPC showed only very minor effects on the gene expression levels, we analysed up regulated genes of RAW 264.7 macrophages exposed to PGPC stress conditions at varying incubation times, which allows a time dependent view on the cellular responses. Furthermore we focused our analysis on biological responses and therefore restricted the analysis to annotation terms referred to biological processes using “GOTERM_BP_ALL” and “PANTHER_BP_ALL” as data sources. Clusters (groups) of enriched annotation terms sharing common genes were identified using medium classification stringency setting based on kappa statistics. As a result similar enriched annotation terms were grouped reflecting the same (or related) biological theme (e.g. “GO:0006915~apoptosis”, “GO:0012501~

programmed cell death"). Annotation clusters were further pooled sharing very similar and significantly enriched annotation terms (FDR < 20%) and interpreted with reflecting biological processes (see Figure 25). We selected 36 highly significantly regulated genes often listed in annotation clusters and considered as potential key elements of the obtained biological processes and performed a literature search. 19 of these 36 candidate genes were selected for RT-qPCR verification and further biochemical interpretation. Heatmaps of expression levels (log_2 fold changes) of deregulated genes were visualized using Genesis [317].

Quantitative RT-PCR

Microarray results of 19 genes (for gene list and primer sequences see supplemental information) were verified by quantitative real-time PCR. 1 µg of total RNA of biological replicates from microarray experiments was reverse transcribed into cDNA with GoScript™ Reverse Transcription System from Promega (Wisconsin, USA) according to the technical manual. cDNA was diluted to 1 ng/µl, 4.5 ng cDNA, primers and SYBR® Green (Applied Biosystems®; Carlsbad, USA) were transferred in a 96-well plate and RT-qPCR measurement was performed with ABI PRISM®7000 Sequence Detection System and HPRT as the reference gene. Obtained results were analyzed with QPCR webtool, developed at the Institute of Genomics and Bioinformatics [318]. Quantifications were performed in triplicates (technical replicates).

3.4 Results

Here we report on a global microarray analysis of mouse genes that are upregulated or downregulated in response to the truncated oxidized phospholipids PGPC and POVPC. For this purpose, cells were incubated with 50 µM of the according lipid followed by preparation of total RNA and expression analysis using mouse cDNA microarrays (>27k elements). From this data, we determined the total number of genes affected by either lipid. The individual target genes were clustered and visualized as heatmap (Figure 24) displaying the kinetics and relative expression levels. A list of all genes affected by either oxPLs is provided in the supplement. DAVID GO analysis was performed to identify biological functions of genes that were regulated under the influence of PGPC showing that the response of the transcriptome to this lipid largely matches the effects of its parent oxLDL (see Discussion), which is a highly atherogenic particle. Results obtained by microarray analysis for genes that are related to atherogenesis were validated by qPCR.

Figure 23 shows that PGPC influences the expression of a large number of genes. Within 4 hours, 146 genes are upregulated and 47 genes are downregulated under the influence of this lipid in a time-dependent manner. The number of expressed genes steadily increases in these cells upon incubation with this lipid for 4 hours. The individual genes upregulated by PGPC are listed in the heatmap shown in Figure 24. POVPC shows a much smaller effect on the transcriptional level. Only four genes are upregulated and one gene is downregulated if cells are exposed to this compound. It has to be emphasized in this context that POVPC is chemically reactive due to its *sn*-2 aldehyde group whereas the carboxylated lipid PGPC is not. It has been shown that POVPC forms covalent Schiff bases with a series of target proteins in cultured macrophages and it looks as if this oxidized phospholipid mainly acts on the proteome level [88], [85], whereas PGPC affects the transcriptome. Specific proteins

undergoing noncovalent interactions with the latter lipid have not been identified so far except some receptor proteins (e.g. CD36, PAF receptor, PPARγ).

DAVID GO analysis of all genes that are upregulated by PGPC led to the identification of functional annotation term clusters. Many of these clusters contain elements that are relevant to the initiation and development of atherosclerosis that is developmental processes/angiogenesis, apoptosis, negative regulation of cellular processes/cell cycle, response to wounding/procoagulant and inflammatory mechanisms (see Figure 25). The individual biological functions of all genes affected by POVPC and selected genes influenced by PGPC were obtained from data bank search in Pubmed/Gene and are shown in Table 3 and Table 4, respectively. The PGPC-induced genes that are mainly involved in atherogenic processes and their molecular functions are shown in Table 5. Their physiological roles in mediating the toxic and atherogenic signal of this oxidized phospholipid are highlighted in the Discussion section.

Expression of the atherogenic genes in response to PGPC was validated by qPCR. Almost all selected genes (19) could be confirmed by qPCR (18) (see Figure 26). Only one gene could not be confirmed. This score of verified genes is much better than the average values of ~75 % reported in the literature and supports our method of gene selection for qPCR. Instead of verifying the genes that were highest regulated, we verified genes identified as most important for biological processes from DAVID analysis.

So far only a limited number of studies is available that describe the transcriptional effects of PGPC, POVPC, and the complex oxidized PAPC in cultured macrophages. However, the respective results are difficult to compare with ours, since different cell lines, incubation conditions, and procedures for data analysis have been used. In addition, microarrays were used for gene expression analysis that only contained limited sets of genes. Our study

represents the first global analysis of gene expression in a murine macrophage-like cell line. The results of this work largely but not completely match the transcriptional effects that have been reported for gene expression in the same cell line under the influence of oxLDL. Since PGPC is one of the major oxidized phospholipids in this particle, we conclude that it is one of the components that mostly contribute to its atherogenic properties. PGPC contains only one long hydrophobic acyl chain and as a consequence easily exchanges between the phospholipid surfaces of lipoproteins and cells. Therefore, its uptake by cells and its cellular toxicity is to a large extent independent of the expression/activity of specific receptors.

Table 3: Time-dependent transcriptional effects of 50 µM POVPC in RAW 264.7 macrophages as observed with microarray analyses.
↑ upregulated genes; ↓ downregulated genes; - no effect. * These genes were also affected by PGPC.

Gene symbol	RefSeq	POVPC 1 h	POVPC 2 h	POVPC 4 h	Description
Klf6*	NM_011803	↑	↑	-	Transcription factor
Hmgb3*	NM_008253	↑	-	-	HMG box (DNA binding) subfamily; DNA replication, nucleosome assembly and transcription
c- Jun*	NM_010591	↑	-	-	Member of AP-1 complex; Transcription regulator
Rgs2*	NM_009061	-	↑	-	Regulator of G protein signaling
Egr1*	NM_007913	-	↑	-	Transcriptional regulator; Differentiation and mitogenesis
Emp1*	NM_010128	-	↑	-	Epithelial membrane protein; cellular contact [319]
B4galt5	NM_019835	-	↓	-	β-1,4-galactosyltransferase
Atf3*	NM_007498	-	↑	↑	Activating transcription factor; cellular stress response
Pdgfb*	NM_011057	-	-	↑	Platelet derived growth factor
Fam20C	NM_030565	-	-	↑	Potential regulator of differentiation and function of hematopoietic tissue
Lpl	NM_008509	-	-	↑	Triglyceride hydrolase / bridging factor for receptor mediated lipoprotein uptake
Abca1*	NM_013454	-	-	↓	Cholestol efflux pump

Table 4: Comparison of relative gene expression levels (log_2 fold changes) observed by microarray (µa) and RT-qPCR (qPCR) analyses.

Cells were incubated with PGPC for 4 hours and cDNA was prepared for microarray and RT-qPCR analysis as described in "Experimental procedures". Cycle numbers for RT-qPCR were normalized to hypoxanthin-guanin-phosphoribosyltransferase (HPRT) as housekeeping gene. Gene expression levels of oxPL-treated samples are shown as folds of data for control cells. Up- and down-regulated genes are shown in red and blue, respectively.

Gene	RefSeq	µa	qPCR	Description
Atf3	NM_007498	2.95	2.65	cAMP-dependent transcription factor
Atf4	NM_009716	0.79	0.86	cAMP-dependent transcription factor
c-Jun	NM_010591	1.32	1.72	Member of the AP-1 protein complex; transcription factor; cellular stress response
Junb	NM_008416	0.70	0.95	Member of the AP-1 protein complex; transcription factor
Klf2	NM_008452	1.35	4.49	Zinc finger transcription factor; T-cell trafficking
Egr1	NM_007913	0.78	1.01	EGR family of C2H2-type zinc-finger proteins; transcriptional factor
Tnf-α	NM_013693	1.08	1.94	Proinflammatory cytokine
Trib3	NM_175093	0.59	1.49	Putative protein kinase; sensitization of cells to TNF- induced apoptosis; negative regulation of AKT1
Src	NM_009271	0.87	1.12	Tyrosine-protein kinase / proto-oncogene
Ldlr	NM_010700	0.71	0.50	Receptor-mediated endocytosis of LDL
Gadd45b	NM_008655	0.67	0.79	Activation of the p38/JNK pathway; Regulation of growth & apoptosis
Rhob	NM_007483	2.72	3.90	Ras homolog family member B; GTPase; cytokine trafficking and cell survival; Regulation of CD36 expression
Cd9	NM_007657	1.02	0.69	Transmembrane 4 superfamily; differentiation, adhesion, and signal transduction
Rtn4	NM_194052	0.70	0.54	ER associated reticulon family member
Abca1	NM_013454	-1.10	-1.31	ATP-binding cassette transporter; cholesterol efflux
Ccnb1	NM_172301	-0.55	-0.64	Regulatory protein involved in mitosis
Plk1	NM_011121	-0.62	-0.94	Serine/threonine protein kinase; Cell cycle control
Rad51	NM_011234	-0.67	-0.65	Cellular response to DNA damage
Cd36	NM_007643	0.82	0.02	Scavenger-receptor

Table 5: Processes described to be affected by genes regulated in PGPC treated RAW264.7 macrophages.

