Lipid Phosphate Phosphatase 3 Negatively Regulates Smooth Muscle Cell Phenotypic Modulation to Limit Intimal Hyperplasia


Objective—The lipid phosphate phosphatase 3 (LPP3) degrades bioactive lysosphospholipids, including lysosphosphatidic acid and sphingosine-1-phosphate, and thereby terminates their signaling effects. Although emerging evidence links lysosphosphatidic acid to atherosclerosis and vascular injury responses, little is known about the role of vascular LPP3. The goal of this study was to determine the role of LPP3 in the development of vascular neointima formation and smooth muscle cells (SMC) responses.

Methods and Results—We report that LPP3 is expressed in vascular SMC after experimental arterial injury. Using gain- and loss-of-function approaches, we establish that a major function of LPP3 in isolated SMC is to attenuate proliferation (extracellular signal-regulated kinases) activity, Rho activation, and migration in response to serum and lysosphosphatidic acid. These effects are at least partially a consequence of LPP3-catalyzed lysosphosphatidic acid hydrolysis. Mice with selective inactivation of LPP3 in SMC display an exaggerated neointimal response to injury.

Conclusion—Our observations suggest that LPP3 serves as an intrinsic negative regulator of SMC phenotypic modulation and inflammation after vascular injury, in part, by regulating lysosphosphatidic signaling. These findings may provide a mechanistic link to explain the association between a PPAP2B polymorphism and coronary artery disease risk. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: intima ■ lipid phosphate phosphatase 3 ■ proliferation ■ restenosis ■ smooth muscle cells

Phenotypic modulation of smooth muscle cells (SMC) occurs in response to vascular injury and is a critical component of the development of intimal hyperplasia, a defining feature of atherosclerotic and restenotic lesions.12 Lysosphosphatidic acid (LPA) is a key component in serum that promotes dedifferentiation, proliferation, and migration of cultured SMC. On the basis of SMC responses to LPA and the related lysosphospholipid sphingosine-1-phosphate (SIP), these lipid mediators have been proposed to regulate SMC phenotypic modulation and the development of intimal hyperplasia in vivo. Both LPA and SIP elicit cellular effects through G-protein–coupled receptors. In the case of LPA, at least 6 and potentially 8 G-protein–coupled receptors exist that act through G_{i}, G_{12/13}, G_{q}, and G_{s} to stimulate intracellular signal pathways to modulate cAMP production,12,13 the activity of phospholipase D activity, Rho GTPases,14 phospholipase C and protein kinase C,15 extracellular signal-regulated kinases (ERK),16 and the initiation of Ca^{2+}-transients.17 Similar complex signaling systems exist for SIP.18,19 Evidence from studies using genetic and pharmacological approaches to target specific LPA and SIP receptors supports their role in regulating neointimal growth in response to vascular injury in mice. The development of intimal hyperplasia likely results from effects of LPA or SIP on multiple cell types, including recruitment of inflammatory and progenitor cells and stimulation of resident SMC.20-25 Moreover, LPA heightens atherosclerotic plaque burden in apolipoprotein E–deficient (ApoE^{−/−}) mice in an LPA_{1}- and LPA_{3}-dependent manner.26

The signaling actions of LPA, and potentially SIP, can be terminated by enzymatic dephosphorylation catalyzed by lipid phosphate phosphatases (LPPs), which are cell surface integral membrane proteins. LPP3, encoded by the Ppap2b gene, is essential in mice for vascular development and gastrulation27 and is expressed during development in heart cushions and valves.28 Studies in cultured cells implicate LPP3 as both a regulator of vascular cell LPA responsiveness and a ligand for integrin αVβ3.29,30 However, because of the early embryonic lethality observed in mice lacking LPP3, a specific role for the enzyme in vascular cell function remains unknown. The identification of PPAP2B among 13 loci recently associated with coronary artery disease (CAD) in humans suggests a causal relationship between LPP3 and vascular disease susceptibility.
In the present work, we identified SMC as a source of vascular LPP3 expression and sought to define a pathophysiologic role for SMC LPP3 in adult vascular tissue. We used genetic approaches to alter LPP3 expression and activity in SMC and investigate the consequences on vascular injury and isolated cellular responses. Using gain and loss-of-function approaches, we provide evidence that LPP3 regulates lysosphospholipid signaling responses in SMC. Our findings implicate both LPP3 and lysosphospholipids as pathophysiologically important regulators of SMC biology and may provide mechanistic insight into the association of an intronic single-nucleotide polymorphism in PPAP2B with CAD risk.

Methods
An expanded Materials and Methods section is available in the online-only Data Supplement data.

Mice
All procedures conformed to the recommendations of the Guide for the Care and Use of Laboratory Animals (Department of Health, Education, and Welfare publication number National Institutes of Health 78-23, 1996) and were approved by the Institutional Animal Care and Use Committee. The production and initial characterization of mice in which the Ppap2b gene encoding LPP3 was flanked by lox-P sites (Ppap2bfl/fl) has previously been described.32,33 Ppap2bfl/fl mice were backcrossed for >10 generations to the C57Bl/6 background and then crossed with C57Bl/6 mice expressing Cre recombinase under the control of the SM22 promoter3 to obtain congenic SM-Ppap2bfl/fl mice. The mice survived in the expected numbers. Mating of homozygous Ppap2bfl/fl with SM-Ppap2bfl/fl mice yielded 50% SM-Ppap2bfl/fl offspring with <2% mortality. Mice were housed in cages with HEPA-filtered air in rooms with 12-hour light cycles and fed Purina 5058 rodent chow ad libitum. Systolic blood pressure and heart rate were measured for 5 consecutive days noninvasively in conscious mice using the CODA blood pressure analysis tail cuff system (Kent Scientific Corporation, Torrington, CT) daily after training for 1 week. Mean intra-arterial pressure was measured by placement of a 1.4 Fr Millar catheter in the carotid artery of isoflurane-anesthetized mice.

