Oxidized Phospholipids Induce Phenotypic Switching of Vascular Smooth Muscle Cells In Vivo and In Vitro

Nataliya A. Pidkovka, Olga A. Cherepanova, Tadashi Yoshida, Matthew R. Alexander, Rebecca A. Deaton, James A. Thomas, Norbert Leitinger, Gary K. Owens

Abstract—Atherosclerosis is a vascular disease characterized by lipid deposition and inflammation within the arterial wall. Oxidized phospholipids (oxPLs), such as 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (oxPAPC) and its constituents 1-palmytoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC) and 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC) are concentrated within atherosclerotic lesions and are known to be potent proinflammatory mediators. Phenotypic switching of smooth muscle cells (SMCs) plays a critical role in the development, progression, and end-stage clinical consequences of atherosclerosis, yet little is known regarding the effects of specific oxPLs on SMC phenotype. The present studies were focused on determining whether oxPLs regulate expression of SMC differentiation marker genes and the molecular mechanisms involved. Results showed that POVPC and PGPC induced profound suppression of smooth muscle (SM) α-actin and SM myosin heavy chain expression while simultaneously increasing expression of MCP-1, MCP-3, and cytolysin. OxPLs also induced nuclear translocation of Krüppel-like transcription factor 4 (KLF4), a known repressor of SMC marker genes. siRNA targeting of KLF4 nearly blocked POVPC-induced suppression of SMC marker genes, and myocardin. POVPC-induced repression of SMC marker genes was also significantly attenuated in KLF4 knockout SMCs. Taken together, these results suggest a novel role for oxPLs in phenotypic modulation of SMCs and indicate that these effects are dependent on the transcription factor, KLF4. These results may have important novel implications for the mechanisms by which oxPLs contribute to the pathogenesis of atherosclerosis. (Circ Res. 2007;101:792-801.)

Key Words: atherosclerosis ■ smooth muscle cell phenotypic switching ■ oxidized phospholipids

Vascular smooth muscle cells (SMCs) are a predominant cell type in atherosclerotic plaques and are crucial in the development of vascular disease.¹ Unlike either terminally differentiated skeletal or cardiac muscle cells, SMCs within adult animals retain remarkable plasticity and can undergo profound and reversible changes in phenotype in response to changes in local environmental cues.² During early atherosclerosis, SMCs undergo phenotypic switching which is characterized by decreased expression of SMC differentiation marker proteins, a high rate of cellular proliferation, and increased synthesis of extracellular matrix proteins.³ In contrast, in late disease stage, SMCs within the fibrous cap of vulnerable atherosclerotic plaques exhibit reduced rates of proliferation and matrix production. These cells also undergo apoptosis and activation of matrix metalloproteinases, which may contribute to plaque rupture, thrombosis, and myocardial infarction.² Despite the importance of SMC phenotypic switching in the pathogenesis of atherosclerosis, virtually nothing is known regarding specific factors and mechanisms that control this process.

Oxidized lipids, particularly oxidized phospholipids (oxPLs), accumulate in atherosclerotic lesions and there is evidence that changes in the plasma oxPLs/apoB ratio may reflect the extent of atherosclerotic disease burden.³ The presence of antibodies to oxPLs in patients with atherosclerosis, diabetes, hypertension, antiphospholipid syndrome, and other chronic diseases also underlines the potential importance of these molecules.⁴-⁶ OxPLs promote chronic inflammation at least in part by inducing expression of inflammatory cytokines in endothelial cells.⁷,⁸ The major bioactive lipids in minimally modified low-density lipoprotein (MM-LDL) are derived from the oxidation of arachidonate-containing phospholipids such as 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC), namely: 1-palmytoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC), 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC), and 1-palmitoyl-2-(5,6-epoxyisoprostane E2)-sn-glycero-3-phosphocholine (PEIPC).⁹-¹² These oxidized phospholipids are found at high concentrations within fatty streak lesions of cholesterol-fed rabbits and antibodies to these factors are present in the sera of apoE-null mice.⁵,¹¹,¹³ Unregulated uptake of oxidized LDL via scavenger LDL receptor pathways contributes to development of foam cells within atherosclerotic lesions. MM-LDL and its derivative

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phospholipids including POVPC and PGPC have also been shown to increase adhesion and trafficking of monocytes to endothelial cells and to be involved in development of experimental atherosclerosis.4–6,14 Finally, oxPLs have been implicated in control of end-stage disease consequences including plaque rupture.2,15 Although there is extensive evidence that oxidized lipids promote vascular inflammation and contribute to development of atherosclerosis,7,8,16,17 virtually nothing is known regarding how these atherogenic compounds impact SMC differentiation or phenotypic switching. The aims of the present study were to: (1) determine whether oxPLs regulate SMC differentiation marker genes in cultured SMCs; (2) determine whether POVPC has an effect on SMC differentiation marker genes in vivo; and (3) test the hypothesis that the suppressive effects of oxPLs on SM α-actin and SM myosin heavy chain (SM MHC) expression are mediated at least in part by the potent repressor of SMC differentiation marker genes Krüppel-like transcription factor 4 (KLF4).

