Oxidized Phospholipids Induce Type VIII Collagen Expression and Vascular Smooth Muscle Cell Migration

Olga A. Cherepanova, Nataliya A. Pidkovka, Olga F. Sarmento, Tadashi Yoshida, Qiong Gan, Eser Adiguzel, Michelle P. Bendeck, Judith Berliner, Norbert Leitinger, Gary K. Owens

Abstract—Phenotypic switching of vascular smooth muscle cells (VSMCs) is known to play a critical role in the development of atherosclerosis. However, the factors present within lesions that mediate VSMC phenotypic switching are unclear. Oxidized phospholipids (OxPLs), including 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphorylcholine (POVPC), are active components of minimally modified low density lipoprotein and have been previously shown to induce multiple proatherogenic events in endothelial cells and macrophages, but their effects on VSMCs have been largely unexplored until recently. We previously showed that OxPLs induced phenotypic switching of VSMCs, including suppression of SMC differentiation marker genes. The goal of the present studies was to test the hypothesis that OxPLs alter extracellular matrix production and VSMC migration. Results showed that POVPC activated expression of several extracellular matrix proteins in VSMC. POVPC increased expression of type VIII collagen a1 chain (Col8a1) mRNA in cultured VSMCs and in vivo in rat carotid arteries by 9-fold and 4-fold, respectively. POVPC-induced activation of Col8a1 gene expression was reduced by small interfering RNA–mediated suppression of Krüppel-like factor 4 (Klf4) and Sp1, and was abolished in Klf4-knockout VSMCs. POVPC increased Klf4 binding to the Col8a1 gene promoter both in vivo in rat carotid arteries and in cultured VSMCs based on chromatin immunoprecipitation assays. Moreover, POVPC-induced VSMC migration was markedly reduced in Klf4- or type VIII collagen–knockout VSMCs. Given evidence that OxPLs are present within atherosclerotic lesions, it is interesting to suggest that OxPL-induced changes in VSMC phenotype may contribute to the pathogenesis of atherosclerosis at least in part through changes in extracellular matrix composition. (Circ Res. 2009;104:609-618.)

Key Words: POVPC ■ PGPC ■ PEIPC ■ type VIII collagen ■ Klf4 ■ vascular smooth muscle cell migration

Atherosclerosis is a progressive cardiovascular disease that remains the leading cause of death in the Western world. One of the critical factors implicated in the development of atherosclerosis is an accumulation of cholesterol, lipoproteins, and lipid derivatives within large arteries. Oxidized low density lipoprotein (OxLDL) has been shown to elicit a host of responses that contribute to the development of atherosclerosis including inflammation, proliferation, apoptosis, unregulated uptake of LDL, and regulation of gene expression.1,2 Indeed, there is a growing body of evidence that specific oxidized phospholipids (OxPLs) including OxPAPC (1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine), which are the active part of minimally modified OxLDL and found in high concentrations within atherosclerotic lesions, are the primary stimuli of different proatherogenic events including increased monocyte adherence to endothelial cells, as well as the induction of production of various inflammatory cytokines by macrophages.1,3–5 However, although phenotypic switching of vascular smooth muscle cells (VSMCs) is known to play a key role in the development, progression, and end-stage clinical consequences of atherosclerosis (reviewed by Owens et al6), very little is known regarding the effects of specific OxPLs on VSMCs. Recently we showed that the oxidized phospholipid OxPAPC, as well as the active components of OxPAPC, POVPC (1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphorylcholine), and PGPC (1-palmitoyl-2-gluaroyl-sn-glycero-3-phosphorylcholine), induce profound phenotypic switching of VSMCs including suppression of multiple SMC differentiation marker genes in vivo and in vitro.7

Phenotypic switching of VSMCs results in an increase in the rate of cell proliferation and migration and a decrease in the expression of SMC-specific marker genes.6 It has long been recognized that expression of extracellular matrix (ECM) proteins by phenotypically modulated VSMCs within...
Atherosclerotic lesions play a critical role in the pathogenesis of this disease, as well as its clinical consequences. The normal vessel wall ECM is composed predominantly of type I and III collagens, elastin, and proteoglycans with smaller amounts of fibronectin, laminins, and type IV collagen. However, the composition of ECM dramatically changes during the development of atherosclerosis. High levels of fibronectin and type VIII collagen are synthesized locally by resident VSMCs in diffuse intimal thickening and atherosclerotic plaques. Furthermore, there are major changes in the levels of various laminins and proteoglycans. Of major importance, the new synthesized ECM proteins not only play a key role in determining the biomechanical properties of lesions but also modulate the phenotype of VSMCs, including regulation of VSMC proliferation and migration and further ECM synthesis through integrin-mediated signaling.

It was recently reported that OxLDL stimulates collagenous protein production in VSMCs. However, virtually nothing is known regarding the effects of specific lipid oxidation products on expression of ECM proteins and what role these processes could play in modulating ECM expression by intimal VSMCs within atherosclerotic lesions. The goal of the present studies was to test the hypothesis that OxPLs including POVPc alter the expression of ECM genes within VSMCs. Results showed that POVPc induced upregulation of several ECM genes including type VIII collagen both in vitro and in vivo and that this expression was mediated, at least in part, by Krüppel-like factor (KLF)4. Moreover, the increase in type VIII collagen expression was required for POVPc-induced VSMC migration, demonstrating that altered ECM expression has important functional consequences.

