Cyclooxygenase-2–Derived Prostaglandin E₂ Promotes Injury-Induced Vascular Neointimal Hyperplasia Through the E-prostanoid 3 Receptor

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Rationale: Vascular smooth muscle cell (VSMC) migration and proliferation are the hallmarks of restenosis pathogenesis after angioplasty. Cyclooxygenase (COX)-derived prostaglandin (PG) E₂ is implicated in the vascular remodeling response to injury. However, its precise molecular role remains unknown.

Objective: This study investigates the impact of COX-2–derived PGE₂ on neointima formation after injury.

Methods and Results: Vascular remodeling was induced by wire injury in femoral arteries of mice. Both neointima formation and the restenosis ratio were diminished in COX-2 knockout mice as compared with controls, whereas these parameters were enhanced in COX-1>COX-2 mice, in which COX-1 is governed by COX-2 regulatory elements. PG profile analysis revealed that the reduced PGE₂, by COX-2 deficiency, but not PGI₂, could be rescued by COX-1 replacement, indicating COX-2–derived PGE₂ enhanced neointima formation. Through multiple approaches, the EP3 receptor was identified to mediate the VSMC migration response to various stimuli. Disruption of EP3 impaired VSMC polarity for directional migration by decreasing small GTPase activity and restricted vascular neointimal hyperplasia, whereas overexpression of EP3α and EP3β aggravated neointima formation. Inhibition or deletion of EP3α/β, a Gαi protein–coupled receptor, activated the cAMP/protein kinase A pathway and decreased activation of RhoA in VSMCs. PGE₂ could stimulate phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase3β signaling in VSMCs through Gβγ subunits on EP3α/β activation. Ablation of EP3 suppressed phosphatidylinositol 3-kinase signaling and reduced GTPase activity in VSMCs and altered cell polarity and directional migration.


Key Words: EP3  ■  neointima formation  ■  polarity  ■  prostaglandin E₂  ■  vascular smooth muscle cell migration

Percutaneous transluminal coronary angioplasty is commonly used for the treatment of coronary heart disease. However, restenosis after angioplasty and stent deployment, considered as a wound healing response to mechanical injury, continues to be problematic in coronary interventional treatment, although application of drug-eluting stents has dramatically increased the success rate compared with that of bare metal stents. Directional migration of vascular smooth muscle cells (VSMCs) from media to intima and their subsequent proliferation are the important processes for neointimal hyperplasia–lumen narrowing, followed by deposition of extracellular matrix, which together lead to self-remodeling of vessels (restenosis).²

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De-endothelialization promotes platelet activation and infiltration of inflammatory cells, which could initiate VSMC migration by releasing proinflammatory mediators, such as monocyte chemotactic protein-1, interleukin-6, and prostaglandins (PGs).³ Elevated systemic inflammation markers, such as monocyte chemotactic protein-1, C-reactive

Original received February 6, 2013; revision received March 29, 2013; accepted April 16, 2013. In March 2013, the average time from submission to first decision for all original research papers submitted to Circulation Research was 14.5 days.

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The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.113.301033/-/DC1.

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Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.113.301033
protein, and C3 complement, are associated with the increased risk of clinical angiographic restenosis.4 Thus, investigators have proposed systemic anti-inflammatory approaches to prevent clinical restenosis.5

Cyclooxygenase (COX) is a key enzyme for PG biosynthesis. Both isofoms, COX-1 and COX-2, are expressed in the vasculature6 and can contribute substantially to the generation of vascular PG, including PGE\(_2\),7 COX-2, an inducible isozyme and the dominant source of PGs in inflammation, is upregulated in vascular inflammation, such as atherosclerosis,8 aortic aneurysm,9 and balloon-injured arteries.10,11 Thus, elevated levels of PGE\(_2\), along with the induction of COX-2 in the vascular wall, is associated with the instability of plaque in the progression of atherosclerosis.12 Pharmacological inhibition of COX-2, but not COX-1, reduces vascular neointimal hyperplasia in response to mechanical injury.10,11 Moreover, systematic inhibition of the production of PGE\(_2\) caused by genetic disruption of microsomal prostaglandin E\(_2\) synthase-1 (mPGES-1) attenuates neointima formation after vascular injury.15 These findings strongly suggest that COX-2-derived PGE\(_2\) might contribute to the pathogenesis of vascular restenosis.

