Role of cAMP-Phosphodiesterase 1C Signaling in Regulating Growth Factor Receptor Stability, Vascular Smooth Muscle Cell Growth, Migration, and Neointimal Hyperplasia

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ABSTRACT

**Rationale:** Neointimal hyperplasia characterized by abnormal accumulation of vascular smooth muscle cells (SMCs) is a hallmark of occlusive disorders such as atherosclerosis, post-angioplasty restenosis, vein graft stenosis, and allograft vasculopathy. Cyclic nucleotides are vital in SMC proliferation and migration, which are regulated by cyclic nucleotide phosphodiesterases (PDEs).

**Objective:** Our goal is to understand the regulation and function of PDEs in SMC pathogenesis of vascular diseases.

**Methods and Results:** We performed screening for genes differentially expressed in normal contractile versus proliferating synthetic SMCs. We observed that PDE1C expression was low in contractile SMCs but drastically elevated in synthetic SMCs in vitro and in various mouse vascular injury models in vivo. Additionally, PDE1C was highly induced in neointimal SMCs of human coronary arteries. More importantly, injury-induced neointimal formation was significantly attenuated by PDE1C deficiency or PDE1 inhibition in vivo. PDE1 inhibition suppressed vascular remodeling of human saphenous vein explants ex vivo. In cultured SMCs, PDE1C deficiency or PDE1 inhibition attenuated SMC proliferation and migration. Mechanistic studies revealed that PDE1C plays a critical role in regulating the stability of growth factor receptors, such as PDGF-receptor-beta (PDGFRβ) known to be important in pathological vascular remodeling. PDE1C interacts with LDL-receptor-related-protein-1 (LRP1) and PDGFRβ, thus regulating PDGFRβ endocytosis and lysosome-dependent degradation in an LRP1-dependent manner. A transmembrane-adenylyl-cyclase (tmAC)-cAMP-PKA cascade modulated by PDE1C is critical in regulating PDGFRβ degradation.

**Conclusions:** These findings demonstrated that PDE1C is an important regulator of SMC proliferation, migration, and neointimal hyperplasia, in part through modulating endosome/lysosome dependent PDGFRβ protein degradation via LRP1.

**Keywords:** Cyclic nucleotide, phosphodiesterase inhibitor, smooth muscle cells, neointimal hyperplasia.
Nonstandard Abbreviations and Acronyms:

DES       drug-eluting stent
ECM       extracellular matrix
Epac      exchange protein activated by cAMP
GPCR      G-protein coupled receptor
FRET      fluorescent resonance energy transfer G-protein coupled receptor
IP        immunoprecipitation
LCA       left carotid artery
LDL       low-density lipoprotein
LRP1      LDL receptor-related protein 1
tmAC      transmembrane adenylyl cyclase
NO        nitric oxide
PDE       cyclic nucleotide phosphodiesterase
PDGFR     platelet-derived growth factor receptor
PGI2      prostacyclin
SMC       smooth muscle cell
PKA       cAMP dependent protein kinase
PKG       cGMP-dependent protein kinase
PKI       PKA inhibitor
RCA       right carotid artery
RTK       receptor tyrosine kinases
sGC       soluble guanylate cyclase
SM-MHC    smooth muscle myosin heavy chain
V-ATPase  vacuolar-type H (+)-ATPase.
INTRODUCTION

Intimal hyperplasia and luminal stenosis are the key characteristics of a number of different vascular disorders, such as atherosclerosis, post-angioplasty restenosis, vein graft stenosis, and allograft vasculopathy.\(^1\) Under normal conditions, SMCs residing in the media of vessels are quiescent with a very low turnover rate and insignificant secretory activity. These SMCs are highly differentiated cells that possess a contractile phenotype by expressing large amounts of contractile proteins, and principally function to maintain vascular tone. However, SMCs also retain a degree of plasticity to allow phenotypic modulation. For example, during vascular injury, SMCs undergo profound metamorphosis changing from a quiescent/contractile phenotype to an active/synthetic phenotype.\(^4\) Synthetic SMCs down-regulate contractile proteins and up-regulate growth factors, growth factor receptors, extracellular matrix (ECM) components, ECM proteins and inflammatory mediators.\(^5\)\( ,\)\(^6\) Inhibiting intimal SMC-like cell proliferation has been used as a therapeutic strategy to antagonize pathologic vascular remodeling. To prevent restenosis after percutaneous coronary intervention, the most effective therapy is local delivery of anti-proliferative reagents via drug-eluting stents (DES), containing drugs such as sirolimus\(^7\) and paclitaxel.\(^8\) These drugs inhibit cell proliferation by targeting mTOR or microtubule formation. However, these drugs also attenuate re-endothelization, and can lead to increased in-stent thrombosis.\(^9\) In addition, although DES is effective for focal lesions, DES cannot treat vascular disorders with diffuse neointimal lesions. Thus, developing novel and systemically safe drugs is currently in high demand.

Endothelial dysfunction or damage triggers underlying SMC phenotype transition and pathological vascular wall remodeling. Prostacyclin (PGI\(_2\)) and nitric oxide (NO), two major factors released from the healthy endothelium, stimulate the production of cAMP and cGMP, respectively, in adjacent SMCs. Cyclic AMP and cGMP have a variety of biological effects in vascular SMCs, such as promoting SMC relaxation and inhibiting SMC proliferation, migration, and ECM synthesis.\(^10\)\( ,\)\(^11\) Cyclic nucleotide phosphodiesterases (PDEs), by catalyzing the hydrolysis of cAMP and cGMP to 5’AMP and 5’GMP, regulate the amplitude, duration, and compartmentalization of intracellular cyclic nucleotide signaling. Dysregulation of PDE expression/activation have been implicated in a number of diseases.\(^12\)\( ,\)\(^13\) To date, more than 60 different PDE isoenzymes derived from 22 genes have been identified and grouped into 11 broad families (PDE1-PDE11) based on distinct kinetic, regulatory, and inhibitory properties.\(^14\) PDEs are expressed in a cell/tissue-specific manner and only a few enzymes are expressed in any single cell type. Importantly, different PDE isoforms serve to control distinct cyclic nucleotide signaling and fulfill distinct functions. Over the past decades, PDEs have been proven to be ideal and feasible drug targets, as exemplified by drugs such as sildenafil, milrinone, cilostazol, and roflumilast. Thus, selectively targeting individual PDE isoforms may represent a feasible and appealing strategy for modulating specific cyclic nucleotide pools without affecting global intracellular cyclic nucleotides.

To understand the specific cyclic nucleotide signaling pathway responsible for the synthetic SMC phenotype, we performed initial discovery screening for PDE isozymes that are differentially expressed in synthetic SMCs compared to contractile SMCs. We found that the PDE1C isozyme is markedly upregulated in synthetic SMCs. Consistent with in vitro findings, PDE1C is nearly undetectable in medial SMCs of normal arteries and veins in vivo, but is markedly induced in synthetic SMC-like cells of vascular lesions from various animal injury models and human disease vessels. PDE1C belongs to the calcium/calmodulin (Ca\(^{2+}\)/CaM)-stimulated PDE family comprising 3 gene products: PDE1A, 1B, and 1C.\(^15\) In the vasculature, PDE1A activity is primarily associated with SMCs but not endothelial cells,\(^16\)\(^ ,\)\(^17\) suggesting a specific role of PDE1C in synthetic SMCs. We also used in vitro and in vivo approaches to prove that PDE1C plays a causative role in synthetic SMC proliferation/migration and neointimal hyperplasia. Furthermore, we identified the molecular mechanism by which PDE1C enhances the protein stability of growth factor receptors via attenuating endosome/lysosome-mediated degradation. Our data suggest that PDE1C may represent a novel therapeutic target for treating cardiovascular diseases associated with SMC hyperplasia.
METHODS
Animal care and use was in accordance with institutional guidelines. The global PDE1C knockout mice were kindly provided by Haiqing Zhao (Johns Hopkins University) and backcrossed to C57BL/6 mice for at least 9 generations. FVB/NJ mice were obtained from Jackson Laboratories. Carotid artery intima/media thickening was induced in vivo by blood flow cessation through complete ligation of the left common carotid artery for two weeks.18 For the in vivo animal study with PDE1 inhibitor (IC86340), the compound was applied perivascularly through pluronic gel. Human saphenous veins were collected from discarded unused portions in coronary artery bypass surgeries, and were cultured in vitro for 7 days in the presence of vehicle or IC86340.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

RESULTS
PDE1C is highly expressed in synthetic/proliferative SMCs in vitro.

To identify the PDE isoforms differentially expressed in synthetic versus contractile SMCs, we performed preliminary discovery screening among all PDE genes in contractile SMCs (freshly isolated medial layers) and synthetic SMCs (cultured SMCs) via qRT-PCR. We found that PDE1C expression is selectively associated with synthetic SMCs (data not shown). To confirm this finding, we analyzed PDE1C expression in isolated contractile and synthetic SMCs from rat aortas (Fig. 1A), human aortas (Fig. 1B), and human saphenous veins (Fig. 1C). As expected, SMC markers such as smooth muscle myosin heavy chain (SM-MHC) and calponin are drastically decreased in synthetic SMCs compared to contractile medial SMCs (Fig. 1A-C, middle and right panels), providing validation of a phenotype change. Importantly, we observed a marked increase of PDE1C in all three types of growing synthetic SMCs compared to corresponding contractile SMCs from different species and different vascular beds (Fig. 1A-C, left panels). Consistently, PDE1C protein levels were also increased in synthetic SMCs (Supplemental Fig. I). Our results corroborate previous findings demonstrating that PDE1C was highly expressed in human aortic proliferating SMCs but is not detected in quiescent SMCs isolated from the tunica media.19, 20

To further demonstrate the phenotype-dependent expression of PDE1C, we used in vitro models of SMC phenotype modulation via differentiation- and growth-medium as previously described.21 We found that PDE1C was downregulated about 80% when cells were grown in the differentiation-medium compared to growth-medium (Fig. 1D, left panel). Concurrent phenotype modulation was verified by a drastic increase in the SMC contractile marker protein SM-MHC and calponin in the differentiation-medium (Fig. 1D, middle and right panel). This observation is consistent with the previous finding that PDE1C was significantly down-regulated in SMCs cultured on dishes coated with fibrillar/polymer type I collagen (exhibiting a contractile-like phenotype) compared to SMCs cultured on non-coated plastic dishes (exhibiting a synthetic phenotype).20

PDE1C is highly induced in SMC-like cells in rodent and human disease vessels.