### Materials and Methods

The animal protocols were approved by the Animal Care and Use Committee at the University of Virginia. An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

### Results

**POVPc Activated Expression of Multiple ECM Genes in Cultured SMCs**

As an initial screen of ECM genes modulated by OxPLs in SMCs, we performed Affymetrix GeneChip microarray analyses to compare gene expression profiles between POVPc- and vehicle-treated rat aortic SMCs. Results showed that POVPc significantly increased the expression of type VIII collagen (3.54-fold increase, *P*=0.01) and the chondroitin...
sulfate proteoglycan versican (3.04-fold increase, \(P < 0.01\)). Results also showed trends for increases in several collagens, including type XV, XVIII, XXII, and XXVII, and integrin receptors \(\alpha_v\) and \(\alpha_8\) and for decreased expression of procollagen type XI, although these changes did not achieve statistical significance (\(P < 0.15\); Figure I, A, in the online data supplement). POVPC also increased expression of fibronectin 3 by 3-fold, and laminin \(\alpha_2\) by 6-fold but did not change the expression of type I collagen, as determined by real-time RT-PCR analyses (supplemental Figure I, B).

Taken together, these results clearly demonstrate that POVPC induces selective changes in the expression of multiple ECM genes in cultured rat aortic SMCs. Because type VIII collagen is implicated in cell migration and growth, as well as atherosclerosis, it was chosen to be the major focus of subsequent studies.

**POVPC Induced Type VIII Collagen mRNA and Protein Synthesis in Cultured SMCs**

Cultured rat aortic SMCs were treated with variable concentrations of OxLDL (10 to 100 \(\mu g/mL\)), OxPAPC (1 to 100 \(\mu g/mL\)), POVPC, PGPC (1 to 25 \(\mu g/mL\)), and PEIPC (0.1 to 3 \(\mu g/mL\)) or vehicle for 24 hours, followed by quantitative real-time RT-PCR analyses. OxLDL and OxPAPC both induced an approximately 4-fold increase in type VIII collagen \(\alpha_1\) chain (Col8a1) gene expression when applied at a concentration of 100 \(\mu g/mL\) (Figure 1A and 1B). POVPC was both more potent and efficacious than OxLDL and OxPAPC in inducing Col8a1 gene expression with a maximum increase of more than 9-fold at a concentration of 10 to 25 \(\mu g/mL\) (Figure 1C). PGPC also increased Col8a1 expression by 4-fold at a concentration of 25 \(\mu g/mL\) (Figure 1C).

To extend our recent studies showing POVPC- and PGPC-induced suppression of SMC differentiation marker genes,\(^7\) we tested the effect of PEIPC, which has been shown to be the most potent component of OxPAPC for activating endothelial cells.\(^{15}\) Of interest, PEIPC markedly suppressed expression of the SMC marker genes smooth muscle (SM) myosin heavy chain (MHC), SM \(\alpha\)-actin, and SM 22\(\alpha\) by 80% to 90% at a concentration of 0.5 to 1 \(\mu g/mL\) (supplemental Figure II). PEIPC also induced a 3-fold increase in type VIII collagen gene expression at concentrations of either 0.5 or 1 \(\mu g/mL\) (Figure 1D). By contrast, VSMCs treated with vehicle or nonoxidized control phospholipids such as

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**Figure 2.** POVPC induced type VIII collagen protein synthesis and secretion in cultured rat aortic SMCs. For Western blot analysis, VSMCs were treated with either POVPC (10 \(\mu g/mL\)) (P) or vehicle (V) for 48 to 72 hours. Conditioned media were collected and concentrated, and 20 \(\mu g\) of total proteins were fractionated by electrophoresis under non-denaturing conditions on a 7.5% polyacrylamide gel (A) or denaturing conditions on a 10% gel (B). Type VIII collagen protein was detected with a guinea pig polyclonal antibody (B) or mouse monoclonal antibody (A) raised against bovine type VIII collagen. To validate the specificity of anti-type VIII collagen antibodies, cultured VSMCs were transfected with Col8a1 siRNA oligonucleotides (sicoll8#3) or control siRNA (siControl), followed by either POVPC or vehicle treatment for 24 hours (C). The expression of Col8a1 gene was measured by quantitative real-time RT-PCR. \(P < 0.05\) vs vehicle. After transfection with siRNA and treatment with either POVPC (10 \(\mu g/mL\)) (P) or vehicle (V) for 48 hours, type VIII collagen protein was detected in VSMC conditioned medium by probing Western blots with a guinea pig polyclonal antibody (D) raised against bovine type VIII collagen.
LDL, PAPC, and DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) (supplemental Figure III) showed no change in Col8a1 gene expression, indicating that oxidation is required for the observed effects.