Vascular Injury
The left common carotid artery was dissected and ligated near the carotid bifurcation. All animals recovered and showed no symptoms of a stroke. At various intervals after carotid surgery,35,36,37 5 mm of carotid proximal to the suture were removed and processed for RNA analysis by qualitative polymerase chain reaction or analysis of protein markers by immunoblotting. Neointimal formation along the length of the vessel was assessed at 4 weeks after surgery using computer assisted morphometry as previously described.38 Digital images were taken with a high-performance digital camera (resolution 3840 x 3072 pixels) attached to a Nikon 80i microscope with an X10 (NA=0.3) or X20 (NA=0.5) objective lens and analyzed with Metamorph software (Molecular Devices, Inc, Sunnyvale, CA).

Statistical Analysis
All results were expressed as mean±SD. In vitro studies were repeated a minimum of 3 times, and results were analyzed by Student t test or ANOVA. Statistical analysis was performed using Sigma-STAT software version 3.5 (Aspire Software, International, Ashburn, VA). A P<0.05 was considered significant.

Results
Upregulation of LPP3 in Neointimal Regions After Vascular Injury
As an initial approach to understanding the role of LPP3 in vascular disease, we profiled LPP3 expression in blood cells and in arteriosclerotic vessels before and after vascular injury. Polymorphonuclear cells, but not other leukocytes or platelets, express detectable levels of LPP3 (Figure I in the online-only Data Supplement). In carotid arteries, LPP3 expression was markedly upregulated in neointimal regions after ligation injury (Figure 1). LPP3 protein (Figure 1A) and mRNA levels (Figure 1B) in carotid arteries were higher most notable ≥14 days after injury. To localize LPP3 expression in injured vessels, immunostaining for LPP3 and the SMC α-actin marker was performed (Figure 1C and 1D). The findings indicated that LPP3 expression co-localized in areas that contain SMCs after arterial injury (Figure 1C, brown staining) and, in particular, in the neointimal region at 28 days after injury (Figure 1D, yellow overlap). These results suggest that SMC are at least one source of neointimal LPP3. Indeed, isolated, cultured SMC from murine aorta expresses LPP3 (Figure II in the online-only Data Supplement).

LPP3 Negatively Regulates LPA Signaling Responses in SMC
The LPP family of cell-surface-associated lipid phosphatases hydrolyze and inactivates LPA and the related lysosphospholipid S1P. Both bioactive lipids have been implicated as mediators of phenotypic modulation of vascular SMC responses in vivo and in vitro. The time course of upregulation of LPP3 expression in injured vessels is consistent with a role for the enzyme in downregulating lipid signaling, and thereby halting lysosphospholipid-mediated inflammation, proliferation, and migration. We used isolated SMC to test the hypothesis that LPP3 alters SMC responses by attenuating LPA-mediated signaling. LPP3 was overexpressed in SMC using a recombinant lentiviral vector (Figure IIA in the online-only Data Supplement). In comparison with control cells infected with green fluorescent protein-expressing virus, LPP3-overexpressing SMC cultured in serum (which contains μmol/L levels of LPA), displayed ~2-fold less growth after 6 days (P<0.05; Figure IIB in the online-only Data Supplement). The effect of LPP3 was specific in that overexpression of LPP3 had no effect on growth in response to platelet-derived growth factor. In cells cultured in serum, LPP3 overexpression reduced ERK activity by ~50% (Figure IIC and IID in the online-only Data Supplement) and Rho activity by ~75% (Figure IIE in the online-only Data Supplement). LPP3 also attenuated LPA-stimulated ERK activation (Figure IID in the online-only Data Supplement) and prevented migration of cells in response to LPA (Figure IIF in the online-only Data Supplement). These effects were not observed in cells overexpressing a catalytically inactive LPP3 mutant (Figure IID and IIF in the online-only Data Supplement), indicating that the lipid phosphatase activity of LPP3 is required to attenuate SMC responses.

Next, the consequences of LPP3 deficiency on LPA signaling in SMC were determined by generating mice lacking SMC LPP3. This was accomplished by crossing mice in which exon 3 and 4 of the Ppap2b locus (encoding LPP3) was flanked by loxP sites (Ppap2bfl/fl) with mice expressing Cre recombinase under the control of the SM22 promoter (SM22-Cre) to generate SMC-LPP3-deficient mice (SM-Ppap2bfl/fl). SM-Ppap2bfl/fl mice are viable and fertile with no obvious phenotypic abnormalities. Immunoblot analysis of aortic SMC confirmed the absence of upregulated LPP3.
of LPP3 in the aorta of SM-Ppap2b<sup>−/−</sup> mice (Figure IIIA in the online-only Data Supplement). In SMC from SM-Ppap2b<sup>−/−</sup> mice, LPA phosphatase activity associated with LPP3 was <10% of that observed in cells from Ppap2b<sup>+/+</sup> mice (Figure IIIB in the online-only Data Supplement), and levels of LPP3 identified in immunoprecipitates from cultured aortic SMC were also reduced (Figure IIIC in the online-only Data Supplement).