Upregulated genes	**Cell response**	**References**
Tnf-α, Atf3, c-Jun, Jun-B	Apoptosis via TNF-α pathway	[306], [320], [321], [322], [323], [324]
c-Jun, Jun-B	Apoptosis via aSMase/Casp3	[58], [59]
Trib3	Apoptosis	[325], [326]
Atf4	Unfolded protein response and angiogenesis	[102], [327], [328]
Egr-1	Procoagulant mechanisms and plaque stability	[329], [330], [331]
CD9, RhoB, Src, c-Jun, Ldlr	OxLDL/LDL uptake and foam cell formation	[332], [333], [334], [335], [336]
Downregulated genes		
Abca1	Cholesterol efflux	[337], [338]
Ccn B1, Ccn F, Ccn A2, Cdc 5, Cdc A7, Ccd 6, Plk1	Proliferation and cell cycle	[339], [340], [341]

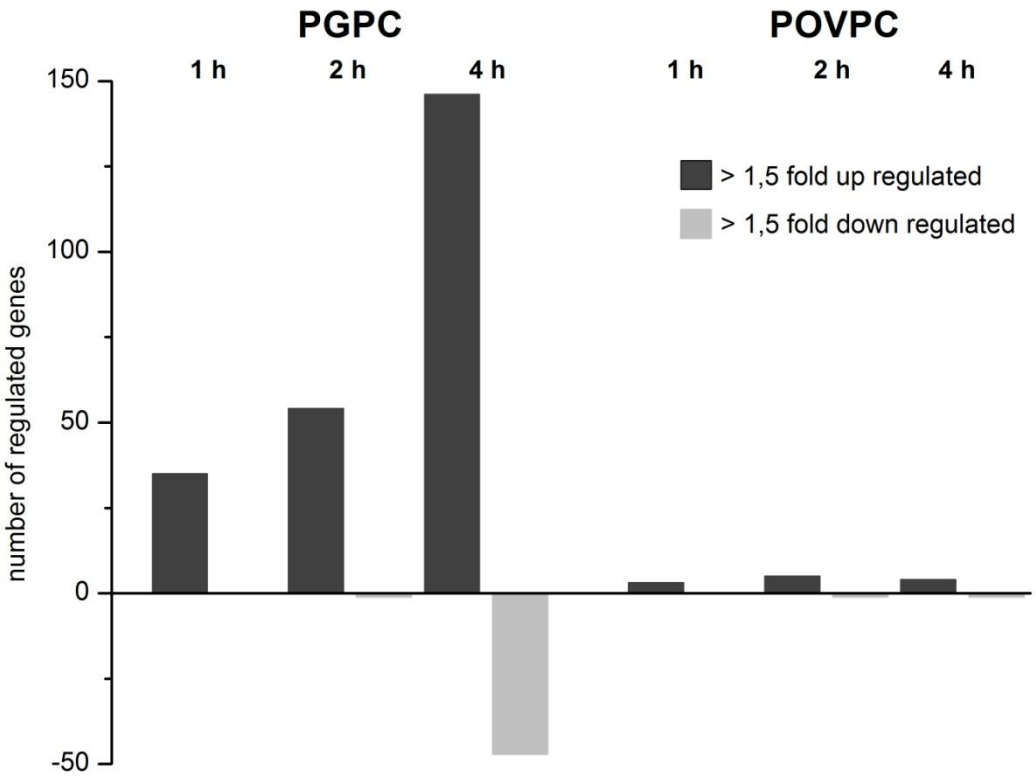


Figure 23: Time-dependent effects of PGPC and POVPC on overall gene expression in RAW 264.7 macrophages.

Number of significantly up- and down-regulated genes relative to control cells (FC>1.5; FDR<5%) are shown. PGPC: 146 up- and 47 down-regulated genes; POVPC: 4 up- and 1 down-regulated genes.

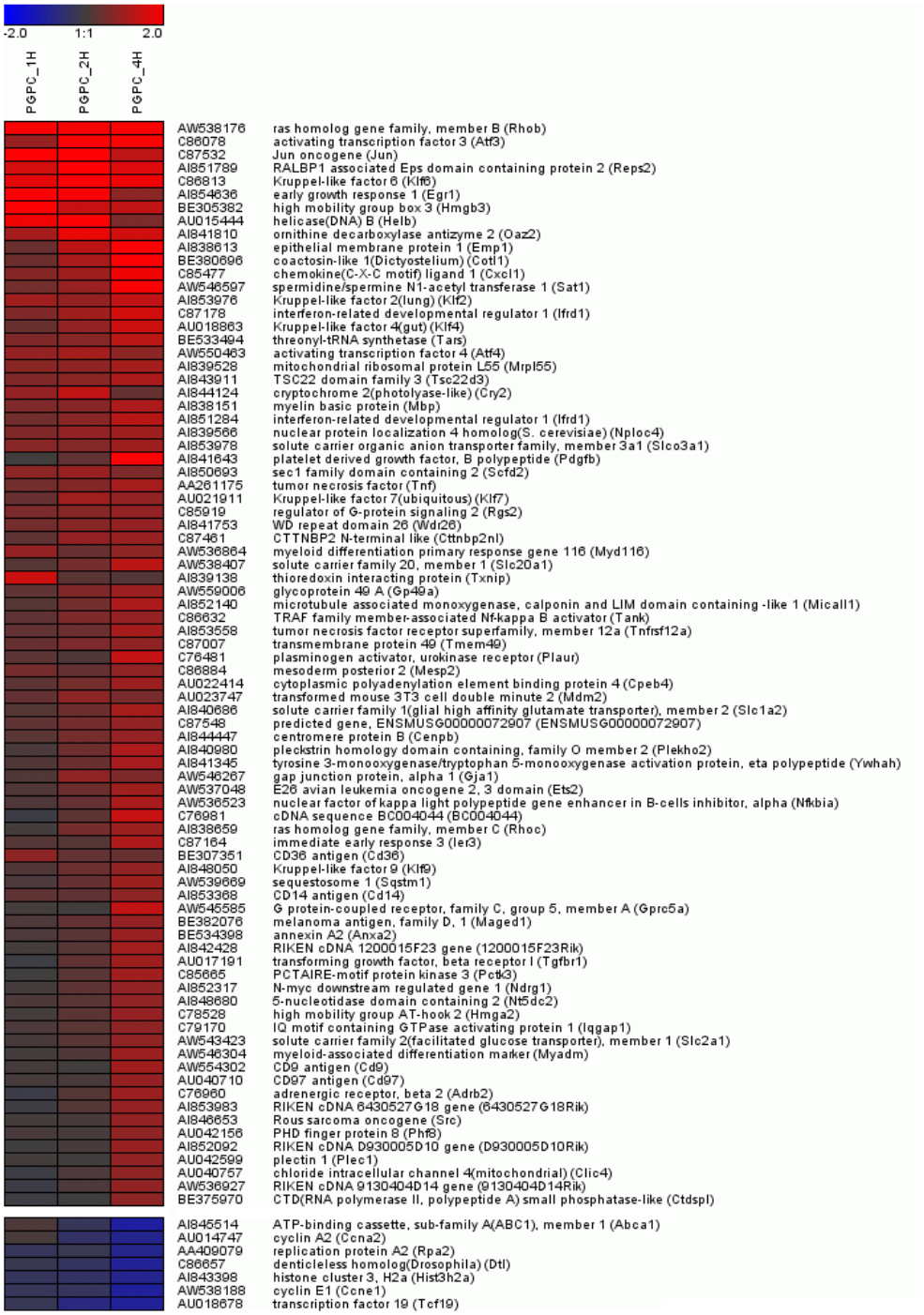


Figure 24: Time dependent Heatmap of the effect of PGPC on gene expression in RAW 264.7 macrophages.

Data are shown for unique genes with a FDR < 5 % and $\log_2$ fold change >= 0.8 or $\log_2$ fold change <= - 0.8.