To determine whether POVPC-induced increases in type VIII collagen mRNA were associated with increased protein secretion, Western blot analyses were performed on conditioned media collected from vehicle- or POVPC-treated cultured aortic SMCs. (Figure 2). We tested 2 antibodies raised against bovine type VIII collagen: a guinea pig polyclonal antibody and a mouse monoclonal antibody. Major bands at 50, 65, and 85 kDa and a high-molecular-mass product >250 kDa were visualized by the monoclonal antibody in the SMC conditioned media under nonreduced conditions (Figure 2A). The polyclonal antibody showed bands at 50, 65, 75, 85, and 125 kDa under reduced conditions (Figure 2B). These bands are consistent in size with purified type VIII collagen.16 The multiple bands that we and others16–18 observed for type VIII collagen reflect the well-known molecular heterogeneity of type VIII collagen. Moreover, as noted by Korsching and Rauterberg,16 detection of type VIII collagen protein molecular mass variants differs depending on the source and type of detection antibody. A distinct band at 85 kDa was observed with both antibodies under reduced and nonreduced conditions after 48 to 72 hours of treatment with POVPC as compared to the vehicle-treated control (Figure 2A and 2B). To further validate the specificity of type VIII collagen antibodies, cultured SMCs were treated with a Col8a1 small interfering (si)RNA. These results confirmed that the 85-kDa band corresponded to the α1 chain of type VIII collagen (Figure 2C and 2D).

POVPC Induced Type VIII Collagen α1 Chain mRNA Expression in Rat Carotid Arteries In Vivo

To determine whether POVPC can also activate expression of Col8a1 in vivo, we applied 30 μg of POVPC or DMSO vehicle to the adventitial surface of rat carotid arteries using an F-127 pluronic gel.3,7 Results of real-time RT-PCR demonstrated that the treatment of carotid arteries with POVPC for 24 hours increased expression of Col8a1 mRNA by nearly 3-fold relative to vehicle-treated vessels (Figure 3A). In contrast, no changes in expression of type VIII collagen mRNA were seen in various control tissues, including untreated carotid arteries, or within the liver or aorta of the same rats, indicating that effects were not systemic (Figure 3B and 3C).

Klf4 Was Required for POVPC-Induced Col8a1 Gene Expression In Vivo and In Vitro

Previous studies have shown that POVPC induces Klf4 synthesis in VSMCs and that Klf4 is required for POVPC-induced repression of SMC marker genes.7 Mutagenesis experiments determined that a Klf4 consensus binding motif has a sequence of 5'-G/A-G-G-C/T-G-C/T-3' (or 5'-G/A-C-G/A-CC-T/C-T/C-3').19 Of interest, we found a potential Klf4-binding site (ACACCTT) in the proximal Col8a1 promoter that is completely conserved in the Col8a1 genes in human, mouse, and rat (supplemental Figure IV). The effects of siRNA oligonucleotides specific to Klf4 were used to determine whether Klf4 is required for POVPC-induced increases in Col8a1 gene expression. The specificity of the siRNA has been documented previously.7 Klf4 siRNA, but not a control siRNA, blocked POVPC-induced Col8a1 gene expression in cultured SMC at both the mRNA (Figure 4A) and the protein level (Figure 4C). The siKlf4 also blocked POVPC-induced expression of the LAMA2 gene (supplemental Figure V), which also contains a conserved consensus KLF4 binding site in its 5' promoter region.

To further assess if Klf4 is required for POVPC-induced expression of type VIII collagen, we tested effects of POVPC in Klf4 knockout (KO) mouse aortic SMCs. SMCs were isolated from the aorta of Klf4-floxed mice20 and infected with an adenovirus expressing Cre-recombinase
(Klf4 KO) or control adenovirus (control). Control or Klf4 KO SMCs were then treated with POVPc (1 to 10 μg/mL) or vehicle, followed by RNA extraction and real-time RT-PCR analysis of Col8a1 gene expression (Figure 4B). Results showed significant induction of expression of Col8a1 in control but not Klf4 KO mouse aortic SMCs.

To determine whether POVPc-induced expression of Col8a1 in vivo was also dependent on Klf4, rat carotid arteries were treated simultaneously with POVPc plus control or Klf4 siRNAs using the pluronic gel system. Of major interest, results showed that the siKlf4 but not the control siRNA partially blocked POVPc-induced expression of Col8a1, as well as POVPc-induced suppression of SM α-actin and SM MHC (supplemental Figure VI).

It has been previously reported that Sp1 transcription factor regulates Klf4 expression in colon cancer cells.21 Figure 5 demonstrates that an Sp1 siRNA, but not a control siRNA, decreased both the POVPc-induced expression of Klf4 and Col8a1 genes (Figure 5A and 5B), as well as the synthesis of both Klf4 (supplemental Figure VII) and type VIII collagen (Figure 4C) proteins in cultured VSMCs. Thus, we hypothesized that POVPc increased Col8a1 gene expression through Sp1-dependent activation of Klf4.

The Klf4 promoter contains 3 Sp1 binding sites located in the region between 150 and 50 bp upstream of the transcriptional start site.21 To determine whether Sp1 is required for POVPc-induced Klf4 expression, VSMCs were transfected with different reporter plasmids that contained various lengths of the Klf4 promoter (2200, 515, or 51 bp of the promoter region upstream from the transcriptional site cloned into the pGL3-luciferase vector). POVPc markedly increased both the 2200- and 515-bp Klf4 promoters but not the 51-bp region lacking the 3 Sp1 binding sites (supplemental Figure VIII). These results provide evidence suggesting that POVPc-induced expression of Klf4 in VSMCs is Sp1-dependent.