The absence of LPP3 in SMC promoted LPA-mediated ERK activation by increasing and prolonging ERK phosphorylation (Figure 2A). In comparison with Ppap2b<sup>+/+</sup> cells, SM-Ppap2b<sup>−/−</sup> cells demonstrated an ≈2-fold increase in LPA-induced ERK activation that persisted up to 60 minutes (P<0.001; Figure 2A left). Additionally, SM-Ppap2b<sup>−/−</sup> cells responded to lower levels of LPA, with an ≈3-fold increase in ERK activation at 10 minutes in response to 0.25 μmol/L LPA (P<0.001; Figure 2A right).

Expression of murine or human LPP3, but not a catalytically inactive LPP3 mutant, rescued the phenotype of SM-Ppap2b<sup>−/−</sup> cells by reducing phosphorylation of ERK in response to LPA (Figure 2B). Consistent with these observations, LPP3 deficiency in SMC resulted in a 1.9-fold increase in LPA-mediated proliferation (P<0.001; Figure 2C), which was not a result of a nonspecific enhancement of proliferation, because responses to platelet-derived growth factor were unaffected (not shown).

Rho activation in response to LPA was also enhanced in SM-Ppap2b<sup>−/−</sup> cells (P<0.001) and was corrected by overexpression of either murine or human LPP3, but not a catalytically inactive mutant (Figure 3A). In keeping with heightened ERK and Rho activation, SMC-lacking LPP3 demonstrated a 2.3-fold increase in migration toward either LPA or fetal bovine serum (P<0.001; Figure 3B). Migration of SM-Ppap2b<sup>−/−</sup> cells was reduced to levels observed in Ppap2b<sup>+/+</sup> cells by re-expression of either murine or human LPP3 (Figure 3C) and abolished by Rho kinase and ERK inhibitors (Figure 3D).

To exclude the possibility that the results obtained in the SM-Ppap2b<sup>−/−</sup> cells were a result of developmental abnormalities that altered SMC phenotype, engineered short hairpin RNAs were used to target Ppap2b expression and thereby reduce LPP3 expression in wild-type SMC (Figure IVA in the online-only Data Supplement). Decreased LPP3 expression with this approach significantly increased LPA-stimulated ERK activity 1.7-fold (P<0.001; Figure IVB in the online-only Data Supplement) and Rho activation 1.8-fold (P<0.001; Figure IVC in the online-only Data Supplement). Together, these results indicate that lowering LPP3 expression enhances the proliferation and migration of SMC, particularly under conditions where LPA is exogenously supplied, or when LPA is present in the serum component of the culture medium.

Direct measurements of intact cell LPA phosphatase activity demonstrated that exogenously applied LPA was degraded 3-fold more slowly by SM-Ppap2bΔ than control cells (Figure 4A). If the enhanced LPA-signaling of LPP3-deficient cells was a result of a reduction in LPA degradation, then the SM-Ppap2bΔ cells should respond normally to a poorly hydrolyzable receptor-active LPA mimetic, such as the ester-linked thiophosphate derivative (1-oleoyl-2-O-methyl-rac-glycerophosphothionate). In support of this idea, we found that 1-oleoyl-2-O-methyl-rac-glycerophosphothionate-stimulated ERK activation responses of Ppap2b<sup>−/−</sup> and SM-Ppap2bΔ cells were similar (Figure 4B).
Having established that LPP3 downregulates SMC responses to LPA, we next investigated whether the enzyme regulates SMC responses to S1P. In a proliferation assay, expression of murine LPP3, but not a catalytically inactive LPP3 mutant, reduced SMC proliferation elicited by S1P (Figure VA and VB in the online-only Data Supplement). In keeping with this observation, SM-\(Ppap2b\)\(^{-}\) cells displayed exaggerated responses to S1P (Supplemental Figure 5C) but not platelet-derived growth factor (Figure VD in the online-only Data Supplement). Together, these results indicate that LPP3 modulates SMC responses to the lysophospholipid mediators, LPA and S1P, likely by hydrolyzing and thereby inactivating their signaling capabilities.

Consequences of Genetic Deletion of SMC LPP3

Our in vitro analysis of LPP3 function in SMC is consistent with a model in which LPP3 attenuates lysophospholipid-triggered signaling responses. We and others have reported that LPA and S1P contribute to the development of intimal hyperplasia by stimulating SMC migration and vascular inflammation.\(^4,5,21\) Therefore, upregulation of LPP3 expression after vascular injury could serve as an intrinsic mechanism to limit neointimal formation. To investigate this possibility, we examined injury responses in the \(Ppap2b^{\text{fl/fl}}\) and SM-\(Ppap2b\)\(^{-}\) mice. Consistent with SMC serving as a major source of vessel-associated LPP3, SM-\(Ppap2b\)\(^{-}\) mice displayed attenuated upregulation of LPP3 after carotid ligation injury (Figure 5A). LPP3 expression in adventitial adipose cells was not affected by SM22-Cre expression (Figure 5A arrowheads). Dual staining for SMC \(\alpha\)-actin and LPP3 indicated colocalization in \(Ppap2b^{\text{fl/fl}}\) vessels after injury, and this colocalization was lacking in SM-\(Ppap2b\)\(^{-}\) mice (Figure 5B). In the absence of SMC-LPP3, a more extensive neointimal layer formed along the length of the carotid arteries at 4 weeks after injury, as compared with control mice (Figure 5A and 5C). Intima area (Figure 5C), intima/media ratio (Figure 5E), and lumen area (Figure 5F), but not medial area (Figure 5D), were all significantly greater in injured SM-\(Ppap2b\)\(^{-}\) arteries. The findings probably do not reflect subtle differences in genetic background because no differences were observed in injury responses among \(Ppap2b^{\text{h/h}},\) SM22-Cre, and C57BL/6 mice (Figure VI in the online-only Data Supplement).