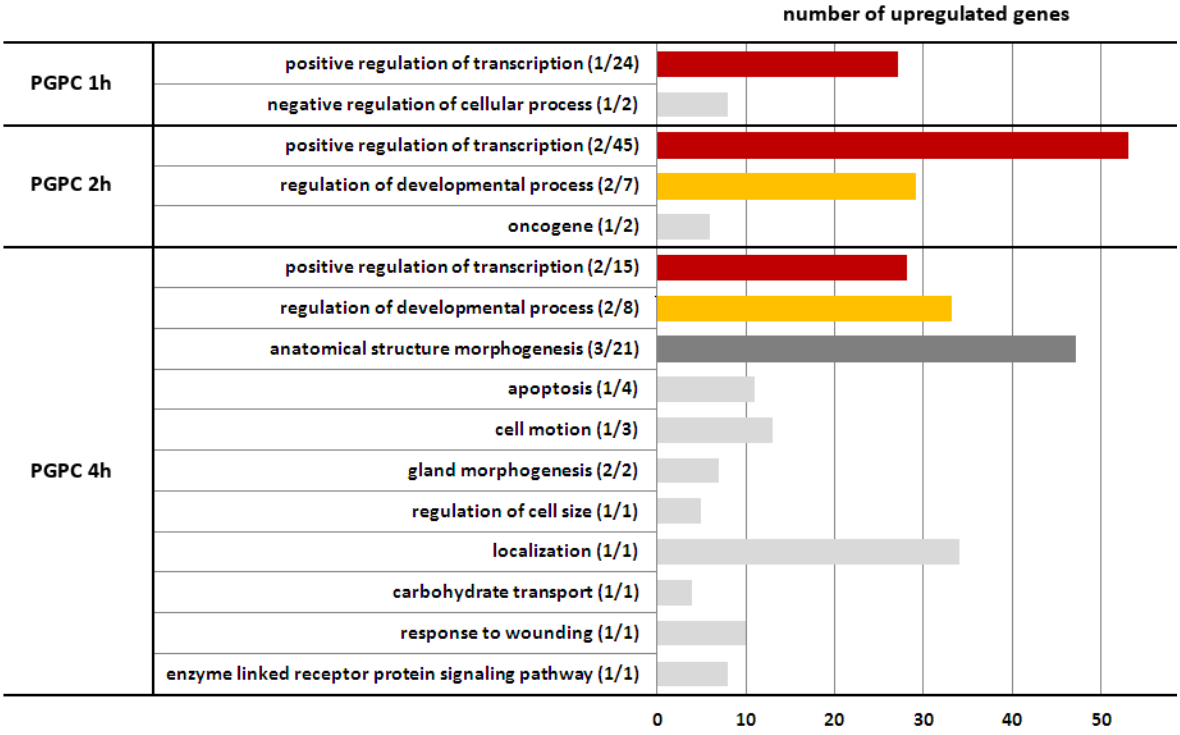


Figure 25: Time dependent effects of PGPC on biological processes in RAW 264.7 macrophages.

An annotation term clustering with annotation terms referred to biological processes of upregulated genes was performed with DAVID. Dominant biological processes were classified applying analysis steps described in “Experimental procedures”.

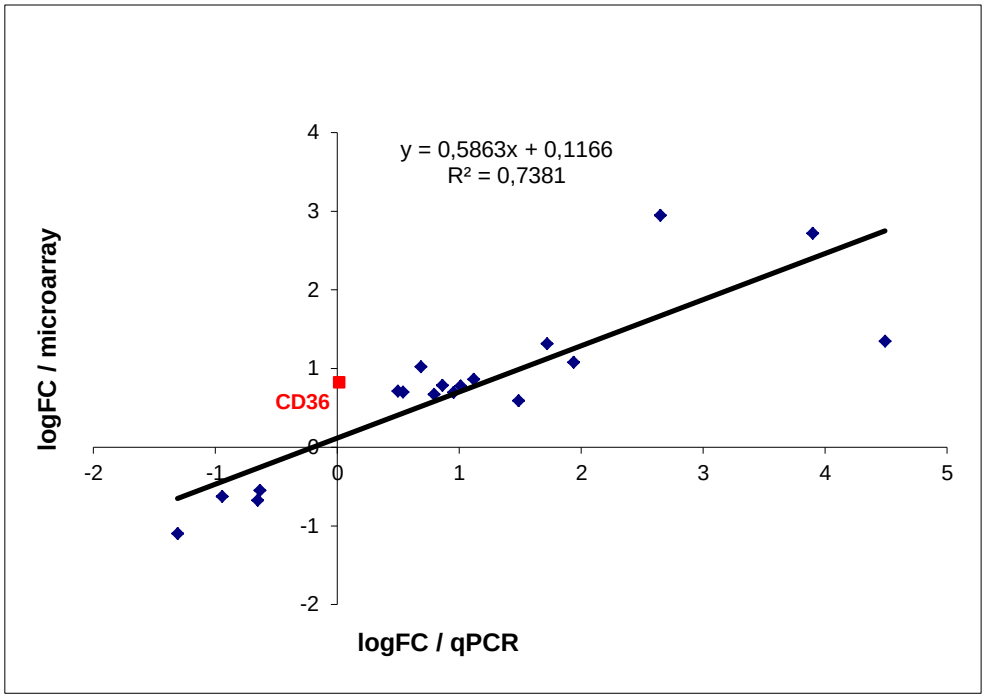


Figure 26: Correlation of gene expression levels determined by microarray and RT-qPCR analysis.

(FC = fold change).

3.5 Discussion

Microarray analysis revealed fundamental differences between the effects of the oxidized phospholipids PGPC and POVPC on gene expression in cultured macrophages as pertinent to the role of cells in atherosclerosis. This is surprising insofar as both oxPLs drive the cells towards the same endpoint, namely apoptosis although mostly different pathways are utilized in this toxic process [170]. POVPC only affected a relatively small number of genes, whereas PGPC up- or down-regulated a several fold larger number of genes. We suppose that this difference is at least in part due to the fact that POVPC is chemically reactive and PGPC is not. POVPC contains an aldehyde function in position *sn*-2 which enables this compound to form Schiff bases with amino groups of proteins [85] and very likely also with amino groups of phospholipids. From fluorescence microscopic observations, it is known

that a labelled POVPC analogue becomes enriched in the plasma membrane due to its adduct formation, whereas a fluorescent PGPC derivative quickly enters the cell interior and mainly localizes to subcellular membranes [88]. As a consequence, the primary signalling processes must be different and the availability for intracellular nuclear receptors too, provided there are any.

Therefore, the following sections mostly deal with the transcriptional effects of PGPC in macrophages encompassing hallmarks of atherogenic events including cell death with emphasis on apoptosis, angiogenesis, cellular uptake and efflux of cholesterol, procoagulant mechanisms, plaque stability, and last but not least cell cycle regulation. Overall, the effects induced by PGPC and to a lesser extent by POVPC support the assumption that they are biologically active components of atherogenic oxLDL which activates or inactivates the same genes that are affected by the oxPL.

PGPC and cell death (upregulated genes: c-Jun, JunB, Tnf-α, Atf3, Trib3)

PGPC stimulated expression of the cell death-associated genes c-Jun, JunB, Tnf-α, Atf3, and Trib3 in mouse macrophages. These findings are in line with the observations that the same genes are activated by oxLDL in vascular cells pointing to a role of the oxPL as a toxic component of the modified lipoprotein. Jovinge et al. reported that exposure of monocytes/ macrophages to oxLDL triggers expression and release of TNF-α [306]. This proinflammatory cytokine activates a series of genes in vascular cells involved in programmed cell death [321], [322]. The activity of TNF-α is intimately associated with the action/expression of the transcriptional factor ATF3 which is induced under stress conditions. Nawa et al. reported that this protein is highly expressed in endothelial cells and macrophages of human atherosclerotic lesions [323]. From *in vitro* experiments it can be inferred that it is involved

in oxLDL-induced death of cultured HUVECs. In addition to ATF3, oxLDL stimulates the expression of TNF-α in these cells. If the ATF3 gene is silenced by antisense cDNA, TNF-α and oxLDL induced apoptosis is substantially decreased [323]. This data supports the assumption that oxLDL activates the TNF-α gene which after secretion stimulates ATF3 expression and cell death according to an autocrine mechanism [323]. This relationship is at least not fully applicable to PGPC toxicity. Nawa et al. measured gene expression after 24 hours incubation of the cells with oxLDL. Our microarray data were collected after much shorter incubation times. ATF3 expression already starts after one hour incubation with the oxPL, whereas activation of the TNF-α gene is observed after 2 and 4 hours. Obviously, different mechanisms may come into play under conditions of such short exposure times perhaps involving more direct interactions of PGPC.