To demonstrate the induction of PDE1C in vivo, we first analyzed PDE1C via Immunohistochemical staining in neointimal lesions from different mouse injury models, including carotid artery ligation, femoral artery wire injury, and vein bypass grafting (Fig. 2A-C). The adjacent sections were immunostained with anti-PDE1C and anti-smooth muscle alpha actin (SM-α-actin, a SMC marker) (Fig. 2A-C, left panels). Carotid artery intima thickening was induced by complete ligation of left
carotid arteries for two weeks and contralateral right carotid arteries were used as control vessels. As shown in Figure 2A, PDE1C expression was very low or almost undetectable in the normal control right carotid artery (inset), but was significantly increased in the neointimal and medial areas of the ligated left carotid artery (middle and right panels). We also examined PDE1C expression in a mouse model of femoral artery with wire injury.\(^{22}\) As shown in Figure 2B, PDE1C staining was significantly increased in the neointimal and medial areas of the injured left femoral artery (middle and right panels) compared to the non-injured right femoral artery (inset). Consistently, PDE1C mRNA was also quantitatively higher in injured compared to uninjured femoral arteries (Supplemental Figure IIA). Moreover, we examined PDE1C in venous neointimal lesions from a mouse model of vein bypass graft with vena cava-to-carotid artery isografting.\(^{23}\) As shown in Figure 2C, we observed a marked induction of PDE1C expression in the graft neointimal lesions (middle and right panels) compared to non-grafted veins (insets). The majority of neointimal cells are stained with SM-\(\alpha\)-actin and PDE1C, suggesting that PDE1C is induced in the SMC-like cells during vascular pathologic states. The immunofluorescent double-staining of PDE1C and SM-\(\alpha\)-actin further indicates that PDE1C is highly induced in SMC-like cells in diseased mouse carotid artery (Supplemental Fig. IIB).

To support the results obtained from murine injury models, cross-sections from human coronary arteries with neointimal lesions were immunofluorescently double-stained with PDE1C and SM-\(\alpha\)-actin. We observed that PDE1C expression was low in medial SMCs, but was highly elevated in the neointimal cells and largely overlapped with SM-\(\alpha\)-actin positive cells (Fig. 2D). These in vivo observations are in line with the findings from cultured SMCs in vitro (Fig. 1). Taken together, our results suggest that PDE1C expression is associated with synthetic SMCs.

**PDE1C ablation attenuates neointimal hyperplasia following vascular injury.**

To determine the causative role of PDE1C in neointimal hyperplasia and pathological vascular remodeling, we used global PDE1C knockout (PDE1C\(^{-/-}\)) mice with backcrossing to C57BL/6 mice for at least 9 generations. This knockout line has normal growth rates and feeding patterns, as well as normal nursing and mating behaviors.\(^{24}\) We examined the effects of PDE1C depletion on carotid remodeling induced by complete carotid artery ligation, a procedure known to induce intimal hyperplasia caused by blood flow cessation.\(^{18,25}\) As shown in Figure 3A, there is no obvious change in the appearance of unligated right carotid artery (RCA) between PDE1C\(^{+/+}\) and PDE1C\(^{-/-}\) mice. Ligation of the left carotid artery (LCA) for 2 weeks developed more significant vascular wall thickening in PDE1C\(^{+/+}\) mice compared to PDE1C\(^{-/-}\) mice. Morphometric analyses revealed that the ligation injury caused a marked increase in neointimal and medial thickening in PDE1C\(^{+/+}\) mice (Fig. 3B). However, these changes were largely attenuated in PDE1C\(^{-/-}\) mice. Because SMC proliferation contributes to neointima hyperplasia, we conducted immunostaining of Ki67 (a marker of cellular proliferation). SMCs were counterstained with SM-\(\alpha\)-actin. There was a significant increase in the number of Ki67-positive SMCs in ligated LCA compared to control RCA in PDE1C\(^{+/+}\) mice, which was significantly decreased in PDE1C\(^{-/-}\) mice (Supplemental Fig. IIIA). Because reactive oxidative stress (ROS) plays a crucial role in the development of vascular diseases, we measured lipid oxidation by immunostaining of 4-Hydroxy-2-Nonenal (4-HNE). We showed that 4-HNE staining intensity per area is significantly decreased in the media-intima of carotid arteries from PDE1C\(^{-/-}\) mice (Supplemental Figure IIIB).

In addition, we tested the effect of a pan-PDE1 inhibitor IC86340 on carotid artery remodeling in FVB mice. IC86340 inhibits all PDE1 isozymes although it is more potent for PDE1C inhibition. IC86340 or vehicle was applied perivascularly through pluronic gel to carotid arteries.\(^{26}\) As shown in Figure 3C, ligation of the artery induced a drastic neointima formation in FVB mice, which was significantly reduced by application of IC86340. The moderate change of media thickness was independent of IC86340. Ki67-positive SMC numbers or 4-HNE staining intensity were also reduced by
IC86340 in ligated LCA compared to vehicle (Supplemental Fig. IIIC and D). These data indicate that PDE1C plays a critical role in neointimal hyperplasia in response to flow-induced vascular injury.

**PDE1 inhibition attenuates human saphenous vein remodeling ex vivo.**

To determine the role of PDE1 in human vascular remodeling, we utilized human saphenous vein (HSV) samples. HSV is used for bypassing stenotic coronary arteries, but late vein graft failure occurs due to remodeling of the vessel wall and the development of stenosis.\(^{27,28}\) When cultured ex vivo, HSV spontaneously undergoes remodeling, which predominantly involves SMC growth, migration, and extracellular matrix.\(^{29}\) As shown in Figure 3D and E, after HSV were cultured ex vivo for 7 days, the thickness of intimal, medial and adventitial layers was markedly increased compared to the same vessels without culture. PDE1 inhibitor IC86340 significantly reduced HSV remodeling in all three layers. This suggests an important role for PDE1 in human vascular remodeling disorders.

**PDE1C deficiency or PDE1 inhibitor antagonizes SMC proliferation and migration.**

Proliferation and migration of SMCs are critical steps in neointimal formation after vascular injury. To examine the role of PDE1C in SMC proliferation, we performed SRB assay (a well-established colorimetric cell viability and proliferation assay) in primary cultured mouse SMCs isolated from PDE1C\(^{+/+}\) and PDE1C\(^{-/-}\) mice. As shown in Figure 4A and B, under serum starvation (SF), the rate of cell growth was similar in PDE1C\(^{+/+}\) and PDE1C\(^{-/-}\) cells. Serum stimulation (10% FBS) or platelet derived growth factor BB (PDGF-BB) markedly increased cell growth in PDE1C\(^{+/+}\) cells, which is reduced more than 70% in PDE1C\(^{-/-}\) cells. We also examined the effect of IC86340 on rat aortic SMCs growth and found that IC86340 significantly reduced PDGF-BB-stimulated SMC growth (Fig. 4C). These observations indicate that blocking PDE1C function attenuates SMC proliferation.

We next examined the effects of PDE1C ablation or inhibition on SMC migration by a modified Boyden chamber assay (Fig. 4D-F). We found that PDGF-BB markedly increased the migration of PDE1C\(^{-/-}\) cells (Fig. 4D-E). However, the migratory capacity was almost completely diminished in PDE1C\(^{-/-}\) SMCs. Similar observations were obtained in cells treated with IC86340 (Fig. 4F). To further explore the role of PDE1C in SMC migration, we performed an ex vivo 3D-collagen gel migration assay with mouse aortic medial explants. SMC migration was assessed by the migration distances of cells from explants. As shown in Figure 4G and H, when mouse aortic medial explants from PDE1C\(^{-/-}\) mice were cultured in a 3D-collagen matrix for 10 days, SMCs migrated out from aortic explants. In contrast, SMC migration was significantly suppressed in aortic explants from PDE1C\(^{-/-}\) mice. These data indicate that PDE1C is critical for SMC migration.

To determine whether PDE1C induction is important in SMC phenotype transition, we measured contractile SMC markers in low passage mouse aortic SMCs isolated from PDE1C\(^{+/+}\) and PDE1C\(^{-/-}\) mice. As shown in the supplemental Figure IVA, SM-MHC, calponin, and SM-\(\alpha\)-actin mRNA levels were significantly higher in PDE1C\(^{-/-}\) than PDE1C\(^{+/+}\) cells. Consistently, SM-MHC and SM-\(\alpha\)-actin protein detected by immunostaining were also elevated in PDE1C\(^{-/-}\) cells (Supplemental Fig. IVB). These observations suggest that PDE1C induction facilitates SMC phenotype transition.

**PDE1C regulates PDGF\(\beta\) protein levels.**

Synthetic SMCs acquire the capacity to proliferate and migrate according to growth factor receptor expression. PDGF signaling, particularly through PDGF receptor beta (PDGF\(\beta\)), plays crucial roles in SMC proliferation, migration, and neointimal formation following vascular injury.\(^{30-32}\) Therefore, we first examined the role of PDE1C in regulating PDGF\(\beta\) in SMCs. Interestingly, we found that
IC86340 dose-dependently reduced PDGFRβ protein levels in rat SMCs (Fig. 5A). IC86340 inhibits both PDE1A and PDE1C isoforms in SMCs. In order to identify the specific PDE1 isoform involved in IC86340-induced reduction of PDGFRβ protein expression, we used adenoviruses expressing PDE1A shRNA or PDE1C shRNA. When high dose adenoviruses were transfected into cells, PDE1A shRNA and PDE1C shRNA specifically decreased PDE1A and PDE1C expression levels by 60-70%, respectively (Supplemental Fig. VA). As shown in Figure 5B, PDE1C knockdown by its shRNA, but not PDE1A, reduced PDGFRβ protein similar to IC86340, suggesting that the effect of IC86340 on PDGFRβ is primarily through inhibition of PDE1C. To determine whether PDE1C regulates PDGFRβ gene expression, we analyzed PDGFRβ mRNA levels. In an unexpected finding, IC86340 or PDE1C shRNA did not alter PDGFRβ mRNA levels (Supplemental Fig. VB and C). Instead, IC86340 reduced both endogenous PDGFRβ and exogenously expressed Flag-PDGFRβ protein levels (Supplemental Fig. VD and F). This further supports the role of PDE1C in PDGFRβ protein regulation. In addition to PDGFRβ, IC86340 or PDE1C shRNA also reduced the protein levels of PDGFR alpha (PDGFRα) and EGF receptor (Supplemental Fig. VD and E) but did not change their mRNA levels (Supplemental Fig. VB and C). Together, these results suggest that PDE1C likely regulates multiple growth factor receptors. Due to the robustness of the response, the remainder of investigations focused on PDGFRβ.

To further determine the PDGF-mediated cellular signaling response, we pretreated cells with IC86340 for 24 hours to down-regulate PDGFR proteins. After washing out IC86340, cells were acutely stimulated with PDGF-BB for 5 and 30 min. We found that PDGF-BB-mediated Erk1/2 and Akt activation was significantly attenuated in IC86340-pretreated cells (Supplemental Fig. VG). We have previously found that treating SMCs with IC86340 up to 30 min did not affect PDGF-BB stimulated Erk1/2 and Akt (data not shown), suggesting that PDE1C does not directly regulate PDGFR activation.