To determine the molecular underpinnings of the enhanced injury response, we examined the consequences of LPP3 deficiency in more detail. After injury, ERK-activation in arterial tissue, as measured by the ratio of phospho-ERK (pERK)/total ERK (mean±SD) from 4 separate experiments with the value observed in the absence of LPA set as 1. Representative immunoblots are presented under the graphed data. B, Lentivirus-mediated expression of mouse (m) or human (h) LPP3 in SMC attenuated ERK activation in aortic SMC from SM-\(Ppap2b\)\(^{-}\) mice. C, Growth curves, measured by WST-1 cell proliferation assay, for SMC in the absence (vehicle) or presence of 1 \(\mu\)mol/L LPA. Results are presented as means±SD from 3 independent cultures of SMC of each genotype. *\(P<0.05\) by 1-way ANOVA. GFP indicates green fluorescent protein.
with persistent elevation at day 14 (Figure 6A and 6B and Figure VIIA in the online-only Data Supplement). Phospho-
histone H3 staining was also enhanced in injured vessels from SM-Ppap2bΔ mice (Figure VIIIB in the online-only Data Supplement). The stereotypic injury response involves dedifferentiation and redifferentiation of SMC, which can be monitored by an initial decrease followed by a later increase in SMC differentiation markers in the vessel wall. In uninjured SM-∆Ppap2b mice, mRNA levels were observed in aortas from SM-∆Ppap2b knockout mice (data not shown). Together, these observations document a role for SMC LPP3 in attenuating proliferative and inflammatory responses after vascular injury.

Blood pressure was similar in Ppap2bΔ and SM-Ppap2bΔ mice, although heart rates were significantly higher in SM-Ppap2bΔ mice (*P < 0.001; Table I in the online-only Data Supplement). The higher heart rates may reflect lower loss of LPP3 in SM-∆Ppap2b mice (data not shown). Together, these observations suggest that LPP3 expression after arterial injury. After ligation injury, a substantial portion of LPP3 expression seems to occur in SMC with a time course consistent with a role in influencing vascular injury responses. Genetic evidence indicates that LPP3 negatively regulates SMC phenotypic modulation both in vitro and in vivo. Loss of LPP3 in SMC enhances intimal hyperplasia, prolongs SMC dedifferentiation, and promotes vascular inflammation after arterial injury. Studies in isolated cells support a model in which a function of LPP3 is to attenuate localized production or availability of bioactive lipid mediators. Specifically, overexpression of LPP3 reduces SMC migration and proliferation in response to LPA or S1P, whereas attenuation or elimination of LPP3 amplifies SMC responses to LPA. Taken together, our observations suggest that LPP3 expression after vascular injury normally limits cellular responses to LPA and with persistent elevation at day 14 (Figure 6A and 6B and Figure VIIA in the online-only Data Supplement). Phospho-
histone H3 staining was also enhanced in injured vessels from SM-Ppap2bΔ mice (Figure VIIIB in the online-only Data Supplement). The stereotypic injury response involves dedifferentiation and redifferentiation of SMC, which can be monitored by an initial decrease followed by a later increase in SMC differentiation markers in the vessel wall. In uninjured SM-∆Ppap2b mice, mRNA levels were observed in aortas from SM-∆Ppap2b knockout mice (data not shown). Together, these observations document a role for SMC LPP3 in attenuating proliferative and inflammatory responses after vascular injury.

Blood pressure was similar in Ppap2bΔ and SM-Ppap2bΔ mice, although heart rates were significantly higher in SM-Ppap2bΔ mice (*P < 0.001; Table I in the online-only Data Supplement). The higher heart rates may reflect lower loss of LPP3 in SM-∆Ppap2b mice (data not shown). Together, these observations suggest that LPP3 expression after arterial injury. After ligation injury, a substantial portion of LPP3 expression seems to occur in SMC with a time course consistent with a role in influencing vascular injury responses. Genetic evidence indicates that LPP3 negatively regulates SMC phenotypic modulation both in vitro and in vivo. Loss of LPP3 in SMC enhances intimal hyperplasia, prolongs SMC dedifferentiation, and promotes vascular inflammation after arterial injury. Studies in isolated cells support a model in which a function of LPP3 is to attenuate localized production or availability of bioactive lipid mediators. Specifically, overexpression of LPP3 reduces SMC migration and proliferation in response to LPA or S1P, whereas attenuation or elimination of LPP3 amplifies SMC responses to LPA. Taken together, our observations suggest that LPP3 expression after vascular injury normally limits cellular responses to LPA and with persistent elevation at day 14 (Figure 6A and 6B and Figure VIIA in the online-only Data Supplement). Phospho-
histone H3 staining was also enhanced in injured vessels from SM-Ppap2bΔ mice (Figure VIIIB in the online-only Data Supplement). The stereotypic injury response involves dedifferentiation and redifferentiation of SMC, which can be monitored by an initial decrease followed by a later increase in SMC differentiation markers in the vessel wall. In uninjured SM-∆Ppap2b mice, mRNA levels were observed in aortas from SM-∆Ppap2b knockout mice (data not shown). Together, these observations document a role for SMC LPP3 in attenuating proliferative and inflammatory responses after vascular injury.