Klf4 Directly Bound to the Col8a1 Promoter Region In Vitro and In Vivo

We tested whether Klf4 binds to the type VIII collagen promoter region using quantitative chromatin immunoprecipitation (ChIP) assays. Treatment of rat aortic SMCs with POVPc was associated with the recruitment of Klf4 to the Col8a1 gene promoter within 6 hours of POVPc treatment (Figure 6A). To verify our in vitro data, we used the pluronic gel system to determine whether POVPc induced Klf4 binding to the type VIII collagen promoter in vivo. Figure 6B shows that Klf4 was also enriched at the Col8a1 gene promoter in ChIP analyses of chromatin isolated from rat carotid arteries treated with POVPc for 8 hours versus vehicle-treated arteries. In contrast, Klf4 was not changed in ChIP analyses of chromatin isolated from aortas of the same
animals (Figure 6B). Taken together, our data provide strong evidence that POVPC-induced increases in type VIII collagen expression are mediated, at least in part, by Klf4 binding to the type VIII collagen promoter.

POVPC Induced Type VIII Collagen Expression Was Required for Migration of Cultured SMCs

It has been reported that type VIII collagen promotes VSMC migration.22,23 Boyden chamber migration assays were used to determine whether OxPLs increased the migration of cultured SMCs, and if this migration was dependent on both Klf4 and type VIII collagen. As shown in Figure 7A, POVPC and PEIPC, but not PGPC, enhanced the fibronectin-dependent migratory response of VSMCs. Of major interest, POVPC-induced migration of SMCs was inhibited with siRNA to either Klf4 or Col8a1 but not a control siRNA that targets luciferase (Figure 7B). To further test the role of type VIII collagen and Klf4 in POVPC-induced SMC migration, we tested migratory effects of POVPC in type VIII collagen and Klf4 KO mouse aortic SMCs. POVPC induced transmigration in Klf4 control and type VIII collagen wild-type (WT) but not Klf4 KO and type VIII collagen KO mouse aortic SMCs (Figure 7C and 7D). Taken together, results of these studies provide evidence that POVPC-induced migration of cultured SMCs is dependent on Klf4 and type VIII collagen.

Endogenous Col8a1 and Klf4 Gene Expression Was Increased in the Aortas of apoE KO Mice

To determine whether Klf4 expression is correlated with Col8a1 gene expression in vivo, we compared the mRNA levels of Col8a1 and Klf4 in the thoracic aortas of WT mice and apoE KO mice after 13 weeks of Western diet feeding. Results of real-time RT-PCR demonstrated that endogenous mRNA levels of Col8a1 and Klf4 were increased in the thoracic aortas of apoE KO mice versus WT mice (Figure 8A and 8B), whereas mRNA levels of SM MHC and myocardin were decreased (Figure 8C and 8D). In contrast, no changes in expression of Col8a1 and Klf4 mRNA were seen in control liver tissue.

Discussion

In the present study, we tested the hypothesis that OxPLs modulate ECM gene expression in VSMCs. Consistent with this hypothesis, results of real-time RT-PCR analyses of the candidate genes and gene microarray studies showed that POVPC altered expression of a number of ECM or ECM-related genes, including multiple collagens, fibronectin 3, laminin α2, integrins, and the proteoglycan versican. We also demonstrated that OxPLs, and in particular POVPC, markedly upregulated type VIII collagen expression in cultured VSMCs, as well as in vivo within carotid arteries. Previous studies have shown that type VIII collagen expression is dramatically increased within VSMCs in re-
response to vascular injury, as well as in atherosclerotic plaques of the apoE KO mice. Type VIII collagen has been shown to stimulate attachment, focal adhesion formation, and chemotaxis of cultured SMC, as well as to modify matrix metalloproteinase synthesis. Furthermore, it has been previously shown that VSMCs from type VIII collagen–deficient mice exhibit greater adhesion to type I collagen than WT VSMCs. In contrast, WT VSMCs spread more, migrate further, exhibit increased proliferation, and express higher level of matrix metalloproteinase 2 in comparison with type VIII collagen–deficient VSMCs, suggesting that the de novo production of type VIII collagen allows VCMCs to overcome adhesion to type I collagen.

A number of factors have been shown to modulate type VIII collagen expression in cultured VSMCs including platelet-derived growth factor-BB, basic fibroblast growth factor-2, angiotensin II, transforming growth factor β, and colony-stimulating factor, which increase expression, and interferon γ, which decreases expression. However, virtually nothing is known regarding factors that regulate the expression of type VIII collagen or other ECM proteins by VSMCs within atherosclerotic lesions.

Of particular relevance to the present studies, previous studies by Plenz et al showed reduced type VIII collagen expression in the media and adventitia, but increased expression in the intima, of carotid arteries in rabbits fed a high-cholesterol diet. However, no direct evidence was presented that these effects were mediated by OxPLs. Our present results clearly demonstrate that OxPAPC, as well as its component phospholipids PGPC, PEIPC, and POVPC, significantly increase type VIII collagen in vitro. Of major significance, we also showed that POVPC increased type VIII collagen expression within carotid arteries in vivo. Taken together, results suggest that the oxidized arachidonoyl phospholipids may be somewhat unique among modified lipids in inducing expression of type VIII collagen and thus implicate these factors as potential mediators of increased type VIII collagen expression in atherosclerotic lesions. A key unresolved question is whether POVPC or other OxPLs directly mediate SMC phenotypic switching in vivo including inducing alterations in expression of type VIII collagen and other ECM components. However, such experiments are not feasible at present for several reasons including: (1) OxPL receptors have not been identified for precluding use of either pharmacological or genetic loss-of-function approaches; (2) there is a lack of highly specific and efficacious inhibitors to block formation of specific OxPL species; and (3) although there are a number of neutralizing antibodies to specific OxPL species, it is not clear these can maintain efficacious inhibition in vascular lesions over longer time periods.
long periods of time in vivo, and, of course, such approaches would not be selective in inhibiting OxPL responses within VSMCs.