**PDE1C modulates a tmAC-cAMP-PKA signaling critical for regulating PDGFRβ protein level.**

PDE1C is able to hydrolyze both cAMP and cGMP with high affinity in vitro. The most common cAMP and cGMP effector molecules include cAMP dependent protein kinase (PKA), exchange protein activated by cAMP (Epac), and cGMP-dependent protein kinase (PKG). We, therefore, examined the role of cAMP/PKA, cAMP/Epac, and cGMP/PKG in PDE1C-mediated regulation of PDGFRβ protein expression. First, we tested forskolin, a transmembrane adenylate cyclase (tmAC) activator that activates tmAC to produce cAMP. We found that 10 µmol/L forskolin or low dose IC86340 (5 µmol/L) alone had minimal effect on PDGFRβ protein expression (Fig. 5C). However, forskolin and IC86340 (5 µmol/L) together elicited a synergistic effect on reducing PDGFRβ protein (Fig. 5C). At a low dose, PDE1C shRNA alone had little appreciable effect on PDGFRβ protein expression, but forskolin combined with a low dose of PDE1C shRNA also showed a synergistic PDGFRβ protein reduction (Fig. 5D). An inhibitor of tmAC, 2,5-ddA, abrogated the enhanced effects of forskolin/IC86340 or forskolin/PDE1C shRNA on PDGFRβ protein reduction (Supplemental Fig. VIA and B). These results suggest that PDE1C is coupled to a tmAC/cAMP signaling, which is important for the regulation of PDGFRβ protein levels.

To examine whether PKA was involved in IC86340-induced PDGFRβ protein reduction, two different PKA inhibitors H89 and PKI (14-22) were utilized. As shown in Figure 5E and F, both H89 and PKI (14-22) largely suppressed IC86340-induced PDGFRβ protein reduction. Consistently, H89 also blocked the enhanced effects of forskolin/IC86340 or forskolin/PDE1C shRNA on PDGFRβ protein reduction (Supplemental Fig. VIC and D). Additionally, we examined the role of Epac I using Epac I siRNA. As shown in supplemental Figure VIIA, knocking down Epac I expression by 90% did not affect IC86340-induced PDGFRβ protein reduction, suggesting that Epac I does not play a major role in this pathway. Therefore, these results suggest that PDE1C regulates a tmAC/cAMP/PKA signaling pathway that mediates a reduction of PDGFRβ protein.
We next examined the role of cGMP/PKG pathway in regulating PDGFβ protein. We found that knocking down PKG1 via its siRNA did not alter IC86340-induced PDGFβ protein reduction (Supplemental Fig. VIIB). Moreover, elevating intracellular cGMP concentration by SNAP (a nitrite oxide donor), YC-1 (a soluble guanylyl cyclase activator), or CNP (a membrane guanylyl cyclase activator) did not enhance the effect of IC86340 on PDGFβ protein reduction (Supplemental Fig. VIIC-E). These results suggest that cGMP/PKG pathway is unlikely to be involved in PDE1C regulation of PDGFβ protein.

**PDE1C regulates lysosome-dependent PDGFβ protein degradation.**

The fact that inhibiting PDE1C depletion/inhibition decreases both endogenous and exogenous PDGFβ protein but not mRNA prompted us to hypothesize that PDE1C regulates PDGFβ degradation. Both proteasome and lysosomes have been implicated in PDGFβ degradation. Therefore, we first tested the proteasome inhibitor MG132 and found that inhibiting proteasome function did not block the effect of IC86340 on PDGFβ reduction (Data not shown). This suggests that IC86340-induced PDGFβ protein reduction is not mediated by proteasome degradation.

We next tested the role of lysosomes using vacuolar-type H(+)-ATPase (V-ATPase) inhibitor bafilomycin A1 and lysosome pH neutralizer NH4Cl to inhibit lysosomal function. We found that both bafilomycin A1 and NH4Cl significantly blocked IC86340 and PDE1C shRNA-induced PDGFβ protein reduction (Fig. 6A and B). Furthermore, we observed that bafilomycin A1 and NH4Cl also blocked the enhanced effect of forskolin/IC86340 on PDGFβ protein degradation (Supplemental Fig. VIIF). These results suggest that PDE1C-regulated PDGFβ protein degradation occurs through a lysosome-dependent mechanism.

**PDE1C regulates endosome-mediated internalization of PDGFβ.**

It is well known that the internalized receptors within endosomes are either recycled to the plasma membrane or trafficked to late endosome/lysosome for degradation. Therefore, we determined the role of endocytosis in IC86340-induced PDGFβ protein degradation through inhibiting the function of dynamin, a protein that is essential for clathrin-dependent coated vesicle formation and receptor endocytosis. As shown in Figure 7A, IC86340-induced PDGFβ protein reduction was almost completely abolished by dynasore (a cell-permeable inhibitor of dynamin) in SMCs. We then further examined the effect of IC86340 on PDGFβ internalization by first labeling SMC surface proteins with biotin and then detecting intracellular biotin-labeled PDGFβ through streptavidin-immunoprecipitation. As shown in Figure 7B, the levels of internalized PDGFβ were increased ≈3 fold by IC86340 compared to vehicle control. We also examined whether IC86340 induced endosome localization of PDGFβ through double immunostaining of PDGFβ and EEA-1, an early endosome marker protein. As shown in Figure 7C-E, there was a substantial amount of PDGFβ localized on the plasma membrane in the absence of IC86340. However treatment with IC86340 caused a significant reduction of PDGFβ protein on the membrane, and increase of PDGFβ co-localization with EEA1. Moreover, we found that the effect of IC86340 on suppressing PDGF-BB-induced cell proliferation is largely abolished in the presence of dynasore, a dynamin inhibitor for blocking the endocytic pathway (Fig. 7F). Together, these results together suggest that PDE1C inhibition promotes PDGFβ endocytosis and degradation, subsequently attenuating SMC growth.
**PDE1C associates with PDGFRβ.**

Based on the facts that PDE1C couples to tmAC/cAMP and PDE1C regulates membrane PDGFRβ internalization, we hypothesized that PDE1C could be located on the plasma membrane and associated with PDGFRβ. To prove this hypothesis, we first performed immunofluorescent double-staining of PDE1C and PDGFRβ. As shown in Figure 8A, in actively growing SMCs, PDE1C was largely detected in perinuclear areas and the cell membrane. PDE1C is co-localized with PDGFRβ, particularly on the cell membrane. To further confirm the co-localization of PDE1C and PDGFRβ, we exogenously expressed EGFP-PDE1C and Flag-tagged PDGFRβ in SMCs via electroporation. Consistently, EGFP-PDE1C was expressed in both plasma membrane and perinuclear areas, similar to endogenous PDE1C (Fig. 8B). Flag-PDGFRβ and GFP-PDE1C was also co-localized on the cell membrane (Fig. 8B).

In addition, we examined if EGFP-PDE1C and Flag-PDGFRβ is able to be co-immunoprecipitated (Fig. 8C and D). We found that in the cells expressing EGFP-PDE1C and Flag-PDGFRβ (Lane 1), immunoprecipitation (IP) of PDGFRβ using an anti-Flag antibody pulled down EGFP-PDE1C (Fig. 8C) and IP of PDE1C using an anti-EGFP antibody pulled down Flag-PDGFRβ (Fig. 8D). However, we failed to detect the interaction between Flag-PDGFRβ and EGFP (Lane 2), or EGFP-PDE1C and Flag-LacZ (Lane 3). These results strongly suggest an association between PDGFRβ and PDE1C.

Moreover, we examined PDE1C and PDGFRβ expression and localization in neointimal lesions in vivo (Supplemental Fig. VIII). Similar to PDE1C, PDGFRβ staining was very low in the normal mouse carotid artery (Fig. S8A, top panels) as well as the medial layer of human coronary arteries (Fig. S8B). In contrast, PDGFRβ staining was markedly increased in the injured mouse carotid arteries (Fig. S8A, bottom panels) and human intimal lesions (Fig. VIIIB). Most importantly, PDGFRβ and PDE1C staining was highly co-localized in intimal SMCs, supporting the association of PDE1C with PDGFRβ in vivo.

**LRP1 is critical for PDE1-mediated regulation of PDGFRβ protein.**

The LDL receptor-related protein 1 (LRP1) is a large endocytic receptor that modulates the endocytosis and trafficking of a number of membrane receptors and extracellular macromolecules. It has been shown that LRP1 forms a complex with PDGFRβ, which alters PDGFRβ subcellular trafficking. PKA-dependent phosphorylation of LRP1 has been shown to be critical for LRP1-mediated endocytosis. Therefore, we examined the relationship between PDE1C and LRP1 and the role of LRP1 in PDE1 inhibition-induced PDGFβ reduction. Interestingly, immunofluorescent double-staining revealed that LRP1 was co-localized with PDE1C and PDGFRβ in cell membrane as well as in some intracellular structures (Fig. 8E and F). In addition, LRP1 and PDE1C or LRP1 and PDGFRβ co-expression and co-localization were found in mouse or human neointimal lesions in vivo (Supplemental Fig. IX). Moreover, siRNA mediated depletion of LRP1 largely blocked the effect of IC86340 on PDGFRβ reduction (Fig. 8G). IC86340 significantly increased LRP1 phosphorylation detected by the phospho-antibody recognizing PKA substrates, which was attenuated upon PKA inhibition by the PKI peptide treatment (Fig. 8H). These observations indicate that LRP1 is required for PDE1-mediated regulation of PDGFRβ protein, likely via PKA-dependent phosphorylation of LRP1.
DISCUSSION

Experimental evidence has strongly supported the conclusion that PDE1C is a synthetic SMC specific enzyme. Previous studies by Rybalkin et al showed that PDE1C was highly expressed in proliferating human aortic SMCs but not detectable in quiescent human aortas.\textsuperscript{19,20} In the current study, we more comprehensively demonstrated that PDE1C is specifically expressed in synthetic SMCs from multiple species and different vascular beds, not only under in vitro culture conditions but also in vivo in vascular lesions from a number of different small animal injury models as well as in diseased human vessels. More importantly, we provided in vitro, ex vivo, and in vivo evidence demonstrating that PDE1C is critical for SMC growth, migration, and neointima formation. Furthermore, we defined a novel mechanism by which PDE1C negatively regulates PDGFR\textsubscript{\(\beta\)} endocytosis and degradation in an LRP1-dependent manner. PDGF signaling has multiple actions in SMCs, including phenotypic modulation, cell proliferation, migration, and ECM metabolism, all of which contribute to pathological vascular remodeling.\textsuperscript{30-32} PDE1C, through modulating PDGF signaling, is thus capable of serving as an important multifunctional regulator in synthetic SMCs. As shown in the proposed model (Fig. 8I), our experimental evidence suggests that a tmAC-derived cAMP-PKA signaling is critical in promoting PDGFR internalization and endocytosis. PDE1C upregulation antagonizes the tm-AC-cAMP-PKA signaling and thus suppresses PDGFR\textsubscript{\(\beta\)} degradation, which facilitates SMC phenotype modulation and accelerates SMC growth/migration. PKA-dependent phosphorylation of LRP1 might be important in PDE1C-cAMP regulation of PDGFR\textsubscript{\(\beta\)} protein degradation. Taken together, these experimental results strongly implicate that the induction of PDE1C in SMCs is responsible for the pathogenesis of synthetic function, contributing to vascular hyperplasia. PDEs have been proven to be worth targets for drug development. Thus our findings may have great therapeutic impact as it may lead to the development of novel therapeutic strategies using PDE1 inhibitors (ideally PDE1C-selective inhibitors) in treating a number of vascular hyperplastic disorders. Currently, a pan PDE1 inhibitor is under development for treating schizophrenia through targeting the PDE1B isozyme in the brain.\textsuperscript{44}