A major source of the PGE\(_2\) formed in vivo is derived from mPGES-1, and other PGES isozymes do not compensate when mPGES-1 is deleted.7 Along with notably direct suppressor of PGE\(_2\) production, deletion of mPGES-1 reduces the neointimal hyperplasia response to vascular injury and results in cell-specific differential use of the accumulated PGH\(_2\) substrates, such as predominant augmentation of prostacyclin (PGI\(_2\)) in VSMCs and thromboxane A\(_2\) in macrophages.14 Genetic deficiency of the PGI\(_2\) receptor (IP) enhances vascular proliferation response to wire injury; however, deletion of the thromboxane A\(_2\) receptor decreases this response.15 Targeted deletion of mPGES-1 in VSMCs and macrophages differentially modulates the response to vascular injury in mice,16 indicating direct impact of mPGES-1-derived PGE\(_2\) on vascular remodeling, probably through its receptors known as EPs.

To test our hypothesis, we used COX-1>COX-2 mice, in which COX-1 is exchanged for COX-2 under the control of COX-2 regulatory elements,17 and COX-2 knockout (KO) mice were used to address how COX-2-derived PGs are involved in the vascular remodeling in response to mechanical injury. We demonstrate here that PGE\(_2\), derived primarily from COX-2, accelerated vascular neointima formation in a vascular wire injury mouse model by comparing the vascular responses and PG profiles in 2 strains of mice. By screening different pharmacological inhibitors, the EP3 receptor was identified to mediate the VSMC migration response to PGE\(_2\) stimulation. Disruption of EP3, particularly its \(\alpha\) and \(\beta\) splice variants, impaired the polarity of VSMCs required for directional migration through inhibition of the activity of small GTPase activity, including RhoA, Rac1, and Cdc42. The activity of RhoA in VSMCs could be modulated by both EP3\(\alpha\) and EP3\(\beta\) through the cAMP-dependent protein kinase A (PKA) pathway. Blockade of EP3 suppressed phosphatidylinositol 3-kinase (PI3K) signaling, reduced Rac1, RhoA, and Cdc42 activity in VSMCs, altered cell polarity, and restrained directional migration.

**Methods**

The detailed Methods section is available in the online-only Data Supplement.

**Results**

**Deletion of COX-2 Conveys Protection Against Vascular Neointima Formation in Response to Injury**

COX-1 and COX-2 were abundantly expressed in cultured VSMCs. Both mRNA and protein for COX-2, but not COX-1, were significantly upregulated by stimulation with lipopolysaccharide (LPS; Figure 1A and 1B). Similarly, the level of expression of the mRNA of the dominant PGE\(_2\) synthase, mPGES-1, also increased significantly in response to LPS (Figure 1B). In the wire-injured artery model, COX-2 was strongly stained in the infiltrating inflammatory cells (CD68\(^{+}\) and CD11b\(^{+}\)) and in the proliferating VSMCs (\(\alpha\)-actin–positive) in the neointima at different stages (Figure 1C and Online Figure I), indicating that COX-2 activity contributes to the vascular response to the injury.

To test our hypothesis, we performed wire injury in the femoral arteries of wild-type (WT), COX-2 KO, and COX-1>COX-2 mice and evaluated neointima formation at day 28 after injury. COX-2 deficiency, as anticipated, led to significant reduction of the intima-to-media ratio (COX-2 KO 0.95±0.17 vs WT 1.70±0.25; \(P=0.02\)) and the percent-age of luminal narrowing (restenosis index: COX-2 KO 25.59±3.38\% vs WT 46.00±5.62\%; \(P=0.005\)) compared with WT with abundant neointima formation (Figure 1D and 1E). In contrast, the replacement of COX-1 in COX-1>COX-2 mice augmented the intima-to-media ratio (COX-1>COX-2 2.53±0.25 vs WT 1.70±0.25; \(P=0.03\)) and the restenosis index (COX-1>COX-2 64.5±6.4\% vs WT 46.0±5.6\%; \(P=0.04\)).

Immunohistochemical analysis revealed the presence of extensive inflammatory cell infiltration around the vasculature, as well as VSMC migration and proliferation in the intima in response to vascular injury (Online Figure IA, IC, and IE). COX-2 deletion coordinately suppressed the inflammatory response as compared with WT controls, but no overt differences were found between COX-1>COX-2 and WT mice (Online Figure IB and ID).