Materials and Methods

For detailed Materials and Methods, please see the supplemental materials (available online at http://circres.ahajournals.org).

Cell Culture and Treatment of SMCs With Phospholipids

Rat aortic SMCs were isolated and cultured as previously described.14 Cells were grown to 100% confluence and then switched to serum-free media. After culture in serum-free media for 3 days, passages 8 to 15 of postconfluent rat aortic SMCs were treated with vehicle (DMSO), nonoxidized phospholipids 1-palmitoyl-2-arachidonoyl phosphatidylcholine (PAPC), 1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine (DMPC) (Avanti Polar Lipids), oxPAPC, PGPC, or POVPVC (Cayman Chemical).

siRNA Oligonucleotides and Transfection

siRNA oligonucleotides specific for KLF4 (siKLF #13; GUCAAUUGGGUAAUCCU-3′, siKLF4 #8; 5′-CAUCUACA-UUUAUCACU-3′) and EGFP (5′-GAUCGCUACAAAGGU-GAAC-3′) were purchased from MWG-Biotech, and transient transfection of siRNA oligonucleotide was performed using Oligofectamine (Invitrogen) according to the manufacturer’s protocol.

Application of POVPVC to Rat Carotid Arteries Using F-127 Pluronic Gel System

The animal protocol was approved by the Animal Care and Use Committee at the University of Virginia. 150 μL of ice-cold F-127 pluronic gel ( Molecular Probes) containing POVPVC 30 μg (n=6 rats) or vehicle (n=6 rats) was applied to 24 hours to the adventitial surface of rat carotid arteries as described previously.10,26 The Fast Prep FP120 (Q-Biogene) was used to homogenize tissues, and total RNA was prepared from both treated and untreated carotid arteries, aorta, and liver. SM α-actin, SM MHC, myocardin, KLF4, and β-actin expression in the each sample was normalized to 18S rRNA levels and to the contralateral untreated control vessel.

Results

oxPAPC Decreased SMC Marker Gene Promoter Activity and mRNA Level in Cultured SMCs

To determine whether oxPLs alter SMC phenotype, cultured SMCs were transfected with various SMC promoter-reporter constructs and treated for 24 hours with oxPAPC at concentrations present within atherosclerotic lesions.11,13,19 OxPAPC markedly suppressed SM α-actin promoter activity and SM MHC promoter activity by 70% and 30%, respectively, at a dose ≥10 μg/mL (Figure 1A). To determine whether oxPAPC also altered endogenous gene expression, quantitative real time RT-PCR analyses were performed on cultured SMCs treated with oxPAPC for 24 hours (Figure 1B). Results showed that oxPAPC caused dose-dependent suppression of SM α-actin and SM MHC mRNA expression.

POVPVC, a Specific Oxidative Product of PAPC, Decreased SMC Marker Gene Expression While Simultaneously Activating Expression of a Number of Proinflammatory Genes

OxPAPC has been shown to contain a variety of specific oxPLs including POVPVC, which is one of the most abundant and bioactive oxPLs found in atherosclerotic lesions.11,12 POVPVC induced concentration-dependent suppression of SM α-actin and SM MHC promoter activity, respectively (Figure 2A). Similarly, PGPC suppressed SM α-actin and SM MHC promoter activity (supplemental Figure I). Quantitative real-time RT-PCR analyses performed in cultured SMCs showed that POVPVC induced dose-dependent repression of endogenous SM α-actin and SM MHC (Figure 2B) but had no effect on β-actin expression (supplemental Figure IIA). POVPVC dose-dependently decreased protein expression of SM α-actin and SM MHC after 72 hours of treatment (Figure 2C). In contrast, nonoxidized phospholipids such as PAPC and DMPC did not significantly affect SM α-actin and SM MHC promoter activity (supplemental Figure III), indicating that oxidative modification of phospholipids is necessary for downregulation of SM α-actin and SM MHC. Western blot analysis for caspase 3 activation and Bax expression, known markers of apoptosis (supplemental Figure IV), showed that POVPVC concentrations of 50 μg/mL and greater caused apoptosis in SMCs, whereas no evidence of apoptosis was obtained at lower doses. Taken together, these results suggest that oxPAPC, POVPVC, and PGPC induce profound concentration-dependent suppression of SMC marker gene expression.