Results of the present study also provide novel insights regarding mechanisms that regulate type VIII collagen expression, in that we show that effects of POVPC are dependent on Sp1-induced activation of Klf4. Klf4 is a member of the Krüppel family of transcription factors. Studies in our laboratory have shown that Klf4 expression is normally undetectable in SMC but is rapidly increased in vivo following vascular injury. Moreover, it was demonstrated that the platelet-derived growth factor-BB– and POVPC-induced downregulation of SMC marker genes is mediated, in part, by Klf4. In addition, our present results demonstrated that Klf4 and Col8a1 expression are increased in the aortas of apoE KO mice after 13 weeks of Western diet feeding. Finally, results of recent studies in our laboratory showed that conditional knockout of Klf4 in adult mice resulted in a transient delay in downregulation of SMC marker genes, but subsequently enhanced neointimal formation following ligation induced injury of the carotid artery. That is, Klf4 appears to be a key rate-limiting factor for initial phenotypic switching of SMC in response to vascular injury but also plays a key role in negative regulation of SMC growth. We found that Klf4 is required for POVPC-induced Col8a1 expression in vivo and in vitro, as well as LAMA2 expression in vitro. Based on the results of the present studies, it is interesting to speculate that Klf4 might also be a key regulator of ECM expression in VSMCs during atherogenesis.

Of interest, although we demonstrated that Sp1 was required for POVPC-induced increases in Klf4 expression, we did not find a significant increase in Sp1 expression or synthesis after POVPC treatment (OA Cherepanova, GK Owens, unpublished data, 2008), suggesting that effects might involve posttranscriptional modification of Sp1 and/or its ability to bind to the Klf4 promoter. Consistent with this possibility, Sp1 has been shown to undergo various posttranscriptional modifications including phosphorylation and O-glycosylation (reviewed by Black et al) that increase its transcriptional activity, at least in part, by enhancing its binding to DNA.

Previously, we reported that POVPC treatment of cultured rat aortic SMC resulted in enhanced repair in a scratch wound assay. Results of the present studies showed that POVPC and PEIPC, but not PGPC, enhanced VSMC migration. Consistent with these observations, previous studies have shown differential effects of POVPC versus PGPC on monocyte and neutrophil binding to endothelium. Moreover, recent voltage-clamp studies by Leitinger et al suggested that POVPC and PGPC recognized different receptors based on mRNA expression studies in Xenopus oocytes. Taken together, results suggest that the stoichiometry of specific OxPLs within lesions may have important functional consequences through differential effects on VSMCs, as well as other vascular cells.

Figure 8. Endogenous mRNA levels of Col8a1 and Klf4 were increased in the aortas of apoE KO after 13 weeks of Western diet vs WT mice. Total RNA was isolated from the thoracic aortas or livers as a control. Expression of Col8a1 (A), Klf4 (B), SM MHC (C), and myocardin (D) mRNA was measured by real-time RT-PCR. Values represent the means for 5 animals per group ± SEM. *P<0.05 vs vehicle.
Previous studies by Rocnik et al\cite{Rocnik2007} suggested that new collagen synthesis is required for VSMC migration on type I collagen–covered substrata. Results of the present studies showing that POVPC-induced migration of cultured rat aortic SMC was inhibited by siRNA suppression of type VIII collagen and failed to occur in type VIII KO mouse aortic SMC provide evidence that de novo synthesis of type VIII collagen plays an important role in mediating VSMC migration in vitro. Moreover, we also showed that POVPC-induced VSMC migration was dependent on Klf4. These findings are highly intriguing in that they raise the question as to whether Klf4-dependent SMC migration is mediated exclusively through induction of type VIII collagen or whether Klf4 also activates additional signaling pathways important in this response.

Interestingly, we found that POVPC did not induce and actually inhibited VSMC migration at concentrations of $>5 \mu g/mL$. From our recent studies,\cite{Cherepanova2006} we know that this concentration is not toxic for VSMCs. As such, it is possible that activation of additional molecular pathways might be responsible for the inhibition of VSMC migration by the higher concentrations of POVPC.

In conclusion, we provide novel evidence showing that OxPLs activate coordinate expression of a variety of ECM genes in VSMCs including type VIII collagen and that activation of type VIII collagen is dependent on both Klf4 and Sp1. Moreover, we show that increased type VIII collagen expression is required for POVPC-induced migration of VSMCs, indicating that activation of ECM genes has important functional consequences. Future studies are needed to define specific receptors and downstream effector molecules that mediate the effects of specific OxPLs in vivo.

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Disclosures

None.

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Oxidized Phospholipids Induce Type VIII Collagen Expression and Vascular Smooth Muscle Cell Migration.

Short title: OxPLs induce type VIII collagen expression in VSMC

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Expanded Materials and Methods

Synthesis and analysis of oxidized phospholipids.