Receptor tyrosine kinases (RTKs) are subjected to endocytosis. Internalized RTKs have a number of different fates: sustained signaling within early endosomes, being recycled to the plasma membrane, or trafficked to late endosomes/lysosomes for degradation.\textsuperscript{37,45} Receptor endocytosis and subsequent lysosome degradation is one of the important mechanisms to prevent sustained RTK activation on the plasma membrane as well as in early endocytic vesicles.\textsuperscript{46,47} Deregulation of the endocytic pathway and impairment of the degradation system have been found in cell transformation and tumorgenesis.\textsuperscript{45-47} Therefore, it is believed that targeting RTK endocytosis and degradation may represent a promising perspective in cancer therapy.\textsuperscript{37,48} In this study, we identified PDE1C as a novel regulator of PDGFR\textsubscript{\(\beta\)} endocytosis/degradation in vascular SMCs. The role of PDE1C may not be only restricted to PDGFR\textsubscript{\(\beta\)} because we found that PDE1C also regulates other RTKs, such as PDGFR\textsubscript{\(\alpha\)} and EGFR (Fig. Supplemental Fig. V). A previous study showed that in SMCs, human cytomegalovirus decreased both PDGFR\textsubscript{\(\alpha\)} and PDGFR\textsubscript{\(\beta\)} protein, accompanied by increased localization of these receptor proteins in endosomes and lysosomes.\textsuperscript{49} PDE1C is also important for the growth of human malignant melanoma cell lines.\textsuperscript{50} Therefore, our findings in SMCs may also be applicable to tumor cells and suggest that PDE1C inhibition may represent a novel strategy to target RTK degradation in vascular diseases as well as cancer therapy.

LRP1 is a multifunctional scavenger and signaling receptor. It plays diverse roles in a variety of biological processes, including lipoprotein metabolism, clearance of plasma proteins, protease degradation, as well as receptor trafficking and signaling.\textsuperscript{38,39} The finding from our current study suggests that LRP1 is important in PDE1C/cAMP-mediated regulation of PDGFR\textsubscript{\(\beta\)} stability and availability. Previous studies have also shown that LRP1 depletion in SMCs resulted in elevated PDGFR\textsubscript{\(\beta\)} level and activation, increased SMC proliferation and migration, and accelerated atherosclerosis and aortic

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aneurysm in SMC-specific LRP1 knockout mice. Blockade of PDGFR signaling with imatinib (a tyrosine kinase inhibitor) prevented atherosclerosis progression in LRP1 knockout mice. These lines of experimental evidence strongly suggest an important role of LRP1 in negatively modulating PDGF signaling and SMC pathogenesis in vascular diseases. The molecular mechanism by which cAMP regulates LRP1-mediated PDGFRβ endocytosis/degradation has not been fully characterized. It has been shown that LRP1 interacts with stimulatory heterotrimeric G-protein (Gsα) that leads to cAMP production and PKA activation. LRP1-mediated endocytosis of urokinase-type plasminogen activator receptor (uPAR) is regulated by PKA. In addition, PKA-dependent phosphorylation of Serine 76 of LRP1 cytoplasmic tail is critical in receptor endocytosis. These observations suggest a potential role of cAMP/PKA in directly modulating LRP1 function, likely through PKA phosphorylation of LRP1. In the current study, we have shown that an mtAC-PDE1C controlled cAMP/PKA signaling regulates LRP1 phosphorylation and subsequent PDGFRβ endocytosis and degradation. Future studies are necessary to determine the specific phosphorylation site and the role of LRP1 phosphorylation in PDE1C-mediated regulation of PDGFRβ endocytosis and SMC proliferation/migration.

It has long been believed that vascular medial SMCs change from quiescent/contractile to active/synthetic phenotype, thereby contributing to neointimal hyperplasia. However, there is also evidence supporting the possible transdifferentiation of adventitial fibroblasts, the differentiation of progenitor cells/stem cells, or endothelial-to-mesenchymal transition to these SMC-like positive, synthetic SMC-like cells in the neointimal lesions. Thus PDE1C-positive cells in neointimal lesions might have multiple origins. Regardless of the origins, synthetic SMC-like cells are able to proliferate, migrate, and secrete ECM proteases and proteins. In addition, they produce pro-inflammatory molecules, providing an inflammatory microenvironment for leukocyte penetration, accumulation and activation. Therefore, developing novel strategies, impeding the phenotype transition from the contractile to synthetic state will be of great interest. Thus, PDE1C may represent a novel therapeutic target in combating SMC phenotype modulation under disease states. Besides those SMC-like cells, inflammatory cells also contribute to neointimal hyperplasia. We failed to detect PDE1C in mouse peritoneal macrophages and found that PDE1C deficiency does not alter LPS-stimulated cytokine expression in macrophages nor circulating inflammatory molecule levels in mice (our unpublished observations). This is also consistent with the previous findings that the PDE1B isozyme represents the major PDE1 activity in macrophages. Together, these observations suggest that PDE1C does not regulate macrophage function and systemic inflammation in murine animals. Nevertheless, the specific contribution of SMC-origin PDE1C needs to be further determined using SMC-specific PDE1C knockout mice in the future.

**SOURCES OF FUNDING**

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**DISCLOSURE**

None.
REFERENCES


FIGURE LEGENDS

Figure 1. PDE1C expression is drastically upregulated in synthetic SMCs. qRT-PCR results showing mRNA levels of PDE1C, SM-MHC and calponin mRNA in contractile SMCs (freshly isolated medial layers) and corresponding synthetic SMCs (cultured SMCs) from rat aortas (A), human aortas (B), and human saphenous veins (C). Contractile SMCs are freshly isolated medial tissues procured by removing endothelial cells and peeling off adventitial layers. Synthetic SMCs are cultured growing SMCs isolated from the corresponding vessel with the explant method. (D) Cultured rat aortic SMCs were in differentiation medium (medium 231 supplemented with Smooth Muscle Differentiation Supplement (SMDS), Cascade Biologics) or growth medium (medium 231 supplemented with Smooth Muscle Growth S (SMGS), from Cascade Biologics) for 2 days. SM-MHC and calponin are used as contractile SMC markers. Values are mean ± SD of at least four repeats. *P < 0.05.

Figure 2. PDE1C expression is markedly induced in injured vessels of different mouse models as well as human disease vessels. (A-C) Representative immunohistochemistry images showing SM-α-actin and PDE1C staining in injured and uninjured control (inset) vessels from different mouse injury models, including (A) left carotid artery with partial ligation for 14 days in FVB mice or right carotid artery without ligation, (B) left femoral artery with wire injury for 28 days in C57BL/6 mice or right femoral artery without injury, and (C) vena cava subjected to isografting, wherein vena cava was obtained from a donor C57BL/6 mouse and grafted between two ends of the right common carotid artery in a recipient C57BL/6 mouse for 4 weeks. Red: SM-α-actin, Brown: PDE1C, blue: nuclear counterstaining with Hematoxylin. (D) Representative immunofluorescent double-staining images of human coronary artery with neointimal lesions. Red: SM-α-actin, Green: PDE1C, Blue: nuclei stained with DAPI.

Figure 3. PDE1C deficiency or PDE1 inhibition attenuates pathological vascular remodeling. (A-B) Effect of PDE1C deficiency on carotid artery intima/media thickening. A, representative Verhoeff-van Gieson (VVG) staining images of carotid artery cross-sections from PDE1C+/+ and PDE1C-/- mice subjected to left common carotid artery ligation for 14 days. The right common carotid arteries were used as unligated controls. Bar = 50 µm. Blue-black indicates elastic fibers. The middle yellowish part (indicated by asterik) is the blood clot. B, quantitative data of morphometric analyses of neointimal and medial areas measured using ImagePro software. N, neointima; M, media; A: adventitia. Values are mean ± SEM (n=7 for each group). *P < 0.05. (C) Quantitative morphometric data showing the effect of IC86340 on carotid artery intima/media thickening. Values are mean ± SEM (n=8 for each group). (D-E) Effects of PDE1 inhibitor IC86340 on human saphenous vein remodeling. D, Representative images of HSV sections with immunostaining for SM-α-actin (brown). E, quantification of intimal, medial, and adventitial thickness. HSV explants were either no culture or subjected to ex vivo culture for 7 days in the presence of vehicle or IC86340 (30 µM). Bar = 50 µm. Values are mean ± SEM (n=6). I: intima; M: media; A: adventitia. *P < 0.05.

Figure 4. PDE1C deficiency or PDE1 inhibition reduces SMC proliferation and migration in vitro and/or ex vivo. (A-B) PDE1C deficiency inhibited SMC proliferation measured by SRB assay. Mouse SMCs isolated from thoracic aortas of PDE1C+/+ and PDE1C-/- mice were subjected to serum starvation (in serum free medium, SF), followed by the same or addition of 10% FBS stimulation (A) or 50 ng/ml PDGF-BB (B) for 2 days. (C) PDE1 inhibitor IC86340 reduced SMC proliferation. Rat aortic SMCs were subjected to serum starvation, followed by with or without 50 ng/ml PDGF-BB in the presence of vehicle or 15 µmol/L IC86340. (D-E) PDE1C deficiency inhibited SMC migration measured by modified Boyden chamber assay. D, representative images showing transmigrated cells from PDE1C+/+ and PDE1C-/- mice. E, quantitative data of SMC migration. SMC from PDE1C+/+ and PDE1C-/- SMC mice was placed in the upper microchemotaxis chamber, and 25 ng/ml of PDGF-BB was added in the lower polycarbonate filter chamber. The transmigrated cells on the filter membrane were fixed, stained with hematoxylin, photographed, and quantified. (F) PDE1 inhibitor IC86340 reduced SMC migration. Rat...
aortic SMCs were pretreated with 30 µmol/L IC86340 for 24 h, and then subjected to the Boyden chamber assay. Values are mean ± SD (n=3). *P < 0.05. (G and H) PDE1C deficiency impaired smooth muscle explant outgrowth. G, representative images showing outgrowth of SMCs of aortic medial explants from PDE1C+/+ and PDE1C−/− mice in 3D collagen I gel ex vivo. H, quantitative data of migration distance (the distance between the leading front SMCs and the explant tissue). Values are mean ± SD (n=3). *P < 0.05.