A key element of apoptotic signaling is c-JUN. This protein is an important component of the AP-1 complex representing a group of protein dimers that may be composed of members of the Jun family (c-Jun, JunB, JunD) and basic leucine zipper (bZIP) proteins [342]. Members of the Fos (c-Fos, FosB, Fra1, Fra2) and ATF families are the most important bZIP proteins for AP-1 complex formation. Expression of each AP-1 component is independently regulated. As a consequence, cells contain complex mixtures of AP-1 dimers with different functions depending on ambient conditions, e.g. oxidized phospholipid stress. AP-1 activity can be induced and modulated by a broad range of other extracellular stimuli including mitogens, hormones, extracellular matrix, and genotoxic agents [342]. Many of these stimuli including oxPL and oxLDL [58], [59] also activate c-Jun N-terminal kinases which catalyze the phosphorylation and thereby increase the transcriptional activity of Jun proteins. Because AP-1 composition may vary to some extent, its activity is complex and may lead to controversial effects depending on experimental conditions. Nevertheless, increased levels

of JUN and FOS proteins associated with high JNK activity have frequently been correlated with apoptosis [343], [344], [345]. Last but not least, there is a relationship with c-Jun activity and the oxLDL-induced expression of TNF-α and ATF3 [346], [347]. Takashiba et al. found a functional binding site for AP-1 at the TNF-α promoter, suggesting that c-Jun possibly regulates expression of the cytokine [324]. In addition, ATF3 seems to contribute to c-Jun activity, since both components synergistically activate death genes by binding to ATF and AP-1-dependent promoters.

A particular aspect of oxLDL and oxPL-mediated apoptosis of vascular cells is the role of ceramide as lipid second messenger. We found that the modified lipoprotein and the truncated oxidized phospholipids PGPC and POVPC induced apoptosis in cultured vascular smooth muscle cells and RAW macrophages. This phenomenon was associated with activation of aSMase within minutes, the formation of ceramide, the activation of JNK and p38 MAP-kinase and the stimulation of caspase3. Since the respective kinase and caspase activities as well as apoptosis were reduced by a specific aSMase inhibitor, it was concluded that the latter enzyme was causally involved in lipid-induced cell death upstream of the other signaling components [58], [59]. In this context it is interesting to mention that TNF-α and ATF3 activities induced by oxLDL or PGPC also give rise to activation of JNK which phosphorylates apoptotic Jun proteins. However, it has to be emphasized that the aSMase pathway can be activated much faster. Exposure of cultured vascular cells to oxPL or oxLDL activates the sphingolipid hydrolase within minutes, very likely on protein level either due to direct lipid-protein interactions or modulation of enzyme activity by a general membrane effect (see Chapter 2).

More recently, TRIB3 was identified as a new protein which was expressed in macrophages in response to oxLDL in a dose-dependent manner [325]. This protein supposedly inhibits

PKB activity and thus may be considered antiproliferative [326]. Since silencing of the Trib3 gene reduced the cellular susceptibility towards apoptosis, it was concluded that this protein is also causally related to lipoprotein-induced cell death. Our microarray analysis shows that PGPC also upregulates Trib3 expression and therefore further supports the hypothesis that PGPC is a component of oxLDL with apoptotic capacity.

PGPC and angiogenesis (upregulated gene: Atf4)

PGPC stimulated expression of the angiogenesis promoting transcription factor ATF4 in this microarray study. This result is in line with the observation that this gene is activated by a mixture of PAPC oxidation products in vascular cells pointing to a role of PGPC as a toxic component of oxidized LDL [102], [327], [328]. The relationships are as follows:

DAVID GO analysis of our microarray data led to the identification of upregulated genes under the annotation terms “vasculature development“, “blood vessel morphogenesis“ and “blood vessel development”. Bochkov et al. reported that oxPL accumulation in atherosclerotic lesions stimulates plaque angiogenesis [102]. In addition, it has been shown that the density of *vasa vasorum* highly correlates with the amounts of mononuclear cells infiltrating the advanced lesions [348], [349], [350], [351]. On top of these observations, Bochkov et al. found that oxPAPC stimulates expression of VEGF in cultured HUVECs and monocyte-derived macrophages [102]. Since macrophages accumulate in atherosclerotic plaques that are rich in oxPL, it may be speculated that VEGF secretion by these cells stimulates angiogenesis in a paracrine manner.

The same group found that expression of VEGF, and as a consequence angiogenesis, is induced by the unfolded protein response (UPR) and the electrophilic stress response (ESR). Enhanced expression of VEGF by oxPAPC is accompanied by elevated levels of the

transcription factor ATF4. In HUVECs, ATF 4 is a key mediator of UPR, promoting angiogenesis through VEGF activation in an autocrine manner [328]. ATF4 formation itself depends on activation of a stress-activated protein kinase. The eukaryotic translation initiating factor 2-α-kinase3 (PERK) selectively stimulates translation of ATF4, whereas it inhibits synthesis of most other proteins [351].

ATF4 may also be activated by the so-called electrophilic stress response which is induced by e. g. carbonyl groups in oxidized lipids and protects cells from such compounds [328]. ESR is initiated by oxidative modification of KEAP-1 which leads to release of the transcription factor NRF2 from the KEAP-1/NRF2 complex [352]. NRF2 binds to the promoter region of the antioxidant response element (ARE) thereby stimulating expression of ATF4 which contains a putative ARE binding site. Silencing of NRF2 expression by siRNA abolishes oxPAPC-induced upregulation of ATF4 and VEGF [328]. In summary, these data provide evidence for a point of convergence of electrophilic and unfolded protein response pathways involved in the toxicity of oxidized phospholipids as pertinent to angiogenesis.

OxPLs and cholesterol efflux (downregulated gene: Abca1)

PGPC and POVPC downregulated expression of the Abca1 gene, which mediates cellular cholesterol efflux. These results are at variance with the stimulatory effect of oxLDL on the expression of ABCA1 which is due to the presence of another subclass of oxPL (9- and 13-HODE) and oxidized sterols [120]. In this context, it has to be emphasized that the lipid fraction of oxLDL contains a great variety of bioactive lipid oxidation products that may synergistically act or counteract in (vascular) cells.

Both ABCA1 and its counterpart ABCG1 mediate efflux of intracellular cholesterol, but their lipid acceptors are different. ABCA1 promotes sterol transport to lipid-poor apoAI

lipoproteins, whereas ABCG1 directs cholesterol to mature HDL and other lipoproteins [353], [354], [355]. The effects of cell and tissue specific inactivation of ABCA1 expression support the assumption that this gene inhibits foam cell formation and the development of atherosclerotic lesions [337]. According to experiments performed with $LDLR^{-/-}$ mice, which received bone marrow from ABCA1 ko mice, deficiency in this component of the sterol efflux machinery can be compensated at least in part by ABCG1 [338]. Our microarray experiments show that downregulation of ABCA1 by oxPL is not compensated by ABCG1 at least on the expression level at investigated time points.

PGPC and procoagulant mechanisms (upregulated gene: Egr-1)

PGPC persistently upregulated expression of the proatherogenic transcription factor EGR-1, whereas POVPC only transiently activated this gene. The oxPL effect is in line with the observation that oxPAPC and oxLDL also increase expression of EGR-1 in HUVECs and RAW macrophages [329], [330].

EGR-1 (Early Growth Response-1) is a zinc-finger protein linked to maladaptive host response mechanisms and ischemic stress [356]. It is inflammatory and proatherogenic. In addition, its expression is stimulated during development of atherosclerosis in apoE-deficient mice. Further, EGR-1 knock-out mice have a reduced atherosclerotic lesion size. EGR-1 stimulates expression of a series of atherogenic genes including JE/MCP, IL-1b, tissue factor (TF), plasminogen activator inhibitor (PAI-1), VCAM-1, and ICAM-1. It has to be noted that EGR-1 activity seems not to affect cholesterol and triacylglycerol levels [330]. EGR-1 is enriched in fibrous caps of atherosclerotic plaques [331]. The EGR-1-dependent gene products TF and PAI-1 are prothrombotic and are associated with fibrin deposition in these areas of atheromas thus enhancing plaque instability and rupture [329].

OxLDL stimulates EGR-1 expression in a dose-dependent manner in RAW macrophages [330]. OxLDL-dependent EGR-1 expression is mediated by the MEK-ERK1/2 MAP kinase pathway. RAW cells pretreated with the MEK-1 inhibitorPD98059 do not express EGR-1 if challenged with oxidized lipoprotein [330]. From our microarray data, it can be concluded that PGPC and POVPC contribute to the inflammatory procoagulant activity of oxLDL as pertinent to EGR-1. However, it remains open whether POVPC and PGPC utilize the same signaling pathways to trigger these effects.