1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine PAPC (Avanti Polar Lipids, Inc) was oxidized to generate OxPAPC by the exposure of the dry lipid to air for 72 hours. The extent of oxidation was monitored by positive ion electrospray mass spectrometry (ESI-MS) as described previously\textsuperscript{1}. Lipids were stored at -70\textdegree C in chloroform and used within one month after testing for purity. Analysis of OxPAPC was performed by mass spectrometry using a Finnigan LCQ classic, connected 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).

1-palmitoyl-2-(5,6-epoxyisoprostane E2)-sn-glycero-3-phosphorylcholine (PEIPC) was purified from OxPAPC as reported previously\textsuperscript{2}. LDL and OxLDL, were obtained from Biomedical Technologies, Inc. POVPC and PGPC were obtained from Cayman Chemical and 1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine (DMPC) was obtained from Avanti Polar Lipids, Inc.

Cell Culture and treatment of SMCs with Phospholipids

Rat aortic SMCs were isolated and cultured as previously described\textsuperscript{3}. Cells were grown to 100% confluence and then switched to serum-free media. After culturing in serum-free media for three days, passages 8-15 of post-confluent rat aortic SMCs were treated with vehicle, LDL, OxLDL, nonoxidized PAPC, DMPC, OxPAPC, PEIPC, PGPC or POVPC.

Mouse SMCs were isolated from Klf4-floxed mice\textsuperscript{4} and cultured as previously described. At passages three SMCs were infected with adenovirus expressing Cre-recombinase or control
adenovirus, and the recombination of Klf4-floxed allele was confirmed by PCR analysis as previously described\(^5\).

Mice with targeted deletion of both the Col8a1 and Col8a2 genes were generated in the laboratory of Dr. Bjorn Olsen (Harvard Medical School) as described\(^6\) with wild type littermate mice used as a control. Aortic SMCs were isolated from the mice as previously described\(^7;8\). Mouse SMCs at the passage 7-10 were used for the experiments testing the effect of POVPC on SMCs.

**RNA isolation, cDNA preparation and quantitative RT-PCR**

Total RNA was isolated from cultured cells and rat tissues using Trizol reagent (Invitrogen) according to the protocol of the manufacturer. One microgram of RNA was reverse transcribed with iScript cDNA synthesis kit (BioRad). Real-time RT-PCR was performed by iCycler technology (BioRad) using primers (5’-3’) specific for rat Col8a1 gene (forward, GGCAAGAGGTACACACCTACC; reverse, GACCTTGTTCCTCCTCGCAACTG), rat fibronectin 3 (forward, ACACCTCAAGACCAAGTCAC; reverse, TAGTGGCACTGCCTCGCTTC), rat laminin \(\alpha_2\) (forward, ATACTTGGAAGGCCTCAGACTCAG; reverse, CGAAGCAAGATTACCGAGCAG) and mouse Col8a1 gene (forward, GGCAAGAGTACACACCTACC; reverse, GACCTTGTTCCTCGCAACTG). Primers and probe sequences of rat SM \(\alpha\)-actin, SM myosin heavy chain, SM 22\(\alpha\), Klf4, myocardin and 18s rRNA were described previously\(^5;9\). Expression of the genes was normalized to 18s rRNA.
Animals

The animal protocols were 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 30 µg of POVPC (n=6 rats) or DMSO vehicle (n=6 rats) was applied to the adventitial surface of rat carotid arteries for 24 hours as described previously. The apoE knockout mice (male, 8 weeks of age) were obtained from The Jackson laboratory (Bar Harbor, ME). The mice were fed a Western atherogenic diet (Harlan Teklad, Madison, WI) containing 21.2 % fat by weight (0.2% by weight cholesterol, 17.3% by weight protein and 48.5% by weight carbohydrate) for 13 weeks. C57BL/6 mice (male, 21 weeks of age) were use as a control. The Fast Prep FP120 (Q-Biogene) was used to homogenize tissues, and total RNA was prepared from carotid arteries, aorta and liver. Col8a1 expression in each sample was normalized to 18S rRNA levels.

siRNA Oligonucleotides and Transfection

siRNA oligonucleotides specific for Klf4 (siKLF#13; 5’-GUACAAUGGUUUAAUCCCA-3’), Sp1 (siSp1; 5’-UUGAGUCACCCCAUGAGAA-3’), Col8a1 (sicoll8#3; 5’-UCAGGGCCGGUGCUCUAU-3’) were purchased from MWG-Biotech. Luciferase siRNA (Dharmacon) or EGFP siRNA (5’-GAACGGCAUCAAGGUGAAC-3’, MWG-Biotech) were used as the control off-target siRNA. The transient transfection of siRNA oligonucleotide was carried out using Oligofectamine (Invitrogen) according to the protocol of the manufacturer.

For in vivo siRNA uptake studies, 50 µl of ice-cold F-127 pluronic gel containing 100 µg of siGFP was applied to the adventitial surface of GFP mouse carotid arteries. Twenty four hours later the mice were killed, and right treated and left control carotid arteries were harvested.
for paraffin tissue sectioning. Tissue sections were visualized using a fluorescent microscope to assess change in GFP intensity after siGFP administration. For studies of siKlf4 in vivo 150 µl of ice-cold F-127 pluronic gel containing 30 µg of POVPC or DMSO vehicle simultaneously with siKlf4 (100 µg) or siControl (100 µg) was applied to the adventitial surface of rat carotid arteries for 24 hours. Six animals were used for each group. Tissues were homogenized, and total RNA was prepared from carotid arteries, aorta and liver. SM α-actin, SM MHC, Klf4 and Col8a1 expressions in each sample was normalized to 18S rRNA levels. For each animal the result for right treated carotid artery were normalized to the result for left native artery.