To more fully characterize the nature of POVPVC-induced changes in SMCs, we performed gene microarray and RayBio cytokine array analyses of POVPVC- and vehicle-treated cultured rat aortic SMCs. Of major interest, POVPVC induced expression of a number of proinflammatory genes including cytolsin (6.9-fold), chemokine ligand 2 (6.2-fold), TNF (5.8-fold), and MCP3 (3.1-fold; supplemental Figure V).

POVPVC Decreased SM α-Actin and SM MHC mRNA Level in Rat Carotid Arteries In Vivo

To determine whether oxPLs also suppress SMC marker gene expression in vivo, POVPVC or vehicle were applied to the adventitial surface of rat carotid arteries in vivo using an F-127 pluronic gel system.6,20 Results of RT-PCR analyses showed that POVPVC induced marked suppression of SM α-actin (Figure 3A, upper panel) and SM MHC (Figure 3B, upper panel) mRNA levels after 24 hours of treatment relative to vehicle-treated vessels. In contrast, SMC marker gene expression in the aorta and liver was not changed, indicating a lack of systemic effects of POVPVC (Figure 3A).
and B, lower 2 panels). Expression of β-actin was not changed in POVPCTreated vessels (supplemental Figure IIC). These results show that POVPCEnter decreased the expression of SMC differentiation marker genes within vessels in vivo.

POVPCEnter Increased Expression and Nuclear Translocation of the Potent SMC Differentiation Repressor Gene, KLF4
In contrast to the marked suppressive effects of POVPCEnter on SMC differentiation marker genes, we found that POVPCEnter increased expression of KLF4×35-fold (Figure 4A). Increases in KLF4 mRNA expression occurred within 2 hours of stimulation (Figure 4B) and were maximal at a concentration of POVPCEnter 10 μg/mL. In addition, POVPCEnter treatment resulted in rapid nuclear localization of KLF4 as determined by cell fractionation and Western blot analysis (Figure 4C), as well as by examining KLF4 subcellular localization by immunofluorescent microscopic analyses of SMCs transiently transfected with a pcDNA3-KLF4-FLAG expression plasmid and treated with POVPCEnter (Figure 5A and 5B). Results showed that KLF4 was localized predominantly in the cytoplasm in cells treated with vehicle, whereas it translocated into the nucleus with POVPCEnter treatment (Figure 5A and 5B).

KLF4-specific antibodies were used to analyze endogenous KLF4 expression and localization by immunofluorescence. SMCs treated with vehicle exhibited diffuse staining (Figure 5C, upper panel). In contrast, POVPCEnter caused nuclear localization of KLF4 (Figure 5C, lower panel). These results provide evidence that POVPCEnter induces not only increased expression of KLF4 but also its nuclear localization.

siRNAs Specific to KLF4 Inhibited POVPCEnter-Induced Suppression of SMC Marker Genes
To determine whether KLF4 is required for POVPCEnter-induced suppression of SMC marker genes, SMCs were treated with POVPCEnter in the presence of 2 different siRNA oligonucleotides specific for KLF4. Results showed that anti-KLF4 siRNA, but not a control anti-EGFP siRNA, inhibited POVPCEnter-induced suppression of SM β-actin and SM MHC mRNA (Figure 6A). KLF4 mRNA expression was induced by 16.5-fold after POVPCEnter treatment of SMCs, and was nearly completely suppressed by either of 2 KLF4 siRNAs (Figure 6B). The efficacy of the KLF4 siRNA oligonucleotide used in these studies was documented at both the mRNA (Figure 6B) and protein (Figure 6C) levels. In the latter case, total protein extracts of SMCs transfected with either EGFP siRNA or

**Figure 1.** oxPAPCEnter suppressed expression of SMC marker genes in cultured SMCs. A, SM β-actin-luc and SM MHC-luc promoter-luciferase constructs were transiently transfected into SMCs and cells were treated for 24 hours with the indicated range of oxPAPCEnter concentrations. Luciferase activity was measured, and normalized for protein contents and background luminescence, and then expressed as fold-increase over vehicle. Values represent the mean±SD. *P<0.05; **P<0.001 vs vehicle. B, SMCs were treated for 24 hours with the indicated range of oxPAPCEnter concentrations. Expression of SM β-actin and SM MHC mRNA was measured by real-time RT-PCR and normalized to 18S rRNA, and expressed as fold-increase over vehicle. Values represent the mean±SD. *P<0.05; **P<0.005 vs vehicle. Each experiment is representative of 3 independent studies.