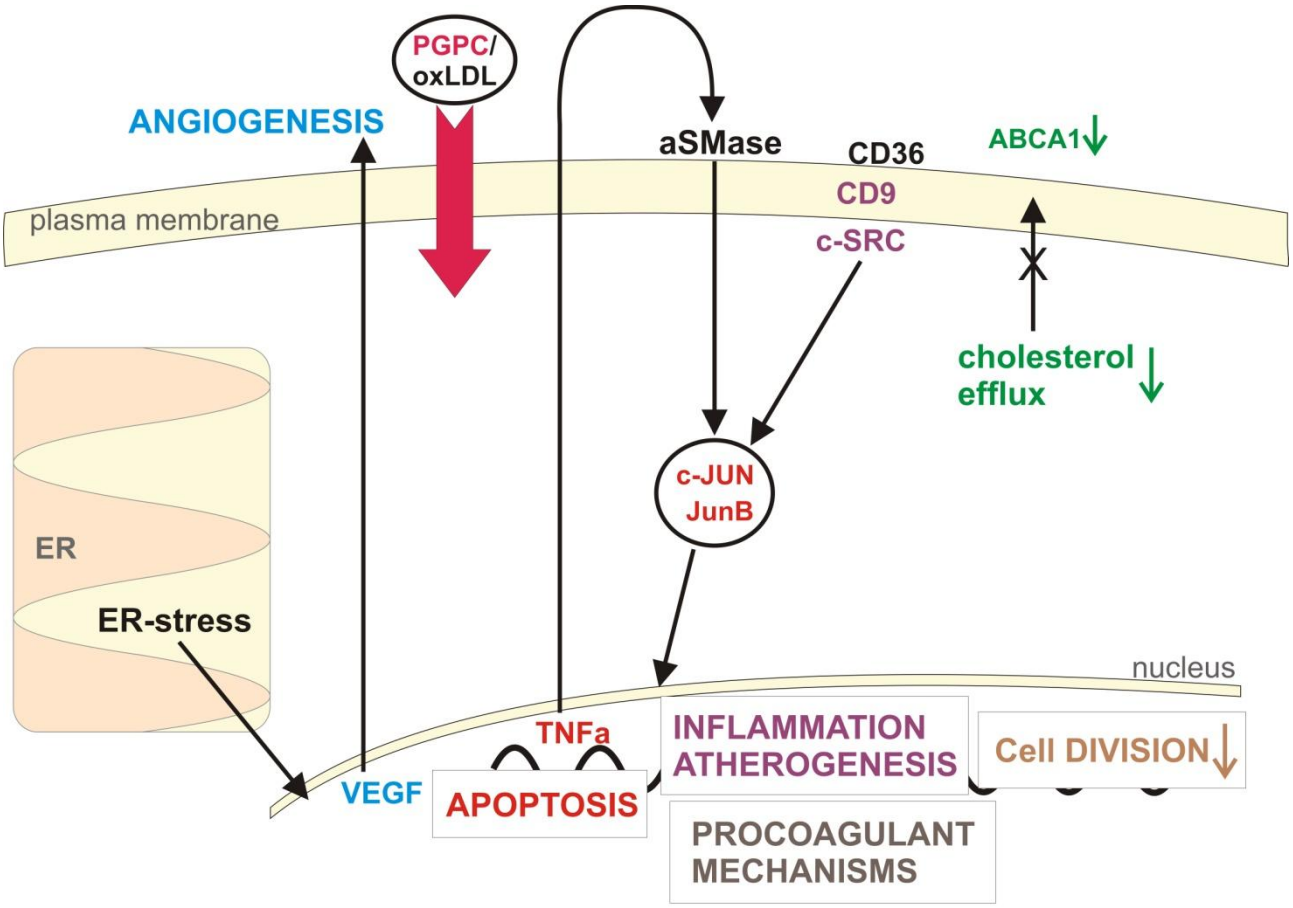
PGPC, atherogenesis and inflammation (upregulated genes: Cd9, Src, c-Jun, and JunB)

PGPC upregulated expression of the proatherogenic and inflammatory genes Cd9, Src kinase, c-Jun, and JunB. POVPC did not affect these genes in our study. These genes are related to scavenger receptor-mediated uptake of oxLDL in macrophages and its intracellular signaling, since they act in concert with CD36. CD36 is the major scavenger receptor for oxLDL in these cells, whereas scavenger receptors class A may be involved to a lesser extend [37]. CD36-mediated signaling in response to oxPL may follow two routes. After lipoprotein internalization, its oxidized lipid components can directly bind to nuclear receptors thereby triggering gene expression [120]. Alternatively, CD36 activates a signaling cascade involving Src kinases, the MAP kinases c-Jun N-terminal kinase-1 and -2, (see Figure 7) and finally the AP-1 components c-Jun and JunB [332], [107] (for activities of AP-1 see inflammatory and apoptotic response to PGPC). CD36 signaling in response to oxLDL is directly modulated by CD9 which binds to the cytoplasmic side of the receptor [333]. It has to be noted that the effects described above are cell-specific. For instance, the responses of peritoneal macrophages to CD36 are mediated by the Src kinase lyn and JNK, whereas fyn and the MAP kinase p38 are involved, respectively, in endothelial cells [332].

CD36-mediated oxLDL uptake by macrophages is not regulated and leads to accumulation of toxic lipoprotein material inside the cells. This process induces the formation of foam cells which is a hallmark of atherosclerosis. In the literature it is emphasized that masking positively charged lysines of apoB by lipid oxidation products (aldehydes) is the reason for the switch from the “beneficial“ apoB receptor to the ”detrimental“ scavenger receptor. In this context, a similar function of PGPC also has to be highlighted. This oxPL directly confers net negative charges to the LDL surface and thus makes it prone to CD36 binding and its consecutive intracellular signaling [85]. On top of that, PGPC enhances oxLDL activity by upregulating important genes that propagate its signals.

PGPC and regulation of cell cycle (downregulated genes: Cyclin B1, Cyclin F, Cyclin A2, Cdc5, Cdca7, Cdc6, Plk1 (polo-like kinase))

PGPC downregulated expression of the cell cycle-dependent proteins Cyclin B1, Cyclin F, Cyclin A2, Cdc5, Cdca7, Cdc6, and PLK1. It has been reported that oxLDL elicits similar effects in vascular smooth muscle cells and fibroblasts [339]. Thus, PGPC is likely to contribute to the activity of the modified lipoprotein on cell cycle components, too. PLK1 seems to play a particular role in this respect. This kinase is a member of a protein family (PLK1-4) that promotes entry of the cell cycle to mitosis. It induces degradation of Wee1 kinase which phosphorylates and thereby inhibits the Cdc2/ Cyclin B1 complex (maturation factor). As a consequence, the cell enters cell cycle arrest and eventually becomes subject to apoptosis which represents the endpoint of oxPL toxicity in macrophages [340], [341].



Angiogenesis:
ATF4 ↑

Apoptosis:
TNF-α ↑, ATF3 ↑, c-Jun ↑, JunB ↑, TRIB3 ↑

Inflammation and atherogenesis:
CD9↑, c-SRC ↑, c-Jun ↑, JunB ↑

Cholesterol efflux:
ABCA1 ↓

Procoagulant mechanisms:
EGR1 ↑

Cell Division:
CycB1 ↓, CycA1 ↓, Cdc5 ↓, Cdc6 ↓, CdcA7 ↓, PLK1 ↓

Figure 27: Biological processes and corresponding genes regulated by PGPC in RAW 264.7 macrophages.

Conclusion

OxLDL is an atherogenic particle that contains a large variety of biologically active lipids and modified apolipoprotein epitopes that are responsible for its activity. This study made an attempt to identify the contributions of two major oxidized phospholipids, namely PGPC and POVPC that localize to the surface of the particle. We determined the effects of the chemically defined compounds on gene expression in cultured macrophages and found that they largely contribute to oxLDL activities that are responsible for its atherogenicity. OxLDL contains in addition to PGPC and POVPC a large variety of other oxidized phospholipids with potential and perhaps different bioactivity. Many of these compounds still await the development of (bio)chemical methods to prepare sufficient amounts for biomedical research.

Acknowledgement:

This work was supported by the Austrian Science Fund (FWF; projects F3006-B5 and I308-B12).