Blood pressure was similar in Ppap2bΔ and SM-Ppap2bΔ mice, although heart rates were significantly higher in SM-Ppap2bΔ mice (*P < 0.001; Table I in the online-only Data Supplement). The higher heart rates may reflect lower loss of LPP3 in SM-∆Ppap2b mice (data not shown). Together, these observations suggest that LPP3 expression after arterial injury. After ligation injury, a substantial portion of LPP3 expression seems to occur in SMC with a time course consistent with a role in influencing vascular injury responses. Genetic evidence indicates that LPP3 negatively regulates SMC phenotypic modulation both in vitro and in vivo. Loss of LPP3 in SMC enhances intimal hyperplasia, prolongs SMC dedifferentiation, and promotes vascular inflammation after arterial injury. Studies in isolated cells support a model in which a function of LPP3 is to attenuate localized production or availability of bioactive lipid mediators. Specifically, overexpression of LPP3 reduces SMC migration and proliferation in response to LPA or S1P, whereas attenuation or elimination of LPP3 amplifies SMC responses to LPA. Taken together, our observations suggest that LPP3 expression after vascular injury normally limits cellular responses to LPA and with persistent elevation at day 14 (Figure 6A and 6B and Figure VIIA in the online-only Data Supplement). Phospho-
histone H3 staining was also enhanced in injured vessels from SM-Ppap2bΔ mice (Figure VIIIB in the online-only Data Supplement). The stereotypic injury response involves dedifferentiation and redifferentiation of SMC, which can be monitored by an initial decrease followed by a later increase in SMC differentiation markers in the vessel wall. In uninjured SM-∆Ppap2b mice, mRNA levels were observed in aortas from SM-∆Ppap2b knockout mice (data not shown). Together, these observations document a role for SMC LPP3 in attenuating proliferative and inflammatory responses after vascular injury.

Blood pressure was similar in Ppap2bΔ and SM-Ppap2bΔ mice, although heart rates were significantly higher in SM-Ppap2bΔ mice (*P < 0.001; Table I in the online-only Data Supplement). The higher heart rates may reflect lower loss of LPP3 in SM-∆Ppap2b mice (data not shown). Together, these observations suggest that LPP3 expression after arterial injury. After ligation injury, a substantial portion of LPP3 expression seems to occur in SMC with a time course consistent with a role in influencing vascular injury responses. Genetic evidence indicates that LPP3 negatively regulates SMC phenotypic modulation both in vitro and in vivo. Loss of LPP3 in SMC enhances intimal hyperplasia, prolongs SMC dedifferentiation, and promotes vascular inflammation after arterial injury. Studies in isolated cells support a model in which a function of LPP3 is to attenuate localized production or availability of bioactive lipid mediators. Specifically, overexpression of LPP3 reduces SMC migration and proliferation in response to LPA or S1P, whereas attenuation or elimination of LPP3 amplifies SMC responses to LPA. Taken together, our observations suggest that LPP3 expression after vascular injury normally limits cellular responses to LPA and
S1P and that reduced expression of LPP3 enhances SMC phenotypic modulation and vascular inflammation, likely in part by increasing their signaling. Although lack of LPP3 clearly impairs LPA degradation and inactivation, additional non-LPA-dependent mechanisms of LPP3 action could also affect SMC phenotypic modulation. For example, LPP3 may have nonenzymatic functions mediated by integrin binding or catenin signaling.27,38,39

A role for LPP3 in human vascular disease was suggested by the recent demonstration that an intronic, common single-nucleotide polymorphism variant in PPAP2B, rs17114036, strongly associates with atherosclerotic CAD, independent of traditional risk factors, such as total-high-density lipoprotein and low-density lipoprotein cholesterol, diabetes mellitus, body mass index, hypertension, or smoking. In this study, we offer support for a possible mechanistic link between this new CAD locus, LPP3 expression, and vascular disease. Our findings support a model in which genetic variants associated with lower LPP3 expression would be associated with exacerbated vascular injury responses.
Emerging evidence supports a role for lysophospholipid mediators in regulation of vascular development and function. Our data add to the weight of the evidence that LPP3 regulates vascular cell function. Our findings provide functional evidence for a novel role for LPP3 and lipid signaling in adult vascular cells. If the enhanced inflammation and neointimal formation observed in mice lacking LPP3 in SMC reflects processes that occur in the development of atherosclerosis in humans, then our results may provide a mechanism by which alterations in LPP levels (or activity) could alter risk for CAD and MI in humans.