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KLF4 siRNA and treated with PDGF-BB were used as positive controls because our laboratory has previously shown KLF4 induction by PDGF-BB.\textsuperscript{21,22} Endogenous KLF4 protein level was markedly increased in control cells transfected with nonspecific siRNA (siEGFP) and treated with POVPC (Figure 6C). This increase was virtually eliminated by the KLF4 siRNA. Expression of β-actin mRNA was not changed after siEGFP or siKLF4 transfection (supplemental Figure IIB). We also tested the contribution of KLF4 to POVPC-induced suppression of SMC marker genes in primary cultures of mouse aortic SMCs. Aortic SMCs were isolated from the aorta of KLF4 floxed mice,\textsuperscript{23} and cells

Figure 2. POVPC decreased expression of SMC marker genes in cultured SMCs. A, SM α-actin-luc and SM MHC-luc promoter-luciferase constructs were transiently transfected into SMCs, and SMCs were treated for 24 hours with the indicated range of POVPC concentration. Luciferase activity was measured and normalized to protein content and a promoterless construct, and expressed as fold-increase over vehicle. Values represent the mean±SEM. \( *P<0.005; **P<0.001 \) vs vehicle. B, SMCs were treated for 24 hours with the indicated range of POVPC concentration. Expression of SM α-actin and SM MHC mRNA was measured by real-time RT-PCR and normalized to 18S rRNA, and expressed as fold-increase over vehicle. Values represent the mean±SEM. \( *P<0.01; **P<0.005 \) vs vehicle. C, SMCs were treated for 24 and 72 hours with the indicated range of POVPC concentration. Total protein lysates of SMCs (0.3 \( \mu \)g and 10 \( \mu \)g) were subjected to Western blot analysis for SM α-actin and SM M HC protein expression, respectively.
infected with adenovirus expressing Cre-recombinase (KLF4 KO SMCs) or control adenovirus (Control SMCs). Treatment with 10 μg/mL POVPC dramatically decreased expression of SM α-actin and SM-MHC in control mouse aortic SMCs (Figure 6D). In contrast, effects of POVPC on SMC marker gene expression were markedly attenuated in KLF4 KO SMCs. Taken together, the preceding results provide evidence that KLF4 is a key down-stream effector of POVPC-induced SMC phenotypic switching.

POVPC Markedly Suppressed Expression of the SMC Selective SRF Coactivator Myocardin in a KLF4 Dependent Manner

To further investigate mechanisms by which POVPC suppresses expression of SMC differentiation marker genes, we determined its effects on expression of the highly potent SRF coactivators, myocardin, and the myocardin-related factors, MKL1 and MKL2. Of major interest, POVPC treatment decreased myocardin mRNA expression by 40% and was blocked by the 2 KLF4 siRNAs (Figure 7A). Conversely, POVPC had no effect on MKL1 and MKL2 mRNA levels and the KLF4 siRNAs had no effect on expression of these genes (Figure 7B). We also tested the effect of POVPC on myocardin and KLF4 expression in rat carotid arteries in vivo by the application of pluronic gel with POVPC to rat carotid arteries for 6 hours. Although results did not achieve statistical significance, there was a trend toward POVPC increasing KLF4 expression while simultaneously decreasing myocardin expression (supplemental Figure VI). These results provide evidence that POVPC-induced suppression of SMC marker genes is attributable, at least in part, to KLF4-dependent suppression of myocardin but not MKL1/2 expression.

Discussion

Despite advances made in understanding mechanisms that promote proliferation and migration of SMCs associated with vascular disease, the mechanisms by which SMC marker gene expression is repressed in phenotypically modulated SMCs within lipid-rich atherosclerotic lesions are poorly understood. In this report, we provide novel evidence showing that a number of oxPLs including POVPC and oXPAPC, which are known to induce vascular inflammation, also regulate SMC phenotypic switching in vitro and in vivo as evidenced by downregulation of SMC differentiation marker genes. Results of the present studies are also the first, to our knowledge to identify a specific factor that is capable of inducing phenotypic switching of SMC in vivo (Figure 3).
Observations in the present studies showing that various oxPLs that are present within atherosclerotic lesions induce marked suppression of SMC marker genes raise the possibility that these factors may play a key role in SMC phenotypic switching in the pathogenesis of atherosclerosis in addition to their well-documented role in controlling vascular inflammation.7,8,16,17 However, further studies will be needed to directly test the role of oxPL-induced SMC phenotypic switching in atherosclerotic disease progression in vivo, and in mediating late stage clinical events such as plaque rupture.