Supplemental information

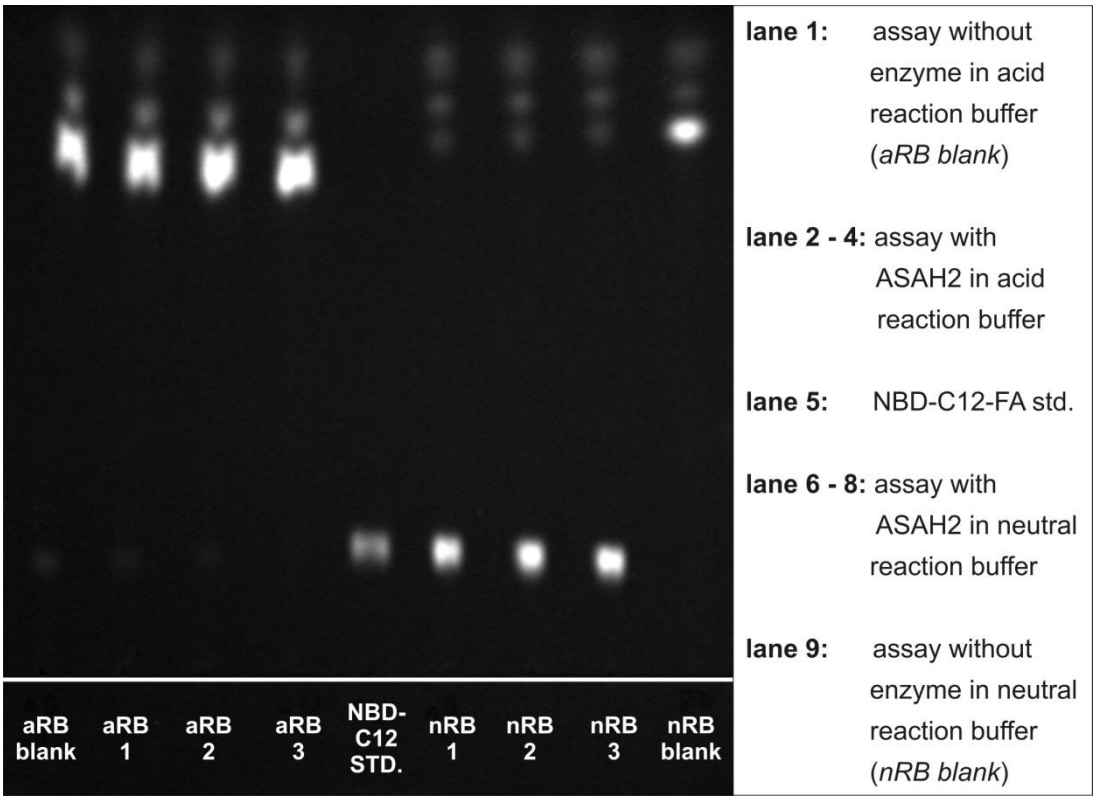


Figure 28: Verification of nCDase activity assay.

NCDase activity assay was developed and optimized with ASAH2 from R&D Systems. Reproducibility and specificity for nCDase activity in nCDase-reaction buffer was demonstrated. Distinguishability of aCDase and nCDase activity was achieved by proper buffer conditions.

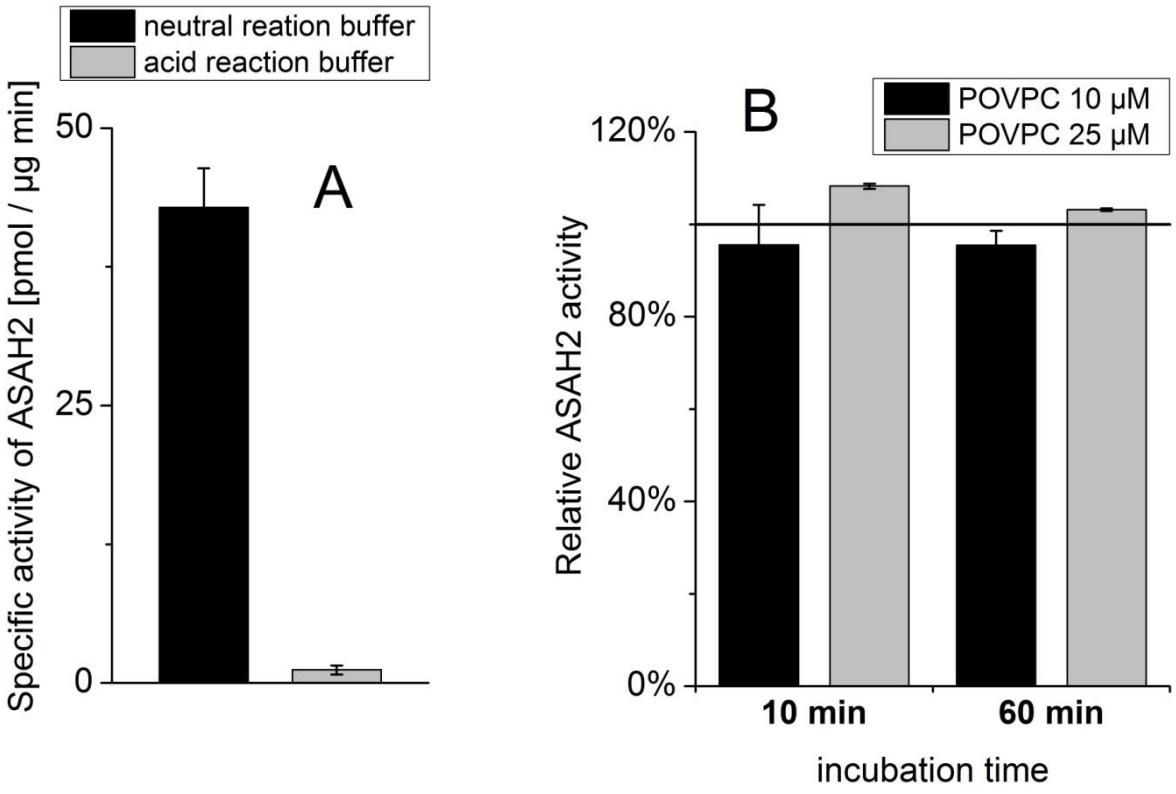


Figure 29: *In vitro* experiments with human recombinant neutral ceramidase ASAH2.

Panel A: Activity of ASAH2 in acid or neutral reaction buffer.

ASAH2 activity was determined in acid and neutral reaction buffer, to proof buffer selectivity. Activity of ASAH2 was approximately 100 times higher in neutral than in acid reaction buffer.

Panel B: Effect of POVPC on the activity of ASAH2.

ASAH2 was incubated *in vitro* with suspensions of 10 µM and 25 µM POVPC in reaction buffer for 10 min or 60 min. No changes in activity could be detected.

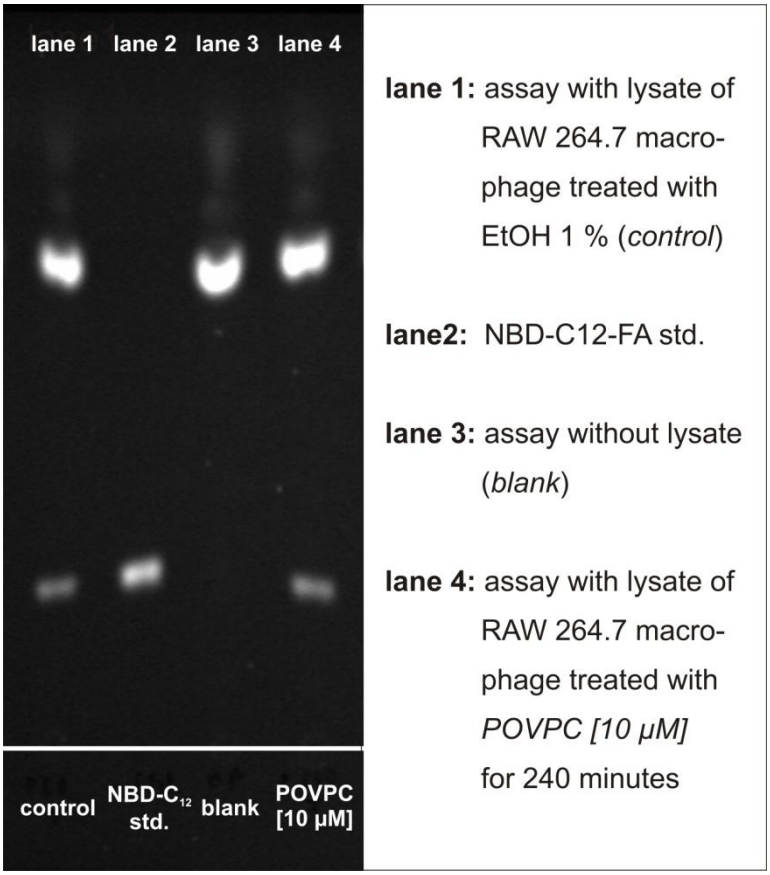


Figure 30: Verification of aCDase activity assay with cell lysates of RAW 264.7 macrophages.

Cleavage of C_{12}-fatty acid from NBD labelled C_{12}-ceramide was determined as described in Experimental procedures. RAW 264.7 macrophages were incubated with 10 µM POVPC in DMEM media for 4 h and aCDase activity was determined in cell lysates.

Table 6: Effects of PGPC and POVPC on gene expression in RAW 264.7 macrophages.