Figure 5. PDE1C regulates PDGFRβ protein levels via a cAMP-PKA dependent mechanism. (A) PDE1 inhibitor IC86340 dose-dependently decreased PDGFRβ protein levels. Rat aortic SMCs were treated with indicated doses of IC86340 for 24 h. (B) Knockdown of PDE1C but not PDE1A decreased PDGFRβ protein. Rat aortic SMCs were transfected with adenoviral vectors expressing a high dose of adenovirus encoding LacZ-shRNA, PDE1A-shRNA, or PDE1C-shRNA for 3 days. (C) Forskolin enhanced the effect of IC86340 on PDGFRβ protein reduction. Rat aortic SMCs were treated with a low dose of IC86340 (5 µmol/L) with or without 10 µmol/L forskolin for 24 h in DMEM containing 0.1% FBS. (D) Forskolin augmented the effect of PDE1C knockdown on PDGFRβ protein reduction. Rat aortic SMCs were transfected with a low dose of adenovirus encoding LacZ-shRNA, PDE1A-shRNA, or PDE1C-shRNA for 3 days, followed with or without 10 µmol/L forskolin treatment for 24 h in DMEM containing 0.1% FBS. (E) PKA inhibitor H89 blocked IC86340-induced PDGFRβ protein reduction. Rat aortic SMCs were treated with a high dose of IC86340 (15 µmol/L) with or without of 5 µmol/L H89 for 24 h in DMEM containing 0.1% FBS. (F) Specific PKA inhibitor PKI (14-22) blocked the effect of IC86340 on PDGFRβ protein reduction. Rat aortic SMCs were treated with a high dose of IC86340 (15 µmol/L) with or without 5 µmol/L PKI (14-22) for 24 h in DMEM containing 0.1% FBS. PDGFRβ protein levels and β-actin equal loading were analyzed by immunoblotting with the anti-PDGFRβ and β-actin antibody, respectively. The blots were analyzed by densitometry. Fold changes normalized to the left lane. Values are mean ± SD (n=3). *p<0.05.

Figure 6. Role of lysosomes in PDE1C-mediated regulation PDGFRβ protein degradation. (A) Lysosome inhibitors blocked the effect of IC86340 on PDGFRβ protein reduction. Rat aortic SMCs were pretreated with lysosome inhibitor NH₄Cl (20 mmol/L) or Bafilomycine A (50 nmol/mL) for 0.5 h, followed by treatment with 15 µmol/L IC86340 for additional 24 h in DMEM containing 0.1% FBS. (B) Lysosome inhibitors blocked the effect of PDE1C knockdown on PDGFRβ protein reduction. Rat aortic SMCs were transfected with a high dose of adenovirus encoding LacZ-shRNA, PDE1A-shRNA, or PDE1C-shRNA for 3 days, and treated with 20 mmol/L NH₄Cl or 50 nmol/L Bafilomycine A for 24 h in DMEM containing 0.1% FBS. Protein levels of PDGFRβ and β-actin equal loading were determined by immunoblotting and analyzed by densitometry. Fold changes normalized to the left lane. Values are mean ± SD (n=3). *p<0.05.

Figure 7. PDE1 inhibitor IC86340 induces PDGFRβ protein degradation via endocytosis. (A) Dynamin inhibitor dynasore blocked the effect of IC86340 on PDGFRβ protein reduction. Rat aortic SMCs were treated with 15 µmol/L IC86340 with or without 2.5 µmol/L dynasore for 24 h in DMEM containing 0.1% FBS. (B) IC86340 induced PDGFRβ internalization. Rat aortic SMC membrane proteins were labeled with biotin and stimulated with 15 µmol/L IC86340 for 12 h in DMEM containing 0.1% FBS. Cell lysates were immunoprecipitated with streptavidin beads, and the recovered internalized biotinylated PDGFRβ were immunoblotted with PDGFRβ antibody. (C-E) IC86340 increased PDGFRβ co-localization with endosomes. Rat aortic SMCs were treated with 15 µmol/L IC86340 for 12 h in DMEM containing 0.1% FBS. PDGFRβ and early endosome marker EEA-1 were determined by immunostaining. C, representative images showing the localization of PDGFRβ in plasma membrane and endosomes. Selected boxes were enlarged for better view. D, quantitative data of PDGFRβ in plasma membrane. E, quantitative data of PDGFRβ in endosomes. (F) Dynasore abrogated the inhibitory effect
of IC86340 on SMC growth. Rat aortic SMCs were treated with IC86340, dynasore or a combination of IC86340 and dynasore, and stimulated with 50 ng/ml PDGF-BB for 48 h. Cell proliferation was measured by SRB assay. Values are mean ± SD of (n=3). *P < 0.05; ns: no significant difference.

**Figure 8.** PDE1C is associated with PDGFRβ. (A) Representative images showing endogenous PDE1C and PDGFRβ co-localized on cytoplasmic membrane. Rat aortic SMCs were immunostained with anti-PDE1C and anti-PDGFRβ antibodies. (B) Representative images showing exogenous PDE1C and PDGFRβ co-localization on plasma membrane. Rat aortic SMCs were co-transfected with Flag-PDGFRβ and EGFP-PDE1C by electroporation, and exogenous Flag-PDGFRβ and EGFP-PDE1C were detected by immunostaining with anti-flag and anti-GFP antibodies, respectively. Cells were visualized using confocal microscopy. (C-D) Co-immunoprecipitation (IP) revealed that PDE1C and PDGFRβ associate together. HEK293A cells were co-transfected with Flag-PDGFRβ and EGFP-PDE1C (lane 1), Flag-PDGFRβ and EGFP (lane 2), or Flag-LacZ and EGFP-PDE1C (lane 3), C, IP with anti-Flag antibody and IB with anti-EGFP antibody. D, IP with anti-EGFP antibody and IB with anti-Flag antibody. The expression of Flag-tagged and EGFP proteins in total cell lysates were immunoblotted with anti-Flag or anti-EGFP antibody, respectively. (E-F) Representative images showing the co-localization of PDE1C and LRP1 (E) or PDGFRβ and LRP1 (F) by immunofluorescent staining. Rat aortic SMCs were immunostained with anti-PDE1C or anti-PDGFRβ together with anti-LRP1 antibodies. (G) Knockdown of LRP-1 attenuated the effect of IC86340 on PDGFR reduction. Rat aortic SMCs were transfected with 50 nmol/L control siRNA (ConsiRNA) or LRP1 siRNA (LRP1siRNA) for 2 days, followed by treatment with 15 µmol/L IC86340 in DMEM supplied with 0.1% FBS for 24 h. The protein levels were determined by western blot. (H) PDE1 inhibitor IC86340 stimulates LRP1 phosphorylation in a PKA-dependent manner. Rat aortic SMCs were treated with vehicle or 15 µmol/L IC86340 for 12 h in the presence of vehicle or 5 µmol/L PKI (14-22) peptide. The phosphorylation of LRP1 was detected by immunoblotting with a phospho-PKA substrate antibody after immunoprecipitation with LRP1 antibody. Values are mean ± SD of (n=3-4). *P < 0.05; ns: no significant difference. (I) Proposed model: a tmAC-derived cAMP-PKA signaling is critical in promoting PDGFRβ internalization and endocytosis in an LRP1-dependent manner.
NOVELTY AND SIGNIFICANCE

What Is Known?

- Abnormal accumulation of vascular smooth muscle cell (SMC) and luminal stenosis are key characteristics of several vascular disorders, including atherosclerosis, post-angioplasty restenosis, vein graft stenosis, and allograft vasculopathy.

- Cyclic nucleotides (cAMP and cGMP) regulate a variety of biological functions of vascular SMCs, such as promoting SMC relaxation and inhibiting SMC proliferation, migration, and ECM synthesis.

- Phosphodiesterases regulate the levels of cyclic nucleotides and dysregulated PDE expression/activity has been implicated in various diseases; however the specific PDE isozymes that responsible for the SMC pathogenesis have not been identified.

What New Information Does This Article Contribute?

- PDE1C is specifically expressed in growing SMCs from multiple species and different vascular beds, not only under in vitro culture conditions but also in vivo in vascular lesions in small animal injury models as well as in diseased human vessels.

- PDE1C is critical for SMC growth, migration, and vascular stenosis, using both genetic and pharmacological approaches.

- PDE1C attenuates cAMP/protein kinase A (PKA)-mediated phosphorylation of LDL-receptor-related-protein-1 (LRP1) and subsequently enhances the protein stability of growth factor receptors, such as PDGF-receptor-beta (PDGFRβ), via negatively regulating endosome/lysosome-mediated degradation.

Inhibiting SMC hyperplasia is a common therapeutic strategy to limit proliferative vascular disorders. The current therapy for treating in-stent restenosis is focused on inhibiting SMC proliferation and inducing SMC death using drug-eluting stents, but these drugs also limit endothelial cell growth and thus increase the risk of fatal thrombosis. Moreover, drug-eluting stents effective for focal lesions cannot be used for vascular disorders with diffuse lesions. Thus, identifying novel molecular targets is important for the development of novel therapeutic strategies. We found that PDE1C isozyme, unexpressed in normal vessels, is highly induced in growing SMCs in cultured SMCs in vitro and in vascular lesions in vivo. Using genetic depletion and pharmacological inhibition, we show that PDE1C plays a causative role in SMC proliferation and migration and intimal lesion formation. These results identify a novel mechanism by which PDE1C promotes SMC growth/migration by increasing the protein stability of growth factor receptors (such as PDGFRβ) by attenuating receptor endocytosis/degradation. These findings could aid the development of novel therapeutic strategies using PDE1 inhibitors (ideally PDE1C-selective inhibitors) for treating vascular hyperplasic disorders.
Figure 2

A  SM-α-actin  PDE1C  PDE1C  
Ligation

B  Wire injury

C  Vein graft

D  SM-α-actin  PDE1C

DAPI  SM-α-actin/PDE1C
Figure 3

A

Unligated

PDE1C\(^{+/+}\)  PDE1C\(^{-/-}\)  PDE1C\(^{+/+}\)  PDE1C\(^{-/-}\)

Ligated

B

Intimal Area (x10\(^4\) \(\mu\)m\(^2\))

- PDE1C\(^{+/+}\)
- PDE1C\(^{-/-}\)

Unligated  Ligated

- *  *

Medial Area (x10\(^4\) \(\mu\)m\(^2\))

- PDE1C\(^{+/+}\)
- PDE1C\(^{-/-}\)

Unligated  Ligated

- *  *

Adventitial area (x10\(^4\) \(\mu\)m\(^2\))