It is possible that the effects of oxPLs on SMC marker gene expression observed in our in vivo studies could be secondary to activation of other cell types as a part of an inflammatory response. A number of inflammation-related genes such as cytolisin, MCP-3, and hemeoxygenase-1 were induced in our cultured SMCs after POVPC treatment (supplemental Figure V). OxPAPC has also been shown to induce a subset of atherosclerotic genes in carotid arteries including MCP-1, IL-6, hemeoxygenase-1, and Egr-1, as well as enhanced adhesion of monocytes.24–26 The distribution of these chemokines was shown throughout the vessel wall as well as the induction of VCAM-1, MCP-1, and keratinocyte derived chemokine (KC), indicating that the oxPAPC effect was not restricted to the adventitial surface of the vessel but rather penetrated the entire vessel wall.7 As such, the effects of oxPLs on SMC we observed in vivo might be indirect through one or more of these secondary pathways. However, our observations that purified oxPLs could profoundly repress SMC marker gene expression in both rat and mouse vascular SMC in culture indicate that they are capable of exerting direct effects on SMC phenotype. We also observed that POVPC induced a modest increase in 3H-thymidine incorporation in cultured SMCs (supplemental Figure VII) and that POVPC induced enhanced repair in a scratch wound assay (supplemental Figure VIII), suggesting that oxPLs may also modulate functional properties of SMC important in lesion formation. There is also evidence that oxPLs stimulate angiogenesis via autocrine mechanisms involving VEGF, IL-8, and COX-2–generated prostanoids in endothelial cells.27 Results of our studies showed that POVPC increased VEGF and MCP-1 secretion by SMCs (supplemental Figure VB and VC). As such, it is possible that at least some of the effects of oxPLs on SMCs phenotypic switching may involve production of autocrine growth factors like VEGF.

Results of the present studies demonstrated that POVPC-induced suppression of SMC marker genes was dependent on KLF4, a factor that we have previously shown potently suppresses expression of multiple SMC marker genes, and to be required for PDGF-BB induced phenotypic switching.21,28,29 Significantly, we previously showed that suppression of the SM22α gene in vivo in response to vascular injury30 and experimental atherosclerosis31 was dependent on a G/C repressor element that closely matches the consensus DNA binding motif for KLF4.32 G/C repressor elements also
exist within other SMC marker genes including SM MHC, and the recently identified myocardin promoter contains multiple potential consensus KLF4 binding sites. As such, it is interesting to postulate that KLF4 binding to G/C repressor elements within SMC marker genes, or KLF4 binding sites within the myocardin promoter may contribute to coordinate repression of SMC marker genes under a variety of pathophysiological conditions. We previously demonstrated that KLF4 suppresses SMC marker gene expression through a variety of potential mechanisms including: (1) reducing SRF binding to CArG elements within intact chromatin through induction of histone hypoacetylation; (2) decreasing expression of myocardin; and (3) binding to SRF and reducing its binding to SMC promoter CArG elements. Results of the present studies have extended these findings by showing that POVPc not only activates expression of KLF4, but also induces its rapid nuclear localization.

Whereas KLF4 is required for POVPc-induced suppression of SMC marker genes, it may not be sufficient to fully account for its effects. Multiple repressor pathways have been shown to contribute to PDGF-BB-induced phenotypic switching of SMCs in addition to KLF4. Of interest, we found that ERK inhibitors partially blocked POVPc-induced repression of SMC marker genes (Pidkovka and Owens, unpublished observations, 2005), indicating that this pathway also contributes to effects of oxPLs. In contrast, effects of oxPLs were not blocked by overexpression of an IκB super-repressor dominant negative construct, indicating that NF-κB is not involved in this response (data not shown). These latter observations are consistent with studies showing that oxPLs-induced activation of endothelial cells are also mediated by activation of MAP-kinase signaling, rather than NF-κB pathways.

We and others have shown that the SRF coactivators, myocardin, and the myocardin-related transcription factors, MKL1 and MKL2, markedly stimulate expression of CArG-dependent SMC marker genes. Moreover, siRNA-
induced suppression of each of these genes showed that they act in a cooperative fashion to regulate expression of a large cohort of SMC differentiation marker genes including SMα-H251-actin, SM MHC, and SM22α-H251. Results of the present studies provided evidence that oxPL-induced suppression of SMC marker genes is mediated at least in part by reduced expression of myocardin. However, surprisingly, oxPLs had no effect on expression of MKL1 and MKL2 in our studies, at least at the mRNA level, indicating that suppression of these factors may not contribute to phenotypic switching in this model system, although it is possible that oxPLs may inhibit the function of MKL1 or MKL2 through posttranscriptional control mechanisms. Consistent with this possibility, our laboratory previously demonstrated that PDGF-BB–