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**Nonstandard Abbreviations and Acronyms**

<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>COX</td>
<td>cyclooxygenase</td>
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<tr>
<td>KO</td>
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<td>MTOC</td>
<td>microtubule organizing center</td>
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<td>mPGES-1</td>
<td>microsomal prostaglandin E(_2) synthase-1</td>
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<tr>
<td>PG</td>
<td>prostaglandin</td>
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<td>PGI(_2)</td>
<td>prostacyclin</td>
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<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
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<tr>
<td>PKA</td>
<td>protein kinase A</td>
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<tr>
<td>ROCK</td>
<td>Rho-associated protein kinase</td>
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<tr>
<td>VSMC</td>
<td>vascular smooth muscle cell</td>
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COX-2–Derived PGE2 Is Involved in the Development of Injury-Induced Neointima Formation

Given the differing impact of COX-2 deficiency and the replacement of COX-2 activity by COX-1 on vascular remodeling (Figure 1), we looked further into systemic PG metabolism and vascular PG generation of these strains. Vascular insult increased the excretion of all PG metabolites (Figure 2) without significant influence on the suppression of urinary PGI2 metabolite by deletion of COX-2 (Figure 2C). Similar PG measurements were observed after vascular injury, albeit with higher levels detected (19.5±3.8 ng/mg creatinine in COX-2 KO vs 38.1±4.6 ng/mg creatinine in WT [P<0.05] 35.1±5.4 ng/mg creatinine in COX-1>COX-2 [P<0.05]). Deletion of COX-2 had no impact on the excretion of urine PGD2 metabolite and thromboxane A2 metabolite before and after injury. In cultured VSMCs, PGE2 and PGI2 were the dominant products of COX-1 and COX-2, and substitution of COX-1 for COX-2 can notably rescue PGE2, but not PGI2, production (measured as its stable hydrolysis product), which was decreased by COX-2 deletion (Online Figure II). Thus, insertion of COX-1 in the COX-2 locus restored COX-2–derived biosynthesis of PGE2, not PGI2, in mice. Depletion of PGI2 receptor (IP) in mice aggravated the vascular responses to injury,15 as in COX-1>COX-2 mice with diminished PGI1 (Figure 2). Taken together, we surmise that the decrease of PGE2 in COX-2 KO mice provides protection against injury-induced vascular remodeling. This occurs despite reduced production of vasoprotective PGI1, in which case COX-2 inhibition increases the risk of cardiovascular events in clinic.4

The EP3 Receptor Mediates Migration of VSMCs on PGE2 Stimulation

In response to de-endothelialization induced by mechanical injury, VSMCs migrate directionally from arterial media into the intima and begin proliferating, resulting in neointima formation and restenosis.2 We first monitored the migration of VSMCs from COX-2 KO mice by a traditional scratch wound healing assay. COX-2 deficiency significantly inhibited wound closure at all time points tested (Online Figure III). In particular, during the first 12 hours, cell proliferation is minimal, and wound closure at this time primarily reflects cell spreading and migration.18 Because COX-2–derived
PGE₂ is responsible for this process, and all 4 receptors for PGE₂ (EP1, EP2, EP3, and EP4) are expressed abundantly in cultured VSMCs and arteries and are not significantly altered in response to LPS stimulation (Figure 3A). Using specific antagonists, we determined the identity of the receptor(s) involved in mediating the VSMC migration. The migration of VSMCs challenged with PGE₂ was markedly reduced dose-dependently by pretreatment with the EP3 receptor antagonist L-789,106 (Figure 3B) and 3C), whereas no significant effects were detected using EP1, EP2, and EP4 receptor antagonists (Figure 3B). To rule out the impact of cell proliferation, we performed transwell migration assays of VSMCs within 3 hours of treatment. Similarly, only the EP3 receptor antagonist L-789,106 strikingly attenuated VSMC migration (59% suppression compared with the vehicle control [Figure 3D], which again exhibited dose-dependent inhibition; Figure 3E). Therefore, PGE₂, derived primarily from COX-2 promoted VSMC migration through the EP3 receptor.