**Transient Transfection and Luciferase Assays**

Cultured rat aortic SMCs were transiently transfected with reporter plasmid using FuGENE reagent (Roche Diagnostics Corp.) at approximately 75% confluency according to the manufacturer’s protocol. The Klf4 promoter luciferase constructs included: KLF4-2200-luc, KLF4-515-luc and KLF4-51-luc (2200, 515 and 51 bp of the promoter region upstream from the transcriptional start site) cloned into the pGL3-Luciferase vector. The Klf4 promoter-reporter plasmids were generated and kindly provided by Dr. Chi-Chuan Tseng\(^\text{10,11}\) (Boston University School of Medicine). Luciferase activity was measured as described previously\(^\text{12}\) and normalized to total protein content (Coomassie Plus protein Assay reagent, Pierce).

**Western blots.**

Conditioned media was concentrated using Amicon Ultra-4 centrifugal filters (Millipore) (3500 rpm, 30min, 4°C). For Western blot analyses, 20 µgs of total proteins were fractionated by
electrophoresis under denaturing conditions on a 10% polyacrylamide gel or under non-denaturing conditions on a 7.5% gel and transferred onto a transfer membrane (Millipore). Type VIII collagen protein was detected by probing Western blots with a guinea pig polyclonal antibody or mouse monoclonal antibody raised against bovine type VIII collagen. The antibodies were kindly provided by Dr. Helene Sage (Benaroya Research Institute at Virginia Mason, Seattle).

Whole cell extracts from cultured rat aortic SMCs were treated with POVPC and vehicle prepared using modified RIPA buffer as described previously\textsuperscript{12}. Lysates were subjected to Western blot analyses using Sp1 (Santa Cruz), Klf4 (Chemicon Int.) or GAPDH (Chemicon Int.) antibodies.

**Chromatin immunoprecipitation (ChIP) assays.**

ChIP assays were performed on cultured cells as described previously\textsuperscript{12-14}. SMCs were fixed with 1% formaldehyde at 37°C for 10 minutes to cross-link protein-DNA and protein-protein complexes within intact chromatin.

For *in vivo* ChIP, 150 µl of ice-cold F-127 pluronic gel (Molecular Probes) containing 30 µg of POVPC or DMSO vehicle was applied to the adventitial surface of rat carotid arteries for 8 hours. Animals were euthanized and rat carotid arteries and aortas quickly dissected from surrounding tissue, washed in ice-cold PBS to remove blood and debris, snapped frozen in liquid nitrogen, and stored at -80°C. The organs were later ground with a mortal and pestle with liquid nitrogen cooling, transferred directly to 37°C 1% formaldehyde for 10 min, washed 4 times with ice-cold PBS as described previously\textsuperscript{13}, followed by the additional homogenization using glass beads in a Fast Prep FP120 (Q-Biogene).
The cross-linked chromatin of the harvested cultured SMC and rat tissues was sonicated to shear chromatin into fragments of approximately 200-600 bps. Chromatin-protein complexes were immunoprecipitated with antibodies against Klf4 and salmon sperm DNA/protein A agarose (Upstate) added as a carrier. As a negative control, the antibody was excluded from the immunoprecipitation reaction. Samples were washed, reverse cross-linked, and purified. Recovered DNA was quantified by fluorescence with picogreen reagent (Molecular Probes, Eugen, OR). Real-time PCR was performed to amplify the *Col8a1* gene promoters. Primer sequences for the type VIII collagen promoter were as follows: forward, ATTCCCTCCCACCTCTGTCTG; reverse, AAGCCAAGTAACCGCCCAAG. The data presented in ChIP assays are representative of three independent experiments.

**Migration assays.**

Cell migration assays were performed on Millipore MultiScreen-MIC plates containing 8 μm pores. Cells were grown to 70% confluence and then switched to serum-free media. A cell suspension (1x10^5 cells/ml, 150 μl) was added to the upper well in serum-free media containing 0.1% BSA (Sigma). Concentrations of OxPLs from 0.05 to 10 μg/ml were added to the bottom chambers. For all experiments, either 5 μg/ml fibronectin (Sigma) were added to the bottom chamber in serum-free media with 0.1% BSA (FN-induced chemotaxis) or membranes were covered with type I collagen (trans-migration through type I collagen). The chambers were incubated at 37°C in a CO₂ incubator for 18 hrs, and fixed in 4% formaldehyde. The non-invaded cells were removed from the upper wells and the invaded cells were stained with 0.2 % Crystal Violet solution in 7% ethanol. Cells from 8-10 randomly chosen high-power fields (magnification X20) on the lower surface of the filter were counted.
GeneChip microarray

Total RNA was isolated from rat aortic SMCs treated with DMSO-vehicle or 10 µg/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 Affimetrix’s DMT software and the same comparison were carried out using dChip software.