Figure 6. POVPC-induced suppression of SMC marker genes was mediated by KLF4. A. Cultured SMCs were transfected with KLF4 siRNA oligonucleotides (siKLF4 #13 and siKLF4 #8) or nonspecific control oligonucleotides (siEGFP) with POVPC or vehicle treatment as described in Methods. Total RNA samples were isolated, and SM α-actin and SM MHC mRNA expression analyzed by real-time RT-PCR. Values were normalized for 18S rRNA level. The experiment was repeated 3 times and the representative data are shown. Values represent the mean±SEM. **P<0.001. B. To validate the knockdown efficacy of KLF4 siRNA, the endogenous KLF4 mRNA expression was analyzed by real-time RT-PCR and normalized for 18S rRNA level, and expressed as fold-increase over vehicle. The data are representative of 4 experiments. Values represent the mean±SD. **P<0.005 vs vehicle. C. Twenty micrograms of total protein from lysates of SMCs transfected with KLF4 siRNA or EGFP siRNA were subjected to Western blotting using anti-KLF4 and anti-GAPDH antibody. The band intensity was quantified by densitometry. Relative band intensity was normalized to the band intensity of GAPDH antibody and presented as fold-increase over vehicle. D. SMCs were isolated from the aorta of KLF4 floxed mice and infected with adenovirus expressing Cre recombinase (KLF4 KO SMCs) or control adenovirus (Control SMCs). KLF4 KO SMCs and Control SMCs were treated with 10 μg/mL POVPC for 24 hours and expression of SM α-actin and SM MHC was determined by real-time RT-PCR. Values were normalized to 18S rRNA level and expressed as fold-increase over vehicle. Values represent the mean±SEM. **P<0.01 vs vehicle.
induced suppression of SMC marker genes was mediated through the combinatorial mechanisms including downregulation of myocardin expression, but also through inhibition of the association of myocardin/MKL factors to CArG-containing SMC marker gene promoters within intact chromatin. Moreover, we presented evidence that this occurred by competition between myocardin/MKL factors and phosphorylated Elk-1 for SRF binding. Further studies will be required to directly test whether oxPLs have similar effects.

In summary, results of the present studies provide novel evidence showing that oxPLs such as POVPC profoundly suppress expression of multiple SMC differentiation marker genes and that these effects, at least in cultured SMCs, are dependent on KLF4. In addition, we found that POVPC also induced expression of a number of proinflammatory genes, and increased repair within a SMC scratch wound assay by SMCs. These findings are of major interest, because they are the first to define a class of compounds known to be present at high concentrations within lipid-rich atherosclerotic lesions, that can induce a SMC inflammatory phenotype. Further studies are needed to directly test the role of these compounds in SMC phenotypic switching in experimental atherosclerosis and to extend our understanding of the functional consequences of this process during different stages of disease development, progression, and end-stage events including plaque rupture.

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Disclosures
None.

References
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Oxidized phospholipids induce phenotypic switching of vascular smooth muscle cells

*in vivo and in vitro*

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Materials and Methods

Synthesis and analysis of oxidized phospholipids (oxPLs)

1-palmitoyl 2-arachidonoyl phosphatidylcholine PAPC (Avanti Polar Lipids) was oxidized to generate oxPAPC by exposure of dry lipid to air for 72 hours. The extent of oxidation was monitored by positive ion electrospray mass spectrometry (ESI-MS) as described previously. Lipids were stored at -70°C in chloroform and used within one month after testing for purity. Analysis of oxPLs was performed by mass spectrometry using a Finnigan LCQ classic, connected with an HP HPLC 1100 series. Using ESI-MS, phospholipids were introduced to the ion source of the mass spectrometer by flow injection using a solvent consisting of acetonitrile/water/formic acid (50:50:0.1, v/v/v).

Mouse smooth muscle cell culture

Mouse SMCs were isolated from KLF4 floxed mice and cultured as previously described. At the passage 3, SMCs were infected with adenovirus expressing Cre recombinase or control adenovirus, and recombination of KLF4 floxed allele was confirmed in cells infected with Cre recombinase adenovirus. Mouse SMCs at the passage 7-9 were used for the experiment testing the effect of POVPC on SMC marker gene expression.

RNA Extraction and Reverse Transcription-PCR

Total RNA was prepared from rat tissues and cultured cells using Trizol (Invitrogen) according the manufacturer’s protocol. One microgram of RNA was used
for reverse transcription with iScript cDNA synthesis kit (BioRad) and real-time RT-PCR was performed. Primers and probe sequences of rat SM α-actin, SM MHC, KLF4, myocardin, 18S rRNA were described previously as well as β-actin primer sequences.