Both EP3α and β Splice Variants Are Involved in Regulation of VSMC Migration and Injury-Induced Neointimal Hyperplasia

Using EP3 small interfering RNA directed at a site within exon 2 to disrupt all 3 mouse EP3 splice variants, EP3 expression was reduced by 80% in primary VSMCs, resulting in a 48% decrease in VSMC migration through the transwell assay (Online Figure IVA). Likewise, EP3 deletion caused similar impairment of VSMC migration as the EP3 inhibitor (Figure 4A). Real-time polymerase chain reaction data showed that all PG receptors were expressed in VSMCs, and that EP3 disruption did not significantly alter the expression of other PG receptors, with or without LPS treatment (Online Figure V). The 3 EP3 splice variants are expressed in VSMCs (Online Figure IVB). To identify the EP3 variant that mediates VSMC migration induced by PGE₂, VSMCs from WT mice were subjected to transfection with small interfering RNA specific for each variant. Real-time polymerase chain reaction demonstrated >70% reduction in the mRNA levels at 24 hours after transfection of the respective variants without significant compensation. Knockdown of EP3α and EP3β significantly impaired the capability of VSMC migration (EP3α inhibition 78.00±2.1 and EP3β inhibition 73.03±2.4 vs scrambled control 100.0±1.7; P<0.05; Online Figure IVC). However, silencing of EP3γ did not significantly influence VSMC migration.

We sought to determine whether re-expression/overexpression of EP3 variants could restore the ability of VSMC migration. Primary VSMCs isolated from EP3 KO mice were transfected with mouse EP3α, EP3β, or EP3γ cDNAs that were tagged with the hemagglutinin epitope at the N terminus. Robust induction of the expression of each EP3 variant was observed at 24 hours after transfection (Online Figure IVD). Overexpression of EP3α or EP3β rescued the ability of VSMCs to migration, which was inhibited previously by EP3 deficiency (Figure 4B), and this renewed capability correlates positively with expression of EP3α or EP3β (Figure 4C), whereas EP3γ overexpression showed very little influence on migratory behavior (Figure 4B). These data demonstrate that PGE₂-induced VSMC migration is mainly mediated by EP3α and EP3β. Consistent with these results, in this vascular injury model, EP3 abolition rendered a decrease in the intima-to-media ratio (0.65±0.16 vs 1.34±0.15; P=0.005) and restenosis in EP3 KO mice (index, 23.4±5.0% vs 42.2±3.4%; P=0.007) compared with control mice (Figure 4D). The levels of PG metabolites were not significantly influenced by EP3 disruption in mice, even when subjected to wire injury (Online Figure VI).

To further confirm the involvement of EP3α and EP3β in vascular remodeling, we delivered lentiviral vectors, each constitutively expressing EP3α, EP3β, or EP3γ, to the femoral arteries after wire injury (Figure 4E). After 4 weeks, expression of the EP3α and EP3β transgenes significantly reversed the inhibition of neointimal hyperplasia in EP3-deficient mice (intima-to-media ratio: EP3α overexpression 1.68±0.16 and
EP3β overexpression 1.42±0.08 vs green fluorescent protein control 0.70±0.07; *P<0.05), whereas vascular overexpression of EP3γ failed to significantly alter the vascular response to injury (Figure 4E and 4F). Thus, these data indicate the EP3α and EP3β receptors play important roles in the vascular remodeling in response to injury.

Activation of EP3 Is Required for Polarity of Migrating VSMCs

The positioning of the microtubule organizing center (MTOC) relative to the nucleus toward the leading edge is a hallmark of migrating cell polarity. 19 Confluent cultures of VSMCs were wounded with a pipette tip and immunostained for the MTOC marker γ-tubulin and counterstained with 4',6-diamidino-2-phenylindole to visualize cell nuclei at the wound edge (Figure 5A). The MTOCs that localized between the nucleus and the leading edge were determined as front-polarized (Figure 5B). 20 Before wounding, the MTOC was randomly distributed with 20.1±1.0% of front-polarized cells. Six hours after wounding, the percentage of front-polarized VSMCs increased to 40.4±8.5% (Online Figure VII). Impairment of directional cell migration is accompanied by a loss of cell polarity toward the direction of migration. 20 Antagonism of the EP3 receptor activity by L-798,106 (10 μmol/L) markedly disrupted polarization of VSMCs and randomized the orientation of the MTOC front-polarized cells of EP3 antagonist vs control (13.5±3.4 vs 40.3±8.5; *P<0.05; Figure 5B). Comparable results were observed in VSMCs from EP3 KO mice with ≈16% front-polarized cells (EP3 KO vs WT, 15.5±5.1 versus 42.3±12.1; *P<0.05; Figure 5A and 5C).