Transcription analysis was performed using a genome-wide microarray containing 27648 oligonuceotide probes for murine genes (http://www.genome.tugraz.at/adipocyte/Microarray.html)

Cells were incubated with oxPLs for the indicated times, cDNA was prepared and microarray analysis was performed as described in Experimental procedures. Shown are log_2-fold changes of gene expression of all affected genes for oxPL-treated cells compared to oxPL-free controls.

	Fold changes of gene expression					
	PGPC			POVPC		
Gene symbol	**1 h**	**2 h**	**4 h**	**1 h**	**2 h**	**4 h**
Jun	2,20	2,43	1,32	0,66		
Reps2	1,56	2,72	1,66			
Klf6	1,74	2,63	1,85	0,68	0,84	
Rhob	2,13	1,79	2,72			
Hmgb3	2,06		1,36	0,70		
Txnip	1,48					
Cry2	0,88	1,34				
Atf4	0,88	1,03	0,79			
Helb	1,87	2,03	0,63			
Cd36	0,82					
Tsc22d3	0,73	0,91	1,14			
Egr1	2,18	2,39	0,78		0,59	
Myd116	0,88		0,82			
2610101N10Rik	0,65		0,77			
Pnrc1	0,62	0,70				
Klf2	1,07	1,06	1,37			
Atf3	0,92		2,95		0,96	0,72
Oaz2	1,08	1,79	1,51			
Slc17a7	0,58		0,74			
Scfd2	0,83	1,01	0,73			
Rgs2	0,62		0,88		0,70	
Mrpl55	0,77	0,83	1,04			
Nploc4	0,67	0,86	0,99			
Clk1	0,71	0,78				
Mbp	0,66		1,16			
2900083I11Rik	0,58					
Scmh1	0,63	0,71				
Tars	0,69		1,30			
Slco3a1	0,76		0,99			
Ifrd1	0,67	1,00	1,55			
Ubc|Ubb	0,73					
D430041D05Rik	0,67	1,21				
Sp3	0,63					
Cxcl1	0,74	0,99	1,86			
Prkcb1	0,60					
Emp1		1,33	2,11		0,58	

	1 h	2 h	4 h	1 h	2 h	4 h
Cttnbp2nl		0,90	0,94			
Rasgef1b		1,10				
Mdm2		0,81	0,59			
Zfp655		0,72				
Tmem49		0,65	0,83			
Gp49a|Lilrb4		0,67	0,96			
Mdfic		0,59	0,64			
Cotl1		1,12	2,13			
Pcf11		0,59				
Bet1		0,77				
Trib3		1,15	0,59			
Serpina3g|Serpina3h|Serpina3f|Serpina3n		0,74				
Wdr5		0,61				
Wsb1		0,65				
Abl2		0,61	0,76			
Slc2a4		0,59	0,60			
Klf7		1,01	0,87			
Gja1		0,83	0,66			
Smg7		0,61				
Zfand5		0,72	0,71			
Egr4|Egr3|Egr2		0,68				
Tank		0,66	0,89			
Fbxw17		0,94				
RP24-320O9.1|1700029I01Rik|OTTMUSG00000010657		0,88				
Rcan1		0,77				
Klf4		0,87	1,47			
Trim6		0,74				
Junb		0,63	0,70			
Wdr26		0,75	0,90			
Sat1		0,92	1,96			
Tnf		0,70	1,08			
Zdhhc21		0,83				
Klhl26		0,79				
Cdkn2aip		-0,96				
Gprc5a			1,37			
Pdgfb			2,01			0,71
Plaur			1,39			
BC004044			1,42			0,69
Cd9			1,02			
Ywhah			1,09			
Tnfrsf12a			1,07			
Plekho2			1,13			
Pctk3			1,01			
Anxa2			0,93			
D930005D10Rik			0,92			
Slc20a1			1,30			
ENSMUSG00000072907|Oog1| OTTMUSG00000010009			0,86			
Mesp2			0,84			
1200015F23Rik			1,03			
Cenpb			0,89			
Micall1			1,14			

	1 h	2 h	4 h	1 h	2 h	4 h
6430527G18Rik			**0,90**			
Iqgap1			**0,82**			
Cdc42ep2			**0,85**			
Slc2a1			**0,83**			
Myadm			**0,96**			
Ier3			**1,15**			
Bmpr1a			**0,78**			
Nfkbia			**1,12**			
Cep170			**0,72**			
Ets2			**1,01**			
Ctdspl			**0,82**			
Slc1a2			**1,07**			
Clic4			**0,83**			
Rtn4			**0,70**			
Blcap			**0,70**			
Fgd6			**0,73**			
9130404D14Rik			**0,84**			
Flna			**0,74**			
Slc35a4			**0,71**			
Cdc42ep4			**0,66**			
2900011O08Rik			**0,78**			
Tnfsf12			**0,66**			
Plec1			**0,87**			
Pmp22			**0,69**			
Anxa7			**0,67**			
Smad1			**0,69**			
Ptk2b			**0,67**			
Ldlr			**0,71**			
Cd44			**0,80**			
Phldb1			**0,65**			
Adrb2			**0,96**			
Psmd8			**0,66**			
Stab1			**0,83**			
Klf3			**0,78**			
Trpv2			**0,61**			
Cd14			**0,82**			
Pcyt1a			**0,65**			
Ehd1			**0,71**			
Cpeb4			**0,95**			
1110005A03Rik			**0,69**			
Adamtsl5			**0,72**			
Slc23a2			**0,65**			
Pfkp			**0,58**			
Gadd45b			**0,67**			
S100a10			**0,65**			
Klf9			**0,86**			
Rhbdf1			**0,61**			
Src			**0,88**			
Cd97			**0,89**			
Prkcc			**0,59**			
Pik3c3			**0,58**			
Pdcd10			**0,76**			

	1 h	2 h	4 h	1 h	2 h	4 h
Ndrg1			0,87			
Srgn			0,69			
Apba1			0,68			
Vim			0,60			
Mpeg1			0,64			
Phf8			0,83			
Ncan			0,61			
Tgfbr1			0,99			
Sdhd			0,63			
Sostdc1			0,70			
Rhoc			0,98			
B3gnt2			0,66			
Ahnak2			0,59			
Emp3			0,66			
Nip7			0,75			
Lcp2			0,59			
Lims2			0,64			
Txn2			0,62			
Anxa1			0,71			
Dhrs3			0,62			
Maged1			0,88			
Stx3			0,63			
Rapgef2			0,61			
Scn2b			0,58			
Fem1b			0,62			
Myo1b			0,68			
AK122525			0,65			
Sqstm1			0,80			
Ralgds			0,59			
Kcnk6			0,68			
Rap1b			0,72			
Btg1			0,58			
Hmga2			0,89			
Coq10b			0,61			
Pfpl			0,59			
Tcf19			-1,03			
Cdca5			-0,80			
Rpa2			-0,80			
Ccnb1			-0,74			
Hist3h2a			-0,83			
Uhrf1			-0,77			
Rad51			-0,67			
Top2a			-0,74			
Cubn			-0,64			
Ccne1			-0,98			
Cdca7			-0,65			
Hist1h2ao|Hist1h2ag|Hist1h2ad|Hist1h2ai| Hist1h2ac|Hist1h2af|Hist1h2ah			-0,72			
Fen1			-0,65			
Dtl			-0,91			
Cdc6			-0,60			
Usp1			-0,75			

	1 h	2 h	4 h	1 h	2 h	4 h
Asf1a			-0,68			
Cbx5			-0,63			
Abca1			-1,10			-0,72
Ccna2			-0,71			
Hist1h4m|Hist1h4a|Hist1h4h|Hist1h4b| Hist1h4i|Hist1h4f|Hist1h4k|Hist1h4j|Hist1h4d			-0,77			
Cep55			-0,64			
Chaf1b			-0,66			
Ttk			-0,58			
Plk1			-0,62			
Aspm			-0,62			
Nlrp14			-0,59			
Aurka			-0,68			
Sema6c			-0,77			
Chst10			-0,63			
Exosc9			-0,83			
D10Wsu52e			-0,60			
Sfrs3			-0,60			
Aurkc			-0,77			
Ncaph			-0,68			
Ccnf			-0,69			
Gclc			-0,58			
Ppil1			-0,59			
Mcm6			-0,67			
Prei4			-0,60			
Pscdbp			-0,69			
2810417H13Rik			-0,67			
Arnt			-0,70			
Zfp518b			-0,65			
Deaf1			-0,62			
Hist1h1c|Hist1h1d|Hist1h1e|Hist1h1b			-0,76			
Klc1			-0,67			
Mt2			-0,67			
B4galt5					-0,60	
Lpl						0,62