**Transient Transfection and Luciferase Assay**

SMCs were transiently transfected with reporter plasmid using FuGENE reagent (Roche Diagnostics Corp.) at approximately 75% confluency according to the manufacturer’s protocol. The promoter luciferase constructs included: SM α-actin-luc (−2555/+2813 bp), SM MHC-luc (−4220/+11600 bp), and pGL3 basic plasmid (Promega Corporation). Luciferase activity was measured as described previously and normalized to total protein content (Coomassie Plus protein Assay reagent, Pierce).

For KLF4 Western blot analysis and KLF4 immunofluorescence, SMCs were transfected with expression plasmids pcDNA3-KLF4 and pcDNA3-KLF4-FLAG, respectively.

**Immunofluorescence**

Rat aortic SMCs were seeded at 0.5×10⁴ cells/cm² and cultured as described in the Figure legends. SMCs were fixed, permeabilized and incubated with anti-KLF4 or polyclonal anti-FLAG (Sigma Chemical Co) antibodies. Specific staining was detected with Cy3-conjugated anti-rabbit antibody (Jackson Immunoresearch Laboratories, Inc). Cells were counterstained with 4’, 6-diamino-2-phenylindole (DAPI).
**Cell Extract Preparation and Western blot Analysis**

Whole cell extracts from cultured rat aortic SMCs treated with POVPC and vehicle were prepared using modified RIPA buffer as described previously\(^6\). Lysates were subjected to Western blot analyses using anti-SM \(\alpha\)-actin (Sigma), SM MHC (Biomedical Technologies, Inc.), caspase 3 (Cell Signaling), Bax (Santa Cruz), KLF4 (Santa Cruz), GAPDH (Chemicon International) and Lamin A (Chemicon International) antibodies.

**GeneChip microarray**

Total RNA was isolated from SMCs treated with vehicle (DMSO) or 10 \(\mu\)g/ml of POVPC and subjected to Affymetrix GeneChip microarray (www.affymetrix.com) using chip type: RAE230 2.0. Two group comparisons were carried out using Affymetrix’s DMT software and the same comparisons were carried out using the dChip software. Gene expression was deemed significantly different if the p-value was less than or equal 0.05.

**Scratch wound repair assay**

Rat aortic SMCs were treated with the range of POVPC concentration and subjected to *in vitro* scratch assay as described previously\(^8\). Images were captured at 0 and 18 hours after POVPC treatment using phase-contrast microscope.

**Statistics**
Unless otherwise indicated, all experiments were done with triplicate samples and performed in 2 to 6 independent experiments giving similar results. Data were analyzed for statistical significance using a 2-tailed Student’s t-test. Error bars represent SEM. P<0.05 were considered statistically significant.

Reference List


Supplemental Figure Legends

**Figure S1.** PGPC induced a concentration-dependent decrease in SMC marker gene promoter activity.

SMα-actin-luc (-2555/+2813 bp) and SM MHC-luc (–4220/+11600 bp) promoter-luciferase constructs were transiently transfected into SMCs, and SMCs were treated for 24 hours with the indicated range of PGPC concentration as described in Materials and Methods. Luciferase activity was measured, and values were normalized to protein content and promoterless construct and expressed as fold-increase over vehicle. Values represent the mean ± SEM. *P <0.005; **P<0.001 vs. vehicle.

**Figure S2.** POVPC had no effect on β-actin mRNA expression in SMCs.

(A, B) RNA samples from Figure 2 and Figure 7 were subjected to real time RT-PCR. The mRNA level of β-actin was normalized for 18S rRNA level, and then expressed as fold-increase over vehicle. Values represent the mean ± SEM. (C). Total RNA samples from Figure 3 were subjected to real time RT-PCR. β-actin expression in the each sample was normalized to 18S rRNA level. Values for the POVPC-treated carotid artery were normalized to those for the contralateral right carotid artery. β-actin mRNA level was expressed as fold-increase over vehicle. Values represent the mean ± SEM.
Figure S3. The non-oxidized phospholipids PAPC and 1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine (DMPC) had no effect on SMC marker gene promoter activity in cultured SMCs.

SM α-actin-luc (-2555/+2813 bp) and SM MHC-luc (−4220/+11600 bp) promoter-luciferase constructs were transiently transfected into SMCs, and cells were treated for 24 hours with the indicated range of PAPC and DMPC concentrations as described in Methods. Cell lysates were assayed for luciferase activity. Activity was normalized for protein content and background luminescence, and then expressed as fold-increase over vehicle. Values present the mean ± SEM.