Immunostaining for α-tubulin showed wound-facilitated formation of long and unbranched protrusions, which were oriented toward the direction of migration in intact VSMCs, whereas EP3 inhibition (L-798,106 or Gi protein blocker, pertussis toxin), or deletion of EP3, led to the formation of randomly oriented protrusions (Figure 5D). In migrating cells, protrusion of the leading edge requires the precise regulation of the lamellipodia and F-actin networks. 21 As indicated by F-actin staining, pharmacological inhibition of EP3 by L-798,106 or pertussis toxin sharply reduced the number of leading protrusions during VSMC migration (L-798,106 treatment, 1.29±0.20; pertussis toxin treatment 1.0±0.19 vs control 3.47±0.38; *P<0.05; Figure 5E). Similar suppression of leading protrusions was replicated in VSMCs derived from EP3 KO mice compared with WT (1.11±0.21 vs 3.40±0.36; *P<0.05; Figure 5E). These results suggest the migratory defect of VSMCs from EP3 KO is attributed to abnormal cell polarity.

Activity of Small GTPases Is Impaired in VSMCs From EP3 KO Mice

Rho GTPases are pivotal regulators for cell polarization 22 and directional migration. 21 Therefore, we performed standard pull-down assays using lysates prepared from primary VSMCs obtained from EP3 KO and WT mice and examined GTP-bound RhoA, Rac1, and Cdc42 in response to PGE2 stimulation. RhoA activity was undetectable in untreated cultured VSMCs (data not shown) but was boosted robustly in the presence of PGE2, whereas EP3 disruption attenuated the induction of GTP-RhoA to 68% (Figure 6A). Overexpression of either EP3α or EP3β by transfection of EP3-deficient VSMCs resulted in upregulated RhoA activity on stimulation (Figure 6A). Again, Cdc42 and Rac1 activities that were low in VSMCs could be induced notably by PGE2; Deletion of EP3 resulted in a slight, but significant, increase in their activities, whereas re-expression of either EP3α or EP3β completely restored the suppression in EP3 KO VSMCs (Figure 6B). These observations indicate that either EP3α or EP3β was involved in regulating of RhoA, Cdc42, and Rac1 activation.
EP3α/β Variants Are Coupled Via Gαi Protein to Regulated RhoA Activity and VSMC Migration Through a PKA Signaling Pathway

Evidence indicates EP3α and EP3β are coupled to Gαi.24 To determine whether these G-protein interactions occur in VSMCs, hemagglutinin-tagged EP3α or EP3β was introduced into cultured VSMCs. Coimmunoprecipitation experiments displayed clear binding of EP3α or EP3β with Gαi using a hemagglutinin-tag antibody (Figure 7A), suggesting that either EP3α or EP3β suppresses adenylyl cyclase activity and cellular cAMP levels. As anticipated, the levels of cAMP were significantly decreased by overexpression of either EP3α or EP3β receptors on stimulation with a PGE2 analog misoprostol (Figure 7B). Accordingly, PKA activity was increased by 20% when EP3 was deleted in VSMCs and reduced by 40% and 36% by overexpression of EP3α or EP3β, respectively (Figure 7C).

Various lines of evidence suggest that PKA modulates activity of GTPases and influences cytoskeletal dynamics and cell migration.25 To determine whether PKA is involved in the EP3-mediated RhoA activation, VSMCs were pretreated with a PKA inhibitor (H-89) before PGE2 stimulation. As shown in Figure 7D, overexpression of EP3α or EP3β markedly elevated RhoA activity. Blocking PKA activity by H-89 also enhanced RhoA activation, which was more apparent in EP3 KO cells. Surprisingly, we failed to detect a consistent effect of PKA on Cdc42 and Rac1 by using H-89, even in cells overexpressing EP3 (Online Figure VIII). Along with the increasing repositioning of MTOCs toward a scratch in the presence of the EP3 antagonist, L-798,106, nuclei were stained with 4',6-diamidino-2-phenylindole. The dashed box outlines the region enlarged to the right. Scale bar, 50 μm.