Heart, particularly atrial tissue, robustly expresses LPP3. SM22-Cre–mediated deletion of the floxed Ppap2b allele lowers LPP3 levels in heart, presumably because of recombination in cardiac tissue. This is associated with lower heart rates, which may indicate a role for LPP3 in regulating heart rate and function. Infusion of LPA in rabbits increases ventricular arrhythmia and the proportion of non-phosphorylated connexin 43, which may inhibit junction transmission. Whether LPP3 regulates any of these responses is not known.

In summary, LPP3 expression in arterial tissue, and SMC in particular, increases after arterial injury. Enhanced LPP3 expression attenuates SMC proliferatory and migratory responses and may attenuate vascular inflammation. Mice lacking SMC-LPP display enhanced vascular inflammatory responses and development of intimal hyperplasia after injury. We propose that these observations reflect a role for LPP3 in attenuating local signaling by lysophospholipid mediators. Our observations may suggest novel strategies to attenuate vascular inflammation and suggest that a better understanding of genetic factors and environmental stimuli that alter LPP3 levels is warranted.

Acknowledgments
We thank University of Kentucky laboratory assistants Kelsey Johnson, for animal husbandry, and Adrienne Nguyen, for assistance with histology.

Sources of Funding
This work was supported by grants HL078663, HL0870166, and HL074219 from the National Institutes of Health (NIH; to S.S.S.); GM050388 and 1P20RR021954 (to A.J.M.), a Beginning Grant-in-Aid (0950118G), and scientist development grant (10SDG4190036), from the American Heart Association; and UL1RR033173 from NIH NCRR (to M.P.), a predoctoral fellowship grant from the American Heart Association (to A.K.S.) and NIH T32HL072743 (to P.M.). A portion of this work was presented in Arteriosclerosis Thrombosis and Vascular Biology early career investigator award form at the American Heart Association Scientific Sessions in 2011. This material is the result of work supported with the resources and use of the facilities at the Lexington, KY VA Medical Center.

Disclosures
None.
References


Lipid Phosphate Phosphatase 3 Negatively Regulates Smooth Muscle Cell Phenotypic Modulation to Limit Intimal Hyperplasia
Manikandan Panchatcharam, Sumitra Miriyala, Abdelghaffar Salous, Jessica Wheeler, Anping Dong, Paul Mueller, Manjula Sunkara, Diana Escalante-Alcalde, Andrew J. Morris and Susan S. Smyth

Arterioscler Thromb Vasc Biol. published online October 25, 2012;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2012/10/25/ATVBAHA.112.300527

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2012/10/25/ATVBAHA.112.300527.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
SUPPLEMENT MATERIAL

METHODS

Isolation of SMC

Mouse aortic SMCs were obtained from thoracic aortas by removing the adventitia and endothelium by digestion with collagenase type II (Worthington; 175 U/mL). The media were further digested in solution containing collagenase type II (175 U/mL) and elastase (Sigma; 0.5 mg/mL), which yielded \( \approx 100,000 \) cells per aorta. Cells were grown in DMEM containing 0.5 ng/mL EGF, 5 \( \mu \)g/mL insulin, 2 ng/mL basic fibroblast growth factor, 10% FBS, 100 U/mL penicillin, and 100 \( \mu \)g/mL streptomycin and incubated at 37°C with 5% CO2/95% air. Purity of these cultures was confirmed by immunoreactivity for \( \alpha \)-SM actin (Sigma) in >99% of the cells. Experiments involving SMC were performed by using cells with a passage number 5.

LPP3 Expression

Human and mouse LPP3 cDNAs were obtained from the IMAGE consortium clone collection and the ORF amplified by PCR using a reverse primer that removed the stop codon. The PCR product was subcloned into pENTR D/TOPO (Invitrogen) and then used to generate a vector for expression of LPP3 with a C-terminal GFP tag by recombination with pcDNA DEST47 (Invitrogen). A lentivirus expression vector was made by in vitro recombination with pLenti6.2 nV5 DEST (Invitrogen). Recombinant lentiviruses were obtained by transfection of HEK293FT cells with this vector and appropriate helper plasmids.

RNA Isolation and Quantitative PCR

Total RNA was extracted from carotid arteries and primary SMC using the RNeasy mini kit (Qiagen, Chatsworth, CA) following manufacturer’s instructions. cDNA was prepared with Multiscribe reverse-trancriptase enzyme (High-Capacity cDNA Archive Kit; Applied Biosystems, Foster City, CA), and mRNA expression was measured in a RT-PCR reaction using TaqMan® gene expression assays and TaqMan® Universal PCR Master Mix No Amp Erase® (Applied Biosystems) in an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Threshold cycles (CT) were determined by an in-program algorithm assigning a fluorescence baseline based on readings prior to exponential amplification. An embryo RNA standard was used as a positive control. Fold change in expression was calculated using the \( 2^{-\Delta\Delta CT} \) method using 18s RNA as an endogenous control.

SMC Proliferation

SMC were exposed to indicated concentrations of LPA (1-oleoyl-2-hydroxy-sn-glycero-3-phosphate) dispersed in fatty acid-free BSA or vehicle for the indicated times and then washed Panchatcharam et al. Vascular Smooth Muscle Regulation by LPP3 with ice cold PBS and lysed (10 mM Tris-HCl pH 7.2, 1% Nonidet P-40, 158 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM PMSF, 10 \( \mu \)g/ml aprotinin, 10 \( \mu \)g/ml leupeptin, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate). Total protein in clarified cell lysates (16,000g x 10 min) was determined (BCA protein assay; Pierce) and equal amounts loaded onto 10% SDS-PAGE. Immunoblotting was
performed with antibodies to actin (Santa Cruz Biotechnology) and ERK/phospho-ERK Thr202/Tyr204 (Abcam, Cambridge, MA) and quantified using the Odyssey infrared imaging system (LI-COR). Equal sample loading was confirmed by Coomassie blue staining.