Figure S4. Effect of POVPC on the expression of apoptosis markers.

SMCs were treated for 24 hours with the indicated range of POVPC concentration as described in Methods. Twenty micrograms of total protein from SMC lysates were subjected to Western blot analysis of (inactive) pro-caspase 3 protein (35 kDa) and Bax (23 kDa). Total protein extract from SMCs treated with 20 µM of Camptothecin was used as a positive control for pro-caspase 3 activation and Bax expression. Significant activation of pro-caspase 3 and an increase of Bax protein expression were observed with POVPC concentrations of 50 µg/ml and higher.

Figure S5. POVPC treatment induced a subset of inflammation-related genes in SMCs.

(A) SMCs were treated with 10 µg/ml of POVPC for 24 hours. Total mRNA samples were subjected to Affymetrix GeneChip microarray. Inflammatory-related
subset of genes induced by POVPC treatment is shown. (B) Results of a RayBio® cytokine array analysis on conditioned media from SMCs treated with 20 µg/ml of POVPC for 48 hours. (C) Western blot analysis of MCP-1 secretion in conditioned media from SMCs treated with 20 µg/ml POVPC for 48 hours.

Figure S6. Effect of POVPC on KLF4 and myocardin mRNA expression in vivo.

(A) The Pluronic F-127 gel mixed with POVPC or vehicle was applied onto the adventitial surface of the common carotid arteries of 8 rats and incubated for 6 hours. Total RNA was isolated from both the treated left carotid artery and the untreated right carotid artery as well as from the aorta and the liver. KLF4 and myocardin mRNA expression in the each sample was measured and normalized to 18S rRNA level. Values for the POVPC-treated carotid arteries were normalized to those for the contralateral control right carotid arteries. Values represent the mean ± SEM. We believe the large variance between samples relates to uncertainties regarding the kinetics of POVPC effects on KLF4 and myocardin in vivo, and the transient nature of KLF4 mRNA induction by POVPC (see Figure 4B).

Figure S7. POVPC had minimal effects on $[^3]$H-thymidine incorporation in cultured rat aortic SMCs.

SMCs were treated for 24 hours with the indicated range of POVPC concentration as described in Materials and Methods, and $[^3]$H-thymidine (100 µCi/ml) was added to the medium. $[^3]$H-thymidine incorporation into DNA was evaluated by trichloracetic acid
precipitation and counting in a scintillation counter, and expressed as counts per 1 min. Values represent the mean ± SEM. *P < 0.05; **P<0.001 vs. vehicle.

**Figure S8.** POVPC enhanced the scratch wound repair by SMCs.

SMCs were treated with the indicated POVPC concentrations and subjected to an in *vitro* scratch assay. Images were captured at 0 and 18 hours of POVPC treatment using phase-contrast microscopy.
Supplementary figure S1. Effect of PGPC on SMC marker genes
Supplementary figure S2. Effect of POVPC on \(\beta\)-actin mRNA expression

A

Effect of POVPC concentration on \(\beta\)-actin mRNA level in the carotid artery.

B

Effect of POVPC on \(\beta\)-actin mRNA level in the aorta.

C

Effect of POVPC on \(\beta\)-actin mRNA level in the liver.
Supplementary figure S3. Effect of nonoxidized phospholipids on SMC marker genes expression

A

Relative luciferase activity of SM α-actin promoter (fold over vehicle)

PAPC concentration, µg/ml

B

Relative luciferase activity of SM MHC promoter (fold over vehicle)

PAPC concentration, µg/ml

Relative luciferase activity of SM α-actin promoter (fold over vehicle)

DMPC concentration, µg/ml

Relative luciferase activity of SM MHC promoter (fold over vehicle)

DMPC concentration, µg/ml
Supplementary figure S4. Effect of POVPC on the expression of apoptosis markers

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>POVPC, µg/ml</th>
<th>Camptothecin, 40 µM</th>
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<tr>
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- pro-Caspase 3
- BAX
- GAPDH
Supplementary figure S5. Inflammation-related genes induced by POVPC in SMCs

<table>
<thead>
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<th>Gene</th>
<th>Fold change</th>
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<tbody>
<tr>
<td>Cytolysin</td>
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<tr>
<td>Chemokine (C-X-C motif) ligand 2 (macrophage inflammatory protein 2)</td>
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<td>CD 86</td>
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<td>Transforming growth factor-β</td>
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Supplementary figure S6. Effect of POVPC on KLF4 and Myocardin in vivo
Supplementary figure S7. POVPC effect on [3H]-thymidine incorporation into SMCs
Supplementary figure S8. POVPC enhanced the scratch wound repair by SMCs.