The EP3α/β-Mediated PI3K-Akt-GSK3β Signaling Axis Modulates Migration and Polarization of VSMCs

Phosphorylation of glycogen synthase kinase (GSK)3β is implicated in the control of direction of cell protrusion by regulating the centrosome polarization.25 To explore whether PGE2 activates the PI3K/Akt/GSK3β signaling pathway through the EP3 receptor, we first determined inhibitory phosphorylation of GSK3β at Ser9. We found that in response to PGE2 treatment, GSK3β underwent a rapid phosphorylation within 30 minutes, reaching a peak at 6 hours and fully recovered at 12 hours (Online Figure IXA and IXB). The EP3 antagonist L-798,106 and Gαi blocker pertussis toxin suppressed PGE2-induced phosphorylation of GSK3β at Ser9. We found that in response to PGE2 treatment, GSK3β underwent a rapid phosphorylation within 30 minutes, reaching a peak at 6 hours and fully recovered at 12 hours (Online Figure IXA and IXB). The EP3 antagonist L-798,106 and Gαi blocker pertussis toxin suppressed PGE2-induced phosphorylation of GSK3β at Ser9. We found that in response to PGE2 treatment, GSK3β underwent a rapid phosphorylation within 30 minutes, reaching a peak at 6 hours and fully recovered at 12 hours (Online Figure IXA and IXB).
We next asked whether EP3α/β mediated the PI3K/Akt/GSK3β signaling, because these splice variants were involved in cell migration and polarization. Re-expression of either EP3α or EP3β in VSMCs from EP3 KO mice elevated the level of phosphorylation of Akt and GSK3β induced by PGE₂. This effect was blunted by the PI3K selective inhibitors, wortmannin, and LY294002 but not affected significantly by HIMO or SB216763 (Figure 8F). However, elevation of Rac1 activity by transfection with either EP3α or EP3β transfection with either EP3α or EP3β cDNA was blunted by the selective PI3K inhibitors, wortmannin, and LY294002 but not affected significantly by HIMO or SB216763 (Figure 8B), indicating that the PGE₂-induced Rac1 activation depends on PI3K activity through the EP3α/β receptors (Figure 8F).

Discussion
Vascular remodeling is differentially regulated by PGs derived from COX-1 and COX-2. Pharmacological inhibition of COX-2 suppresses the vascular remodeling in response to balloon injury, whereas genetic knockdown (data not shown) or pharmacological inhibition of COX-1 does not affect this response. We found here that deletion of COX-2 or the downstream EP3 receptor attenuates vascular neointima formation after wire injury in mice; VSMC migration and cell polarization toward directional migration stimulated by PGE₂ could be impaired by pharmacological inhibition or genetic modulation of EP3α or EP3β receptors through downregulation of small GTPases. Activation of the EP3α or EP3β receptors directly fine-tune RhoA activity through inhibitory Gαi signaling, whereas liberated Gβγ subunits regulate Cdc42 and Rac1, and RhoA activities in VSMCs through the PI3K/Akt/GSK3β pathway.

COX-2 expressed in vasculature, including VSMCs, is markedly induced during vascular inflammation. Vascular COX-2 is the dominant source of PGI₂, and PGE₂. Thus, deletion of COX-2 significantly reduces systemic PGE₂ and PGI₂ production. As shown by the data presented in Figure 2 and Online Figure I, COX-1 knocked-in to the COX-2 locus (COX-1>COX-2) could rescue PGE₂ production, but not PGI₂ production, which aggravated the vascular response to injury, consistent with previous observations on the disruption of the IP receptor.

Analogous to pharmacological studies, COX-2 deficiency conferred protection against neointima formation along with decreased production of PGE₂ and PGI₂. Therefore, we reasoned that COX-2-derived PGE₂, from both VSMCs and the infiltrated inflammatory cells, predominantly promoted cell polarity and migration responses were abolished by pretreatment with Wortmannin, HIMO, or SB216763, which specifically inhibit signaling through the PI3K pathway (Figure 8E). Thus, the PI3K/Akt/GSK3β signaling axis is involved in the regulation of VSMC migration and polarity through the EP3α/β receptors.