- PDE1C\(^{+/+}\)
- PDE1C\(^{-/-}\)

Unligated  Ligated

- *

C

Intimal Area (x10\(^4\) \(\mu\)m\(^2\))

- Vehicle
- IC86340

Unligated  Ligated

- *  *

Medial Area (x10\(^4\) \(\mu\)m\(^2\))

- Vehicle
- IC86340

Unligated  Ligated

- *  *

Adventitial area (x10\(^4\) \(\mu\)m\(^2\))

- Vehicle
- IC86340

Unligated  Ligated

- *

D

No culture  Culture + Vehicle  Culture + IC86340

E

Neointima thickness (\(\mu\)m)

- No culture
- Culture + Vehicle
- Culture + IC86340

- *  *

Media thickness (\(\mu\)m)

- No culture
- Culture + Vehicle
- Culture + IC86340

- *  *

Adventitial thickness (\(\mu\)m)

- No culture
- Culture + Vehicle
- Culture + IC86340

- *  *
Figure 4

**A**

![Graph A](Image)

**B**

![Graph B](Image)

**C**

![Graph C](Image)

**D**

![Images D](Image)

**E**

![Graph E](Image)

**F**

![Graph F](Image)

**G**

![Images G](Image)

**H**

![Graph H](Image)
Figure 5
Figure 7

A) Dynasore - - + +
IC86340 (15 μmol/L) - + + +

PDGFRβ
β-Actin

Vehicle
IC86340

% of control
PDGFRβ protein levels

Vehicle
Dynasore

* ns

B) IC86340 (15 μmol/L) - + +

Internalized PDGFRβ
Total PDGFRβ

Vehicle
IC86340

* ns

C) PDGFRβ
EEA-1
PDGFRβ/EEA-1

Vehicle
IC86340

D) PDGFRβ with membrane

Vehicle
IC86340

* ns

E) PDGFRβ with endosome

Vehicle
IC86340

* ns

F) Cell proliferation (folds)

Vehicle
IC86340

* ns
Figure 8

A. Endogenous

B. Exogenous

C. Lane 1 2 3

D. Lane 1 2 3

E. PDE1C LRP1 PDE1C/LRP1

F. PDGFRβ LRP1 PDGFRβ/LRP1

G. ConsiRNA LRP1siRNA

H. Vehicle PKI p-LRP1 LRP1

I. mAC PDE1C LRP1 PDGFR

Endosome PKA Lysosome (Degradation) SMC phenotype modulation SMC growth/migration
Role of cAMP-Phosphodiesterase 1C Signaling in Regulating Growth Factor Receptor Stability, Vascular Smooth Muscle Cell Growth, Migration, and Neointimal Hyperplasia
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Supplemental Methods

Reagents
IC86340 was provided by ICOS Corporation. Forskolin, 8-CPT-cAMP, Dibutyryl-cAMP (Db-cAMP), H89 and SNAP were purchased from Biomol (Plymouth Meeting, PA). Bafilomycine A1, NH$_4$Cl, YC-1, C-type natriuretic peptide (CNP), 2',5'-dideoxyadenosine (2',5'-ddA), PKI (14-22) and other reagents were purchased from Sigma. Recombinant adenoviruses encoding PDE1A, PDE1C, or Lacz shRNA were constructed using BLOCK-iT Adenoviral shRNA Expression System (Invitrogen), as previously described $^1$. Adenovirus encoding PDE1C1 was kindly provided by Dr. Maurice (Queen's University, Canada).

Carotid artery ligation injury model
All animals and the procedures were performed in accordance with experimental protocols that were approved by the University Committee on Animal Resources at the University of Rochester. Global PDE1C knockout mice were kindly provided by Haiqing Zhao (Johns Hopkins University) $^2$ and backcrossed to C57BL/6 for at least 9 generation. FVB/NJ mice were obtained from Jackson Laboratories. 8-week-old male mice were used for all animal experiments. Mice were anesthetized with isoflurane, and complete common carotid artery ligation was performed as previously described $^3$. For PDE1C-WT and PDE1C-KO mice, the left common carotid artery was dissected through a small midline incision in the neck and ligated with 6-0 silk suture, and the right carotid artery served as unligated control. For FVB/NJ mice, after left common carotid artery was ligated with 6-0 silk suture, 50 µl 20% F-127 pluronic gel (BASF) containing 30 µM PDE1 inhibitor IC86340 or vehicle was applied around the carotid artery. At 14 days after surgery, mice were anesthetized by intraperitoneal injection with 80 mg/kg ketamine and 5 mg/kg xylazine and perfused with saline, and then fixed in 10% phosphate-buffered formalin (10% NBF). The arteries were embedded in paraffin, and cross sections were prepared. Elastic fiber was stained by Verhoeff-van Gieson (VVG).

Morphometric analysis
For morphological analysis, the carotid arteries were dissected, fixed with 10% NBF for overnight at 4°C, and embedded in paraffin. 10 sections located at 200 µm intervals from the bifurcation were measured. The intimal, medial, and adventitial areas were analyzed by Image Pro Plus software (Media Cybernetics Inc, Silver Spring). Intimal area was calculated as the internal elastic lamina area minus luminal area, the medial area was the external elastic lamina area minus the internal elastic lamina area, and the adventitial area was the vascular area minus the external elastic lamina area.

Femoral artery wire injury model
12-week-old male C57bl/6J mice were obtained from Jackson Laboratories, and femoral artery wire injury was performed as described previously $^4$. Briefly, left femoral artery was dissected and temporarily looped proximally and distally with 6-0 silk suture. A small branch artery between the rectus femoris and vastus medialis muscles was isolated and looped. Transverse arterioctomy was performed in this branch. A straight spring wire (0.38mm in diameter, No. C-SF-15-15, COOK, Bloomington, IN) was carefully inserted into the femoral artery and left in place for 1 min to denude vessel. At 4 weeks after surgery, mice were anesthetized, perfused with saline, and fixed in 10% phosphate-buffered formalin (10% NBF). The femoral arteries were embedded in paraffin, and cross sections were prepared.

Mouse Vein Graft model
12-week-old male C57bl/6J mice were obtained from Jackson Laboratories, and used for this procedure described previously. Mice were anesthetized with isoflurane. The right common carotid artery of recipient mouse was mobilized free from the bifurcation in the distal end toward the proximal end as far as possible, ligated, cut, and placed two cuffs at the both ends. The vena cava of donor mouse was harvested and connected between the 2 ends of the carotid artery by sleeving the ends of the vein over the artery cuff and ligating them. At 28 days after surgery, mice were anesthetized by intraperitoneal injection with 80 mg/kg ketamine and 5 mg/kg xylazine and perfused with saline, and then fixed in 10% phosphate-buffered formalin (10% NBF). The arteries were embedded in paraffin, and cross sections were prepared.

**Human saphenous veins culture ex vivo**

Human saphenous veins, not required for surgery, were collected from discards after coronary artery bypass graft surgery and they are RSRB (Research Subject Review Board) exempt human samples. Human saphenous vein explants were cultured as previously described. Briefly, the vein segments were opened longitudinally and cut transversely into 0.5 cm lengths. The vein segments were cultured individually with luminal surface facing up in 12-well plates in RPMI 1640 medium supplemented with 30% FBS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin for 7 days with or without 30 µM IC86340. The medium and inhibitor were changed every other day. At the end of the culture, the vein segments were washed with PBS, fixed with 10% NBF for 24 h, embedded in paraffin, and cross-sections (5 µm) were cut. The paraffin sections were stained with VVG and SM α-actin, and thickness of neointima, media and adventitia were measured by Image-Pro Plus software.

**Preparation of contractile and synthetic SMCs from rat and human aortas as well as human saphenous veins**

SMCs from human aortas, rat aortas, and HSV were prepared using an explant method. HSV or human aorta segment was cut longitudinally, the intima was scraped, and the adventitia was removed. Half media was cut into small pieces and cultured in DMEM medium supplemented with 10% FBS at 37°C in a humidified incubator in 5% CO₂, and half media was used to isolate RNA from contractile SMCs. SMCs from HSV and human aortas were identified by typical “hill and valley” growing pattern and SM- α-actin staining, and cells at passage 4 were used, which refers to synthetic SMCs. For rat aortic SMC isolation, rat aorta was dissected and digested with type 2 collagenase (worthington, CLS 2) for 10 min. The adventitia was peeled off, and endothelial layer was scraped. Half media were cut into small pieces and cultured in DMEM medium supplemented with 10% FBS, which refer to synthetic rat SMCs and half media were used to isolate RNA from contractile rat SMCs. Cultured rat aortic SMCs at passage 4 were used.

**Rat aortic SMC culture**

Rat aortic SMCs were prepared using enzymatic digestion of aortas from 10-week-old Sprague-Dawley rats as previously described. SMCs were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS in a humidified incubator (37°, 5% CO₂), and used for the experiments from passages 7 to 12. In the case to study SMCs under the differentiation and growing status, we cultured SMCs for two days either in the differentiation medium (medium 231 supplemented with Smooth Muscle Differentiation Supplement (SMDS, cat. # S-008-5) or in the growth medium(medium 231 supplemented with Smooth Muscle Growth Supplement (SMGS, cat. # S-007-25) from Cascade Biologics.

**Small Interfering RNA Transfection**

The Small Interfering RNA (siRNAs) targeting PKG1 (GCGUUCCGGAAGUUCACUA) and
Epac1 (CCACAGAGCAUGUGCGACAA) were designed through siDESIGN Center with Dharmacon RNAi Technologies or purchased. Rat aortic SMCs were transfected with control siRNA, PKG1 siRNA and Epac1 siRNA by a Gene Pulser Xcell Electroporation system (Bio-Rad) according to the manufacturer's instructions. Briefly, 1x10^6 SMCs were mixed with 400 ng of siRNA in 250 µl of siRNA electroporation buffer in 4 mm Gene Pulser cuvette and pulsed (300 V, 500 µf) as previously described 1. Cells were seeded in six-well plates and washed with PBS next day.

Real-time and semi-quantitative RT-PCR
Total cellular RNA was isolated from SMCs using the RNeasy Mini Kit (Qiagen). mRNA levels of various gene products were measured by real-time or semi-quantitative RT-PCR using Reverse Transcription System (Promega, A3500). Real-time PCR amplifications were performed using iQTM SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA). The relative quantities of mRNA levels were obtained using the comparative Ct method and normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Semi-quantitative RT-PCR amplifications were performed using GoTaq Green Master Mix (Promega, M7123), according to the manufacturer's instruction.