Table 7: List of primers used for RT-qPCR

	Sequence (5' → 3')	**Length**	**T_m [°C]**
Tnf-α (NM_013693.2)			
Forward primer	CCTGTAGCCCACGTCGTAG	19	61.5
Reverse primer	GGGAGTAGACAAGGTACAACCC	22	61.4
Amplicon size	148		
c-Jun (NM_010591.2)			
Forward primer	GCTCCGTGAGTGACCGCGAC	20	60.1
Reverse primer	ACGGCAGTGCTTCCCCCTGA	20	60.1
Amplicon size	133		
Cd36 (NM_007643.4)			
Forward primer	AGCTGCCTTCTGAAATGTGTGGAGC	25	59.7
Reverse primer	ACGTGGCCCGGTTCTACTAATTCAT	25	58.1
Amplicon size	166		
Src (NM_001025395.2)			
Forward primer	CAATGCCAAGGGCCTAAATGT	21	60.9
Reverse primer	TGTTTGGAGTAGTAAGCCACGA	22	61.0
Amplicon size	123		
Cd9 (NM_007657.3)			
Forward primer	CCCTAGCCGCAGCGTGTCTC	20	59.6
Reverse primer	CTGCAATGCCAGCGAGCCAGA	21	60.0
Amplicon size	171		
Egr1 (NM_007913.5)			
Forward primer	GAGCACCTGACCACAGAGTCCTT	23	58.0
Reverse primer	ACTGTTGGGTGCGGGCTCCA	20	60.7
Amplicon size	144		
Trib3 (NM_175093.2)			
Forward primer	TGTGAGAGGACGAAGCTGGTGC	22	58.9
Reverse primer	CACGTAGGCAGGGCACGCAT	20	59.8
Amplicon size	102		

Atf4 (NM_009716.2)			
Forward primer	AAGCCATGGCGCTCTTCACGA	21	59.1
Reverse primer	GTCCCCCGCCAACACTTCGC	20	60.6
Amplicon size	147		

Atf3 (NM_007498.3)			
Forward primer	CCGAGCGAAGACTGGAGCAAA	21	57.1
Reverse primer	GGGACAATGGCGGTCGCACT	20	59.7
Amplicon size	83		

Klf2 (NM_008452.2)			
Forward primer	TGGAGGCCAAGCCCAAACGC	20	60.2
Reverse primer	AAGGCTTCTCACCTGTGTGTGTAC G	25	58.9
Amplicon size	147		

Ldlr (NM_010700.3)			
Forward primer	TCAGACGAACAAGGCTGTCC	20	61.8
Reverse primer	CCATCTAGGCAATCTCGGTCTC	22	61.7
Amplicon size	116		

Junb (NM_008416.3)			
Forward primer	CTATCGGGGTCTCAAGGGTC	20	60.8
Reverse primer	CTGTTGGGGACGATCAAGC	19	60.5
Amplicon size	147		

Gadd45b (NM_008655.1)			
Forward primer	CGCAGCGTCAGGATCGCCTC	20	60.2
Reverse primer	ACGCTGTCGGGGTCCACATT	20	58.3
Amplicon size	73		

Rhob (NM_007483.2)			
Forward primer	GTGCCTGCTGATCGTGTTCA	20	62.8
Reverse primer	GTCCGCCACATAGTTCTCGAA	21	61.9
Amplicon size	79		

Rtn4 (NM_194052.3)			
Forward primer	CGGGCTCAGTGGTTGTTGACCT	22	59.1
Reverse primer	GCTGGCACCAAACACCACTCCA	22	59.1
Amplicon size	71		

Abca1 (NM_013454.3)			
Forward primer	GTTACGGCAGATCAAGCATCC	21	61.1
Reverse primer	TGGAAGGGACAAATTGTGCTG	21	60.8
Amplicon size	103		

Ccnb1 (NM_172301.3)			
Forward primer	GGTGTCTTCTCGAATCGGGGAACCT	25	60.0
Reverse primer	TTCGTGTTCCTAGTGACCCTGAGCG	25	60.2
Amplicon size	71		

Plk1 (NM_011121.3)			
Forward primer	TACATCCCCGCCCGTCTCCC	20	70.0
Reverse primer	GGCAGGGGGTTCTCCACACCTTT	23	60.9
Amplicon size	131		

Rad51 (NM_011234.4)			
Forward primer	CATGCCAGCTCCCCATTGACCG	22	63.4
Reverse primer	CGCTCCGGCCTAAAGGTGCC	20	70.0
Amplicon size	82		

List of abbrevations

AA	Arachidonic acid
ABCA1	ATP-binding cassette transporter
ABCG1	ATP-binding cassette sub-family G member 1
ACAT	A:cholesterol acyltransferase
cAMP	Cyclic adenosine monophosphate
apoAI	Apolipoprotein AI
apoB	Apolipoprotein B
apoE	Apolipoprotein E
ARE	Antioxidant response element
ATF	Activating transcription factor
BMDM	Bone marrow-derived macrophages
CAPP	Ceramide-activated Ser-Thr phosphatase
CCR2	C-C chemokine receptor-2
CD	Cluster of differentiation
aCDase	Acid ceramidase
nCDase	Neutral ceramidase
CE	Cholesteryl ester
Cer	Ceramide
CERK	Ceramide kinase
CerS	Ceramide synthase
CERT	Ceramide transfer protein
C1P	Ceramide-1-phosphate
2-DGE	2-Dimensional gel electrophoresis
EGF	Epidermal growth factor
EGR	Early growth response protein
EPR	Electron paramagnetic resonance
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ESR	Electrophilic stress response
FAPP2	Four-phosphate-adaptor protein 2

FCS	Fetal calf serum
FRET	Fluorescence resonance energy transfer
FPALM	Fluorescence photoactivation localization microscopy
GalCer	Galactosylceramide
GCS	Glucosylceramide synthase
GEF	Guanine-nucleotide exchange factor
GluCer	Glucosylceramide
GM-CSF	Granulocyte macrophage colony stimulating factor
GM1	Monosialotetrahexosylganglioside 1
GPI	Glycophosphatidylinositol
GSL	Glycosphingolipids
GTPase	Guanosine triphosphate hydrolase
HAECs	Human aortic endothelial cells
HDL	High-density lipoprotein
HETE	Hydroxyeicosatetraenoic acid
HNE	4-Hydroxy-trans-2-nonenal
HODE	Hydroxyoctadecanoic acid
HPRT	Hypoxanthin-guanin-phosphoribosyltransferase
HUVECs	Human umbilical vein endothelial cells
IFN-γ	Cytokine interferon-γ
IL	Interleukin
isoP	Isoprostane
JNK	c-Jun N-terminal kinase
KEAP-1	Kelch-like ECH-associated protein 1
KSR	Kinase suppressor of ras
LA	Linoleic acid
LCAT	Lecithin:cholesterol acyltransferase
LDL	Low-density lipoprotein
mmLDL	Minimally modified low-density lipoprotein
oxLDL	Oxidized low-density lipoprotein
LOX-1	Lectin like oxLDL receptor-1
LPL	Lipoprotein lipase

LPS	Lipopolysaccharide
LXR	Liver X receptor
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemotractant protein
M-CSF	Macrophage colony-stimulating factor
MHC class II	Major histocompatibility complex class II
MIP	Migration inflammatory protein
MKK4	Mitogen-activated kinase kinase 4
MSR	Macrophage scavenger receptor
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
eNOS	Endothelial nitric oxide synthase
NRF2	Nuclear factor erythroid 2-related factor 2
ONE	4-Oxo-(2E)-nonenal
PAF	Platelet activating factor
PAI-1	Plasminogen activator inhibitor 1
oxPAPC	Oxidited 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine
PAPS	1-Palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphoserine
Paze-PC	1-Palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine
PC	Phosphatidylcholine
PECPC	1-Palmitoyl-2-(5,6-epoxycyclopentenoneisoprostane)-*sn*-glycero-3-phosphocholine
PEIPC	1-Palmitoyl-2-(5,6-epoxyisoprostane E2 oyl)-*sn*-glycero-3-phosphocholine
PERK	Eukaryotic translation initiating factor 2-α-kinase3
PGPC	1-Palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PI3-K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKB or AKT	Protein kinase B
PL	Phopsholipid
oxPL	Oxidized phospholipid
PMA	Phorbol 12-myristate 13-acetate

POVPC	1-Palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphocholine
Poxno-PC	1-Palmitoyl-2-(9'-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acid
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
siRNA	Small interfering RNA
ROS	Reactive oxygen species
SM	Sphingomyelin
SMase	Sphingomyelinase
aSMase	Acid sphingomyelinase
nSMase	Neutral sphingomyelinase
S1P-R	Sphingosine-1-phosphate receptor
Sph	Sphingosine
S1P	Sphingosine-1-phosphate
SR	Scavenger receptor
SRC	Proto-oncogene tyrosine-protein kinase Src
SREBP	Sterol regulatory element-binding protein
STED	Stimulated emission depletion microscopy
STALL	Stimulation-induced temporary arrest of lateral diffusion
TCR	T cell receptor
TF	Tissue factor
TG	Triacylglycerol
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WHHL	Watanabe heritable hyperlipidemic

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