The activation of Cdc42 and Rac1, key regulators of cell migration, was impaired in EP3-deficient VSMCs. Again, re-expression of either EP3α or EP3β in EP3-deficient VSMCs markedly rescued the decreased activation of Rac1 and Cdc42 (Figure 8C and 8D). Pharmacological inhibition of PI3K (Wortmannin), Akt (HIMO), and GSK3β (SB216763) abrogated the activation of Rac1 induced by PGE₂, in EP3α-expressing, EP3β-expressing, and WT VSMCs (Figure 8B). Similarly, elevation of RhoA activity by transfection of VSMCs with either EP3α or EP3β was blocked by wortmannin, HIMO, and SB216763 (Online Figure X). These results suggest that Rac1 and RhoA activity can be regulated by the EP3α/β-mediated PI3K/Akt/GSK3β signaling pathway (Figure 8F). However, elevation of Cdc42 activity by EP3α or EP3β transfection with either EP3α or EP3β cDNA was blunted by the selective PI3K inhibitors, wortmannin, and LY294002 but not affected significantly by HIMO or SB216763 (Figure 8B), indicating that the PI3K/Akt/GSK3β signaling axis is involved in the regulation of VSMC migration and polarity through the EP3α/β receptors.
vascular neointimal hyperplasia, because impaired PGE₂ synthesis in mice attenuates the vascular response. In this study, COX-2 deletion impaired VSMC migration, whereas exogenous PGE₂ accelerated VSMC migration. Pharmacological inhibition, gene silencing using small interfering RNA, or genetic deletion of the EP3 receptor restricted PGE₂-induced VSMC migration, indicating that COX-2−derived PGE₂ mediated VSMC migration through EP3 α/β activation (Figure 2). Recently, EP3 was shown to promote the migration of other cells, such as airway smooth muscle cells. Deficiency of EP3 attenuated the thickening of the neointima, whereas overexpression of EP3α or EP3β in vasculature augmented its formation in mice, which suggests that EP3-mediated VSMC migration contributes to the vascular remodeling in response to mechanical injury. Because endothelium loss triggers VSMC migration through fenestrae in the internal elastic lamellae into the intima and subsequent proliferation, EP3 may be involved in mediating the early events in restenosis, although other PGE₂ receptor subtypes, such as EP2, or perhaps EP4, might participate in later events, including VSMC proliferation. However, different EP receptors play different roles in stabilizing atherosclerotic plaque.

Small GTPases, including Rho, Rac, and Cdc42, are pivotal for MTOC orientation and for cell migration. Pharmacological inhibition or genetic deficiency of EP3 impairs MTOC reorientation and decreases the activities of Rac1, RhoA, and Cdc42 on PGE₂ stimulation, which lead to restriction of VSMC migration and reduction of vascular neointima formation in response to injury in mice. These observations also are consistent with the notion that repositioning the centrosome stabilizes the chosen direction of movement through regulation of the microtubule system. Cdc42 is believed to be a master regulator of cell polarity by directing MTOCs reorientation toward the direction of movement, despite accumulated evidence showing that Rho and Rac are involved in the coordinate regulation of cell polarization. In the present study, pharmacological inhibition of PI3K signaling diminished Cdc42 activity and disrupted the reorientation of the MTOCs in cells overexpressing EP3α/β receptors. Surprisingly, inhibition of Akt and GSK3β, which act downstream of PI3K, reduced Rac1 and RhoA activity in VSMCs without a detectable impact on Cdc42 and inhibited the reorientation of the MTOCs toward directional migration, suggesting the involvement of Rac1 and RhoA in the microtubule rearrangement and cell polarity as described previously.

EP3α and EP3β splice variants couple with Gαi1, resulting in the inhibition of adenyl cyclase, whereas activation of the EP3γ elevates cAMP, probably through Gαs. Stimulation of G-protein–coupled receptors activates PI3K/Akt/GSK3β signaling by free Gβγ or indirectly by Gα by competitive binding of axin and releasing the GSK3β substrate–β-catenin. Here, we found that the EP3α or EP3β receptors, but not EP3γ, mediated VSMC migration and vascular neointimal hyperplasia in mice. The Gαi-activated PKA pathway, signaling through the PI3K/Akt/GSK3β pathway that mediated cell migration, was shown to occur on activation of EP3α or EP3β receptors through the liberation of their coupled Gβγ subunits in VSMCs. PKA inhibits RhoA-dependent function by inducing Ser188 phosphorylation of RhoA and subsequent cytoplasmic sequestration of GTP-RhoA by Rho GDP dissociation inhibitor. Coincidentally, EP3 inhibition or deletion reduced, whereas overexpression of EP3α or EP3β elevated, RhoA activity through a Gαi-mediated cAMP/PKA pathway. RhoA and its key downstream effector ROCK are involved in neointima formation after vascular injury, probably through regulation of cell shape and migration by modulating stress fiber formation. We also observed a partial but significant increase of VSMC migration through upregulation of RhoA activity by inhibition of PKA, despite