SMC Migration

After serum starvation in DMEM with 0.1% FBS, SMCs (1.8 × 10^4 cells/well) were placed into the upper well of a chamber of a multiwell chambers (Neuroprobe Inc., Gaithersburg, MD) with apolyvinylpyrrolidone-free polycarbonate filter (5 μm pore). The bottom chamber was filled with DMEM with 0.1% FBS and the indicated chemotactic agent (1 μM LPA, 10% FBS, 20ng/ml PDGF) or vehicle. The chamber was incubated for 12-h at 37°C in a CO2 incubator, at which time the filter was removed from the chamber and non-migrated cells were scraped from the upper surface. The membranes were fixed, and the migrated cells stained with Diff-Quik® (VWR Scientific Products). Digital images of the membranes were obtained with a Nikon 80i microscope using a 20x objective (NA = 0.5). The total area (μm²) occupied by migrated cells was determined with Metamorph imaging software.

Rho Activity

Rho activity was measured by incubating SMC lysates with GST-rhotekin Rho binding domain fusion protein immobilized to glutathione-beads. Proteins were separated on a 15% SDS-PAGE and immunoblotted with an anti-Rho antibody (Upstate). Results are expressed as a ratio of active to total Rho in the lysate.

Histology

Immunohistochemistry was performed on frozen sections using CD68 (1:200, Serotec, Raleigh, NC), CD8 (1:50, Serotec) and pERK (1:100, Abcam, Cambridge, MA). Briefly, the carotid artery were directly embedded in OCT and frozen at −20°C. Blocks were cut into 10 μm sections and fixed with chilled acetone in −20°C. To block endogenous peroxidases, slides were immersed in 1% H₂O₂ in methanol for two minutes at 40°C. Non-specific sites were blocked using 1.5% serum from the secondary antibody-derived animal for 15 minutes at 40°C. Slides were then incubated with primary antibodies for 15 minutes at 40°C, then for 15 minutes with biotinylated secondary antibody, and then for 10 minutes with ABC detector (Vector Labs) at 40°C. Hypersensitive response and pathogenicity substrate-chromogen (Biomed) was used as the chromogen, and hematoxylin (Accurate Chemical & Scientific Corp., Westbury, NY) was used for counter-staining. Frozen slides were fixed with chilled acetone in −20°C. Non-specific sites were blocked using 1.5% serum from the secondary antibody derived animal for 20 minutes at RT. Slides were then incubated with primary antibody at RT for one hour and a rhodamine-conjugated secondary antibody (15 μg/ml, Jackson ImmunoResearch, West Grove, PA) at RT for 30 minutes. Slides were viewed and images were taken using a Leica TCS SP5 laser scanning inverted confocal microscope. For each antibody, isotope-matched non reactive IgG served as the negative control.

Quantitation of LPA molecular species by HPLC tandem mass spectrometry.
Plasma was prepared by centrifugation of EDTA-anticoagulated blood, extracted, and separated by reverse phase HPLC as previously described. Sixteen abundant LPA molecular species quantitated using an ABI 4000 Q-Trap hybrid linear ion trap triple quadrupole mass spectrometer. Recovery was determined with C17-LPC as an internal standard and quantitation accomplished by reference to calibration curves determined using a series of synthetic LPAs that were independently quantitated by phosphorous analysis following wet digestion in perchloric acid.