The primers used in qPCR and RT-PCR

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<td>5’-TCGAGATGGTAATGGCCACA-3’</td>
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Rat EGFR  | FP  | CCAAGCCCACTTGAGGATATT
| RP  | CCCTCTGCATGGTATTCTTTCT
GAPDH  | FP  | 5'-TCAAGAAGGTGGTGAAGCAG-3' 
| RP  | 5'-TGGAAGTTGCTGTTGAAGTC-3'

**Cell proliferation**

Cell proliferation was measured using the sulforhodamine-B (SRB) colorimetric assay (Vichai, 2006). 100 microliter of SMCs (5x10^4/ml) from aortas of PDE1C wild-type and PDE1C knockout mice or rat aortic SMCs were seeded in 96-well plates overnight in DMEM supplemented with 10% FBS, serum-starved for 1 day, and stimulated with 10% FBS or 50 ng/ml PDGF-BB for 2 days. Cells were fixed with 10% TCA, stained with 4 mg/ml SRB, and dissolved with 10 mM Tris. Optical density was measured at 515 nm by using a microplate spectrophotometer (VersaMax; Molecular Devices, Sunnyvale, CA).

**Boyden Chamber Assays**

SMCs derived from aortas of PDE1C wild-type and PDE1C knockout mice, or rat aortic SMC were trypsinized and resuspended at a concentration of 5x10^5 cells/ml in DMEM supplemented with 0.5% FBS. 100 µl SMC suspension was placed in the upper insert chamber (6.5 mm diameter, 8.0 µm pore size, polycarbonate membrane, Corning Inc. Corning, NY), and 600 ul DMEM with 25 ng/ml of PDGF-BB was in the 24-well plate. The insert chamber was incubated at 37°C and 5% CO₂ for 6 h. The cells on the upper side of the filter were scraped off with a cotton tip. The filter membrane were fixed in 4% paraformaldehyde and stained with hematoxylin. The images were viewed and captured by microscopes (BX41; Olympus) and a digital camera (Spot Insight 2; Diagnostic Instruments, Inc.).

**3-D Aortic Medial Explant Migration Assay**

3-D collagen gel was made by diluting type I collagen solution (PureCol, INAMED, CA) with 6×DMEM to a final 1x DMEM (final collagen I concentration 2.7 mg/ml). Briefly, the thoracic aortas from PDE1C wild-type and PDE1C knockout mice were dissected, the adventitia was stripped by collagenase type II digestion, and the endothelium layer was removed. The media explants were then cut into 1-mm fragments, suspended in type I collagen solution, and cultured for 10 days in DMEM supplemented with 10% FBS. A mixture of PDGF-BB/FGF-2 (10 ng/ml each; Sigma) was added to explant cultures to trigger SMC outgrowth and migration. Cell migration was quantified at day 10 by measuring the distance migrated by the leading front of SMCs from the explanted tissue, as previously described.

**Receptor internalization**

Receptor internalization was measured by biotinylation assay as described previously. Rat aortic SMCs were grown on 10 cm dishes for overnight, and washed three times with dPBS (GIBCO, 14190; 10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.4, 0.5 mM MgCl₂, 1 mM CaCl₂) on ice. The cells were incubated with 5 ml biotin (1mg/ml; EZ-Link Sulfo-NHS-SS-Biotin from Thermo, Prod # 21331) in dPBS for 20 min at 4°C. After washed three times with cold dPBS to remove excess biotin, the biotinylated cells were incubated with DMEM with 0.1% FBS, and treated with 30 µM IC86340 for 6 h. PDGFRβ trafficking was stopped by rapid cooling the cells on ice, washed once with 10 ml ice-cold dPBS. The surface-bound biotin was stripped off by washing once with 5 ml glutathione cleavage buffer (50 mM glutathione in 75 mM NaCl, 1 mM EDTA, 75 mM NaOH and 10% FBS) and incubate for 30 min at 4 °C with 5ml glutathione cleavage buffer. After washed three times with 5 ml ice-cold dPBS, cells were immediately lysed by adding 500 ul lysis buffer (50 mM Tris, pH 7.4, 150 mm NaCl, 1 mm EDTA, 1% NP40, 0.1%
SDS, 0.5% sodium deoxycholate) containing cocktail protease inhibitor. Protein was measured by Bradford assay. 200 µg protein lysates were immunoprecipitated with streptavidin beads (50 ul; Thermo, 20347) overnight at 4°C. Samples were washed five times with a washing buffer (0.1% SDS and 0.5% sodium deoxycholate in PBS), and the entire sample was loaded and western blot with PDGFRβ antibody. Total cell lysates were also immunoblotted with PDGFRβ antibody.

Western blot
Western blot was performed as previously described \(^1\). Briefly, lysates were prepared in RIPA buffer with protease inhibitor cocktail (Sigma). The concentrations of total proteins were measured by Bradford protein assay (Bio-Rad). Total lysates were loaded on SDS-PAGE, electrotransferred into PVDF membrane. The primary antibodies against AKT (Cell signal, #9272), p-AKT (Cell signal, #9271), β-Actin (Santa Cruz Biotechnology, sc-1616), Erk (Cell signal, #9101), p-Erk (Cell signal, #2232), Flag (Sigma, F3165), GAPDH (Chemicon, MAB374), GFP (Abcam, ab6662), HA antibody (Covance, MMS-101P), LRP1 (Sigma, L2170), PDE1C (a chicken polyclonal antibody raised against the C-terminal 60aa in mouse PDE1C1), PDGFRA (Cell signal, #3164), PDGFRβ (Cell signal, #3169), PKG1 (Stressgen, KAP-PK005), and phospho-PKA substrate antibody (Cell signal, #39624) were used. After washing with PBST, the membranes were incubated with horseradish peroxidase coupled secondary antibodies for 1 h. The signals were visualized using ECL reagents (Amersham).

Immunoprecipitation
HEK293A cells in 10 cm dishes were cotransfected with 12 µg of plasmids encoding Flag-PDGFRβ and EGFP-PDE1C, Flag-PDGFRβ and EGFP, or Flag-LacZ and EGFP-PDE1C using lipofectamin 2000 for 48 h, respectively. For LRP1 phosphorylation, rat aortic SMC were treated with 15 µmol/L IC86340 with or without 5 µmol/L PKI (12-24) peptide for 12 h. Cells were washed with PBS and lysed by a 1x lysis buffer (Cell signal, #9803) with 1 mM PMSF. 20 µl of protein agarose A beads was added to 500 µg protein lysates for pre-cleanup (shake for 30 min at 4°C). After spin 6000 rpm for 0.5 min 4°C, the HEK293 cell or SMC supernatant was immunoprecipitated with 5 µg anti-Flag antibody (Sigma, F3165) or 2.5 µl anti-LRP1 antibody (Sigma, L2070), respectively, for overnight at 4°C, followed by 40 µl protein agarose A beads for 3 h at 4°C. Agarose A beads precipitates were washed with PBS for 5 times, followed by addition of 2 x SDS loading buffer. Samples were boiled for 3 min, spin 13500 rpm for 5 min, and subjected to Western blot with indicated antibodies.

Immunofluorescent staining
Immunofluorescence was performed as previously described \(^6\). Briefly, SMCs were fixed in 4% paraformaldehyde and permeabilized in 0.2% saponin/PBS, and blocked with Dako serum-free blocking solution (Dako). For human tissue immunofluorescence, paraffin sections were deparaffinized, followed by treatment with citrate buffer for antigen retrieval. The immunostaining were performed using antibodies including anti-PDDE1C (a chicken polyclonal antibody raised against the C-terminal 60aa in mouse PDE1C1), anti-PDGFRβ (Santa Cruz Biotechnology, sc-432), anti-EEA1 (BD Biosciences, 610456), anti-SM-α-actin (DAKO, M0851), anti-Flag (Sigma, F3165), anti-GFP (Abcam, ab6662), or anti-LRP1 (Sigma, L2170), followed by secondary antibody Alexa Fluor 488 or 546 (Molecular Probes). Nuclei were stained with DAPI. Cells were visualized with Olympus confocal microscope (IX81) and fluorescent microscope (BX-51) microscope.

Immunohistochemistry
Vessels were embedded in paraffin, and cross-sections were cut at 200 µm intervals. The sections were deparaffinized, followed by treatment with citrate buffer for antigen retrieval and 3% H₂O₂. The sections were blocked with Dako serum-free blocking solution (Dako), and incubated with primary antibody for overnight at 4°C. The primary antibodies included PDE1C (chicken polyclonal antibody), anti-SM-α-actin (Dako, M0851), anti-Ki67 (Dako, M7249), and anti-4HNE (Cosmo Bio, NNS-MHN-020P-EX). Subsequently, the sections were incubated with biotinylated secondary antibodies for 30 min at room temperature. Avidin-biotinylated enzyme complex (Vector Laboratories) and a diaminobenzidine substrate chromogen system (Dako) were used for detection. Sections were counterstained with hematoxylin. As a negative control, matched IgG was used in place of the primary antibody. Slides were viewed with a microscope (BX41; Olympus, Tokyo, Japan) and a digital camera (Spot Insight 2; Diagnostic Instruments, Inc., Sterling Heights, MI).

Statistical analysis
Statistical analyses were performed with the StatView 4.0 package. Differences were analyzed by one-way or two-way ANOVA followed by Schéffe’s correction. P values less than 0.05 were considered significant.

References:

Figure I. PDE1C protein level is increased in synthetic SMCs. Western blotting results showing protein levels of PDE1C and calponin in contractile SMCs (freshly isolated medial layers) and corresponding synthetic SMCs (cultured SMCs) from rat aortas (A), human aortas (B), and human saphenous veins (C). Contractile SMCs are freshly isolated medial tissues procured by removing endothelial cells and pealing off adventitial layers. Synthetic SMCs are cultured growing SMCs isolated from the corresponding vessel with the explant method. (D) Cultured rat aortic SMCs were in differentiation medium (medium 231 supplemented with Smooth Muscle Differentiation Supplement (SMDS) from Cascade Biologics), or growth medium (medium 231 supplemented with Smooth Muscle Growth S (SMGS), from Cascade Biologics) for 2 days. SM-MHC and calponin are used as contractile SMC markers. Values are mean ± SD of triplicate experiments. *P < 0.05.
Supplemental Figure II. (A) PDE1C is elevated in femoral arteries after wire injury. qRT-PCR showing the PDE1C mRNA levels of uninjured right and injured left femoral arteries. C57BL/6J mice were subjected to femoral artery wire injury for 28 days, total RNA were isolated from right uninjured and left injured femoral arteries. The levels of PDE1C mRNA were assessed by qPCR. Values are means ± SD of triplicate (three arteries are pooled together). *P < 0.05. (B) PDE1C are induced in SMC-like cells. Representative images of carotid artery sections subjected to immunofluorescent double staining with SM-α-actin (red) and PDE1C (green). Nuclei were stained wit DAPI (blue). FVB mice were subjected to left common carotid artery (LCA) ligation for 14 days. Right common carotid artery (RAC) was used as the control vessel. The merged images (right panels) show that PDE1C-positive cells are largely overlapped with SM-α-actin-positive cells in the neointimal region (N).
Supplemental Figure III. (A and C) PDE1C deficiency or PDE1 inhibition attenuates SMC proliferation in response to vascular injury. Top panels: representative images of carotid artery sections immunostained with SM-α-actin (pink) or Ki67 (brown). Bottom panels: quantitative data of Ki67 positive cells in intima and media. A, PDE1C+/+ and PDE1C−/− mice were subjected to left common carotid artery ligation for 14 days. C, FVB mice were subjected to left common carotid artery ligation for 14 days in the presence of vehicle or 30 µmol/L IC86340 applied perivascularly via pluronic gel. SM-α-actin and Ki67 immunostaining were performed in cross-sections of carotid arteries. Ki-67-positive cells in the intima and the media were calculated. N: neointima; M: media. (B-D) PDE1C deficiency suppresses ROS production in carotid arteries after vascular injury. Top panels: Representative images of carotid arteries immunostained with 4-HNE (an oxidative stress marker). B, PDE1C+/+ and PDE1C−/− mice were subjected to left common carotid artery ligation for 14 days. D, FVB mice were subjected to left common carotid artery ligation for 14 days in the presence of vehicle or 30 µmol/L IC86340 applied perivascularly via pluronic gel. 4-HNE immunostaining were performed in cross-sections of carotid arteries. Quantification were performed using Image Pro Plus software. Values are means ± SEM (n=3). *P < 0.05.
Supplemental Figure IV. (A) Real-time RT-PCR showing the mRNA expression of SM-MHC, calponin, and SM-α-actin in low-passage aortic SMCs isolated from PDE1C+/+ and PDE1C−/− mice. (B) Immunofluorescence staining of SM-MHC or SM-α-actin in SMCs isolated from PDE1C+/+ and PDE1C−/− mice. Green: SM-MHC; Red: SM-α-actin; Blue: nuclei.
Supplemental Figure V. Role of PDE1C in regulating PDGFRβ levels. (A) PDE1A and PDE1C mRNA levels in rat SMCs treated with a low dose or high dose of adenovirus encoding LacZ shRNA, PDE1A shRNA or PDE1C shRNA. (B-C) PDE1 inhibitor IC86340 or PDE1C shRNA does not affect mRNA levels of multiple growth factor receptors including PDGFRβ, PDGFRα, and EGFR. Levels of mRNA were determined by RT-PCR. (D-E) PDE1 inhibitor IC86340 and PDE1C shRNA reduced the protein levels of multiple growth factor receptors. The protein levels of PDGFRβ, PDGFRα, and EGFR were assessed by immunoblotting. Rat aortic SMCs were treated with indicated doses of PDE1 inhibitor IC86340 for 24 h or transduced with adenovirus encoding a high dose shRNA against LacZ, PDE1A, or PDE1C shRNA. (F) PDE1 inhibitor IC86340 reduced exogenously expressed PDGFRβ protein but not PDE1C protein. Rat aortic SMCs were transfected with Flag-PDGFRβ or EGFP-PDE1C via electroporation for 2 days and then treated with 15 µmol/L IC86340 for 24 h. PDGFRβ and PDE1C were detected by anti-Flag and anti-GFP antibodies, respectively. (G) PDGF-stimulated Erk1/2 and AKT activation was attenuated when PDGFR was downregulated. Rat aortic SMCs were pre-treated with 15 µmol/L IC86340 in DMEM with 0.1% FBS for 24 h, washed, and stimulated with 10 ng/ml PDGF-BB for 5 or 30 min. Phospho-Erk1/2, Erk 1, phospho-AKT, and AKT were measured by immunoblotting.
Supplemental Figure VI

A

\[ \text{2',5'-ddA} \quad \text{Forskolin} \quad \text{IC86340 (5 \, \mu\text{mol/L})} \]

\[ \text{PDGFR}\beta \quad \text{ß-Actin} \]

\[ \text{Vehicle} \quad \text{IC86340 (5 \, \mu\text{mol/L})} \]

\[ \text{PDGFR}\beta \text{ protein levels (\% of control)} \]

\[ \text{Vehicle} \quad \text{Forskolin} \quad \text{Forskolin 2',5'-ddA} \]

B

\[ \text{2,5'-ddA} \quad \text{Forskolin} \quad \text{PDE1C shRNA (L)} \quad \text{PDE1A shRNA (L)} \quad \text{LacZ shRNA (L)} \]

\[ \text{PDGFR}\beta \quad \text{ß-Actin} \]

\[ \text{LacZ shRNA (L)} \quad \text{PDE1A shRNA (L)} \quad \text{PDE1C shRNA (L)} \]

\[ \text{PDGFR}\beta \text{ protein levels (\% of control)} \]

\[ \text{Vehicle} \quad \text{Forskolin} \quad \text{Forskolin 2',5'-ddA} \]

C

\[ \text{H89} \quad \text{Forskolin} \quad \text{IC86340 (5 \, \mu\text{mol/L})} \]

\[ \text{PDGFR}\beta \quad \text{ß-Actin} \]

\[ \text{Vehicle} \quad \text{IC86340 (5 \, \mu\text{mol/L})} \]

\[ \text{PDGFR}\beta \text{ protein levels (\% of control)} \]

\[ \text{Vehicle} \quad \text{Forskolin} \quad \text{Forskolin H89} \]

D

\[ \text{H89} \quad \text{Forskolin} \quad \text{PDE1C shRNA (L)} \quad \text{PDE1A shRNA (L)} \quad \text{LacZ shRNA (L)} \]

\[ \text{PDGFR}\beta \quad \text{ß-Actin} \]

\[ \text{LacZ shRNA (L)} \quad \text{PDE1A shRNA (L)} \quad \text{PDE1C shRNA (L)} \]

\[ \text{PDGFR}\beta \text{ protein levels (\% of control)} \]

\[ \text{Vehicle} \quad \text{Forskolin} \quad \text{Forskolin H89} \]
Supplemental Figure VI. Role of tmAC-cAMP signaling in PDE1C-mediated regulation of PDGFRβ. (A) The tmAC inhibitor blocked the synergistic effect of IC86340 and forskolin on PDGFRβ protein reduction. Rat aortic SMCs were treated with 5 µmol/L IC86340, 10 µmol/L forskolin, or both in the presence or absence of tmAC inhibitor 2′,5′-dideoxyadenosine (2′ 5′-ddA, 10 µmol/L) for 24 h in DMEM containing 0.1% FBS. (B) The tmAC inhibitor blocked the synergistic effect of PDE1C shRNA and forskolin on PDGFRβ protein reduction. Rat aortic SMCs were transfected with a low dose of adenovirus expressing LacZ-shRNA, PDE1A-shRNA, or PDE1C-shRNA for 3 days, followed by treatment with 10 µmol/L forskolin in the presence or absence of 10 µmol/L 2′,5′-ddA for 24 h in DMEM containing 0.1% FBS. (C) PKA inhibitor H89 blocked the synergistic effect of IC86340 and forskolin on PDGFRβ protein reduction. Rat aortic SMCs were treated with 5 µmol/L IC86340, or 10 µmol/L forskolin, or both in the presence or absence of 5 µmol/L H89 for 24 h in DMEM containing 0.1% FBS. (D) PKA inhibition by H89 blocked the synergistic effect of PDE1C shRNA and forskolin on PDGFRβ protein reduction. Rat aortic SMCs were transfected with a low dose of adenovirus expressing LacZ-shRNA, PDE1A-shRNA, or PDE1C-shRNA for 3 days, and then treated with 10 µmol/L forskolin in the presence of absence of 5 µmol/L H89 for 24 h in DMEM containing 0.1% FBS. Percentile changes normalized to the left lane. Values are mean ± SD (n=3). *p<0.05.
Supplemental Figure VII. (A) Epac was not involved in PDE1 inhibition-induced PDGFRβ protein reduction. Rat aortic SMCs were transfected with 50 nmol/L control siRNA or Epac siRNA for 3 days, and then treated with 15 µmol/L IC86340 for 24 h in DMEM containing 0.1% FBS. Right panel showed Epac knockdown by specific siRNA using RT-PCR. (B) PKG1 was not involved in PDE1 inhibition-induced the PDGFRβ protein reduction. Rat aortic SMCs were transfected with 50 nmol/L control siRNA or PKG1 siRNA for 3 days, and treated with 15 µmol/L IC86340 for 24 h in DMEM containing 0.1% FBS. (C-E) cGMP and PDE1 inhibition does not have synergistic effect on PDGFRβ protein. Rat aortic SMCs were treated with either 5 µmol/L IC86340 alone, one of cGMP elevators (100 µmol/L NO donor SNAP, 10 µmol/L sGC activator YC-1, and 100 nmol/L CNP), or both of IC86340 and a cGMP elevator for 24 h in DMEM containing 0.1% FBS. (F) Lysosome inhibitors abrogated enhanced effect of forskolin and IC86340 on PDGFRβ protein reduction. Rat aortic SMCs were treated with 5 µmol/L IC86340 and 10 µmol/L forskolin in the presence or absence of 20 mmol/L NH₄Cl or 50 nmol/L Bafilomycine A for 24 h in DMEM containing 0.1% FBS. Protein levels of PDGFRβ and β-actin equal loading were determined by immunoblotting. Quantitative data show percentile changes normalized to the 1st lane. Values are mean ± SD (n= 3). *p<0.05. ns: no significant difference.
Supplemental Figure VIII. PDE1C and PDGFRβ co-localization in neointimal lesions. (A) Representative images of carotid artery sections subjected to co-immunofluorescent staining of PDE1C (green) and PDGFRβ (red), and nuclei are stained with DAPI (blue). FVB mice were subjected to left common carotid artery (LCA) ligation for 14 days. Right common carotid artery (RAC) was used as the control vessel. N: neointima. (B) Reprehensive co-immunofluorescent staining images of human coronary artery with neointimal lesions. Green: PDE1C, Red: PDGFRβ, Blue: nuclei stained with DAPI. The merged images (right panels) show that PDE1C-positive cells are largely overlapped with SM-α-actin-positive cells in the neointimal region. M: media, N: neointima.
Supplemental Figure IX. Co-expression of LRP1 with PDE1C or PDGFRβ in neointimal lesions. (A-B) Representative images of mouse carotid artery sections subjected to co-immunofluorescent staining of LRP1 (green) together with PDE1C (red) or PDGFRβ (red), and nuclei are stained with DAPI (blue). FVB mice were subjected to left common carotid artery (LCA) ligation for 14 days. Right common carotid artery (RCA) was used as the control vessel (inset). N: neointima. (C-D) Representative co-immunofluorescent staining images of human coronary artery with neointimal lesions. Green: LRP1, Red: PDE1C or PDGFRβ, Blue: nuclei stained with DAPI. The merged images (right panels) show that LRP1-positive cells are largely overlapped with PDE1C- or PDGFRβ-positive cells in the neointimal region. M: media, N: neointima.