Statistics

All experiments were done in duplicate or triplicate for each experimental group and performed in 2 to 6 independent experiments. Data were analyzed for statistical significance using a 2-tailed Student’s t-test. Errors bars represent SEM. A value of p<0.05 was considered statistically significant.
Reference List


Supplemental Figure legends

Supplemental Figure I. POVPC treatment induced a subset of ECM-related genes in rat aortic SMCs. (A) VSMCs were treated with 10 µg/ml of POVPC for 24 hours. Total mRNA samples were subjected to Affymetrix GeneChip microarray analyses. ECM-related subset of genes induced by POVPC treatment is shown. (B) VSMCs were treated with the indicated concentrations of POVPC for 24 hrs. Expression of laminin α2 (Lama2), fibronectin 3 (Fn3), and type I collagen α1 (Col1a1) mRNA were measured by quantitative real-time RT-PCR and normalized to 18s rRNA, and demonstrated as a fold-increase over vehicle. Values represent the mean±SEM. *P < 0.05 Lama2 vs. vehicle, #P < 0.05 Fn3 vs. vehicle

Supplemental Figure II. PEIPC decreased expression of SMC marker genes in cultured rat aortic SMCs. VSMCs were treated with the indicated concentrations of PEIPC for 24 hrs. Expression of the SM α-actin, SM MHC and SM 22α mRNA was measured by quantitative real-time RT-PCR and normalized to 18s rRNA, and demonstrated as a fold-increase over vehicle. Values represent the mean±SEM. P*P < 0.05; **P < 0.01 vs. vehicle.

Supplemental Figure III. The non-oxidized phospholipids LDL, PAPC and DMPC had no effect on type VIII gene expression in cultured rat aortic SMCs. VSMCs were treated with the indicated concentrations of LDL (A), PAPC and DMPC (B) for 24 hrs. Expression of Col8a1 gene was measured by quantitative real-time RT-PCR and normalized to 18s rRNA, and expressed as a fold-increase over vehicle. Values represent the mean±SEM.
Supplemental Figure IV. Schematic of the sequence of 5` promoter region of the Col8a1 gene. The sequence comparison of the proximal mouse (NM_007739), human (NM_001850), and rat (NM_001107100) Col8a1 promoter regions was performed by the BioEdit program. The transcription start sites are indicated by arrows. A putative Klf4 binding site is marked by box.

Supplemental Figure V. siKlf4 partially blocked POVPC-induced LAMA2 gene expression in rat aortic SMCs. VSMCs were transfected with Klf4 siRNA oligonucleotides (siKLF4) or control off-target siRNA (siControl) followed by either POVPC (10 µg/ml) or vehicle treatment for 24 hrs. Expression of LAMA2 gene was measured by quantitative real-time RT-PCR.

Supplemental Figure VI. Pluronic gel-based delivery of siKlf4 but not a control siRNA partially blocked the POVPC-increased expression of the endogenous Col8a1 gene as well as POVPC-induced suppression of SM α-actin and MHC in vivo. Pluronic gel with either POVPC (30 µg) or vehicle and simultaneously with either siKlf4 or siControl was applied to the adventitial surface of rat right common carotid arteries. Total RNA was isolated from the right treated and left native carotid arteries, liver and aorta of the animals. Expression of SM α-actin, SM MHC, Klf4 and Col8a1 were measured by quantitative real-time RT-PCR. For each animal the result for right treated carotid artery were normalized to the result for left native artery.

Supplemental Figure VII. To validate the knockdown efficacy of Klf4 and Sp1 siRNA, 20 µg of total protein from lysates of VSMCs transfected with siRNA and treated with either
POVPC (10µg/ml) (P) or vehicle (V) for 48 hrs, were subjected to the Western blotting using anti-Klf4, anti-Sp1 and anti-GAPDH antibodies.

Supplemental Figure VIII. POVPC markedly increased the activity of Klf4 promoter genes in cultured rat aortic SMCs. KLF4-2200, -515 and 51bp promoter-luciferase constructs were transiently transfected into rat aortic SMCs. VSMCs were treated with either POVPC (10 µg/ml) or vehicle for 24 hours. Luciferase activity was measured, and values were normalized to protein content and expressed as a fold-increase over vehicle. Values represent the mean ± SEM. *P <0.005; **P<0.001 vs. vehicle.
### Supplemental Figure I Cherepanova et al

**A**

<table>
<thead>
<tr>
<th><strong>Rattus norvegicus genes</strong></th>
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**B**

![Graph showing mRNA/18s RNA (Fold over vehicle) vs. POVPC concentration, µg/ml](chart.png)

- **Fn3**
- **Lam2a**
- **Col1a**

* * *

# *
Supplemental Figure II Cherepanova et al

![Supplemental Figure II](image)
Supplemental Figure III Cherepanova et al

A

LDL concentration, µg/ml

Col8a1/18s RNA (Fold over vehicle)

vehicle 10 50 100

B

PAPC/DMPC concentration, µg/ml

Col8a1/18s RNA (Fold over vehicle)

vehicle 1 10 25

PAPC
DMPC
5` Col8a1 promoter region

**Supplemental Figure IV Cherepanova et al**

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Supplemental Figure VII Cherepanova et al

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- **Sp1**
- **KLF4**
- **GAPDH**
Supplemental Figure VIII Cherepanova et al

A

2200 bp KLF4 promoter activity

(Fold over vehicle)

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C

500 bp KLF4 promoter activity

(Fold over vehicle)

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