Literature cited.
Supplemental Figure I. LPP3 expression in blood cells. (A) LPA phosphatase activity was measured in plasma and isolated blood cells. (B) Immunoblot analysis of LPP3 in lysates containing 40 mg total protein from the indicated cells. M/L = monocyte / lymphocyte fraction. Results are presented as mean ± SD from 3 independent experiment. #P < 0.001 by t-test
Supplemental Figure II. Lentiviral mediated overexpression of LPP3 attenuates SMC proliferative and migratory responses. (A) SMC were infected with lentivirus expressing murine (m) LPP3, and LPP3 levels measured by immunoblot analysis. Lentivirus overexpressing GFP were used as control. (B) Effects of mLPP3 overexpression on SMC growth in the presence or absence of PDGF (20 ng/ml) were measured by cell counting (C) ERK activation in SMC in serum after infection with lentivirus overexpressing mLPP3. (D) ERK activation in SMC infected with lentivirus expressing mLPP3, human(h) LPP3, or a catalytic inactive LPP3 variant. Serum starved cells were expose to 1 μM LPA for 10 min. (E) Rho activity in SMC in serum. (F) SMC migration in response to 1 μM LPA. AU, arbitrary units. SC, surface coverage. Results are presented as mean SD from 3 independent cultures of SMC. #P < 0.001 by t-test. *P < 0.05 as compared to corresponding control by one-way ANOVA.
Supplemental Figure III. Generation of mice lacking SMC LPP3. (A) Immunoblot analysis of aortas demonstrate the presence of LPP3 in \( Ppap2b^{fl/fl} \) but not SM-\( Ppap2b^\Delta \) mice. (B) Monoacyl glycerol (MAG) generation from exogenously added LPA was measured in anti-LPP3 IgG immunoprecipitates from SMC isolated from \( Ppap2b^{fl/fl} \) (dark bar) and SM-\( Ppap2b^\Delta \) (open bar) aortas. (C) Immunoblot analysis of LPP3 in control IgG or anti-LPP3 IgG immunoprecipitates from SMC isolated from \( Ppap2b^{fl/fl} \) and SM-\( Ppap2b^\Delta \) aorta. Results are presented as mean ± SD from 3 independent experiment. #P < 0.001 by t-test.
Supplemental Figure IV. RNAi mediated downregulation of LPP3 levels regulates SMC responses. (A) SMC were infected with lentivirus expressing human (h) LPP3 or RNAi constructs (LPP3MIR48 and LPP3MIR49) targeting mouse LPP3. Non-silencing refers to a control construct. LPP3 expression was determined by immunoblot analysis. SF9 insect cell membranes expressing LPP3 were used as a positive control. (B) ERK activation in SMCs was measured 10 min after vehicle or LPA exposure in cells infected with lentivirus expressing the indicated constructs. (C) Rho activation in SMCs in serum was measured in cells infected with lentivirus expressing the indicated constructs. Lysate was incubated with GTPγS (γS) as a positive control. AU indicates arbitrary units. Results are presented as mean ± SD from 3 independent cultures of SMC. *P < 0.05 as compared to corresponding control by one-way ANOVA.
Supplemental Figure V. Effect of LPP3 on S1P-mediated SMC proliferation. (A) *Ppap2b*^fl/fl^ SMC were infected with lentivirus expressing mouse (m) LPP3 or catalytically inactive variant of the enzyme prior to performing the WST-1 cell proliferation assay, in the absence (vehicle) of presence of 1 µM S1P. Lentivirus overexpressing GFP were used as control. (B) SM-*Ppap2b*^Δ^ SMC were infected with lentivirus expressing mouse (m) LPP3 or catalytically inactive variant of the enzyme prior to performing the WST-1 cell proliferation assay, in the absence (vehicle) of presence of 1 µM S1P. Lentivirus overexpressing GFP were used as control (C) Growth curves for SMC in the absence of presence of 1 µM S1P. (D) *Ppap2b*^fl/fl^ and SM-*Ppap2b*^Δ^ SMC growth in the presence or absence of PDGF (20 ng/ml) were measured by WST-1 cell proliferation assay. Results are presented as mean SD from 4 independent cultures of SMC.  \#P < 0.001 by t-test, as compared S1P control.
Supplemental Figure VI. Intimal hyperplasia in strains of mice. Intimal area (A), medial area (B), intima/media ratio (C) and luminal area (D) in µm² along the length of vessels in Ppap2b<sup>fl/fl</sup> (n=7), SM22-Cre (n=3) and C57/B6 (n=4) mice. (E) Uninjured medial area along the length of vessels from Ppap2b<sup>fl/fl</sup> and SM-Ppap2b<sup>Δ</sup> mice (n = 6) as mean ± SD in µm². No statistically significant differences were observed.
**Supplemental Figure VII. LPP3 attenuates inflammation and SMC proliferation following vascular injury.**

(A) ERK activity was measured at the indicated times following arterial injury by measuring phospho-ERK (pERK) and total ERK in vessels isolated from Ppap2b^{fl/fl} and SM-Ppap2b^{Δ} mice. (B) Representative confocal sections of carotid arteries 4 weeks after surgery taken ≈1.2mm from the site of ligation in Ppap2b^{fl/fl} and SM-Ppap2b^{Δ} mice. Sections were immunostained with antibodies to phospho-histone H3 and SM-α-actin. Bar denotes 25µm. (C) Representative sections of carotid arteries from Ppap2b^{fl/fl} and SM-Ppap2b^{Δ} mice after surgery. Sections were stained with antibodies to lymphocytes (CD8) at the indicated times. Bar denotes 25µm.
Supplemental Table I. Blood pressure and heart rate in $Ppap2b^{fl/fl}$ and SM-$Ppap2b^\Delta$ mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>SBP (mm Hg)</th>
<th>Heart Rate (bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Ppap2b^{fl/fl}$</td>
<td>19</td>
<td>96±9</td>
<td>600±17</td>
</tr>
<tr>
<td>SM-$Ppap2b^\Delta$</td>
<td>19</td>
<td>92±7</td>
<td>642±21</td>
</tr>
</tbody>
</table>

Values for systolic blood pressure (SBP) and heart rate are presented as means ± SD. There was not a statistically significant difference in blood pressure ($P = 0.135$) with a significant difference in the heart rate ($P = <0.001$).
Supplemental Figure VIII. LPP3 expression in heart. qPCR was used to measure expression of \textit{Ppap2b} in heart apex (A) and atrium (B) from \textit{Ppap2b}^{fl/fl} (closed bars) and SM-\textit{Ppap2b}\textsuperscript{Δ} (open bars) mice. 18s was used as an internal control. (C) Immunoblot analysis of LPP3 expression in heart apex. GAPDH was used as a loading control. LPP3 expression was normalized to GAPDH staining (n = 3 animals per time point) and graphed as mean ± SD in arbitrary units in which the density of LPP3 in \textit{Ppap2b}^{fl/fl} samples were set to 1. # P < 0.001 by t-test.