Figure 7. EP3α/β are coupled via Gαi protein and regulate RhoA activity and vascular smooth muscle cell (VSMC) migration through the cAMP/protein kinase A (PKA) pathway. A, Western blot analysis of binding of EP3α/β and Gαi. B, Cellular cAMP levels were measured in VSMCs isolated from EP3 knockout (KO) mice transfected with EP3α, EP3β, or control vector constructs with or without the PKA inhibitor H-89 (10 μmol/L). Data are normalized to the control group. *P<0.05 vs EP3KO transfected with control vectors, n=3. The results were repeated 3 times. C, PKA activity was measured in VSMCs from EP3 KO or WT mice transfected with EP3α, EP3β, or control vector constructs treated with or without the PKA inhibitor H-89 (10 μmol/L). Data are normalized to the values of the control group. *P<0.05, n=3. The results were repeated 3 times. D, Western blot analysis of binding of EP3α, EP3β, or control vector constructs with or without pretreatment with the PKA inhibitor H-89 (10 μmol/L). E, Effect of H-89 on reorientation of microtubule organizing center (MTOC) of EP3 KO and WT VSMCs. *P<0.05, n=3. The results were repeated 3 times. F, Effect of H-89 on the migration of VSMCs from EP3 KO and WT mice transfected with EP3α, EP3β, or control vectors constructs. The number of migrating cells was normalized to the control group. *P<0.05 as indicated, n=4.
substantial suppression effect of ROCK inhibitor. Moreover, we failed to detect analogous inhibitory effects of H-89 on the activity of Rac1 and Cdc42 in VSMCs similar to that of RhoA (Online Figure IX). These observations strongly indicate additional non-cAMP/PKA pathways mediated by EP3α/β receptors are involved in the regulation of VSMC migration and activity of small GTPases.

The phosphorylation status of GSK3β, a key downstream substrate of PI3K and Akt, is essential for maintaining cell polarity.27 PGE2 stimulation and wound scratch activated PI3K signaling and induced rapid inhibitory phosphorylation of GSK3β in VSMCs, which could be attenuated by genetic deficiency or pharmacological inhibition of EP3. Moreover, the reduced PI3K signaling in EP3 KO VSMCs could be rescued by the EP3α/β overexpression. Pharmacological inhibition of the EP3α/β-activated PI3K signaling reduced the capability of migration and impaired the repositioning of the MTOCs. These observations demonstrate that PI3K/Akt/GSK3β signaling mediated by the EP3α/β receptors is involved in the regulation of migration and polarization of VSMCs and the vascular remodeling response to injury.

In summary, we showed that COX-2-derived PGE2 accelerates neointima formation in response to injury through the EP3α/β receptors in mice, and that the EP3α/β-mediated Gαi/cAMP/PKA and Gβγ/PI3K/Akt/GSK3β pathways play a role in regulation of the activation of small GTPases and the subsequent VSMC polarization and migration on stimulation with PGE2. Given that selective inhibition of COX-2 confers a cardiovascular hazard attributable to inhibition of COX-2-derived PGI2, inhibition of signaling through the EP3 receptor may be a viable target for prevention of in-stent restenosis.

Sources of Funding

This work was supported by grants from the Ministry of Science and Technology of China (2012CB945100, 2011CB503906, and 2011ZX09307-302-01) and by the National Natural Science Foundation of China (81030004), the Natural Science Foundation of China-Canadian Institutes of Health Research-joint grants (NSFC81161120538 and CIHR-CCI117951), the National Institutes of Health grants (R01 DK46205 and R01 DK37097), The Knowledge Innovation Program of the Chinese Academy of Sciences (KSCX2-EW-R-09), and the Clinic Research Center at Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences (CRC2010007).
Yu was supported by the One Hundred Talents Program of the Chinese Academy of Sciences (2010OHTP10) and Pujiant Talents Program of Shanghai Municipality (11PJ1411100). C.D. Funk is a Canada Research Chair holder and recipient of an Ontario Heart and Stroke Foundation Career Investigator award. R.M. Breyer has a Merit Award from the Department of Veterans Affairs.

Disclosures
None.

References

What Is Known?

• Pharmacological inhibition of cyclooxygenase (COX)-2, but not COX-1, reduces vascular response to mechanical injury.
• Genetic disruption of microsomal prostaglandin (PG)E2 synthase-1 attenuates neointima formation after vascular injury.

What New Information Does This Article Contribute?

• COX-2–derived PGE2 promotes vascular neointimal hyperplasia in response to mechanical injury.
• COX-2–derived PGE2 regulates the polarization and directional migration of vascular smooth muscle cells (VSMCs) via both EP3α and EP3β receptors.
• EP3α/β modulates small GTPase activity in VSMCs through both cAMP/protein kinase A and phosphatidylinositol 3-kinase pathways.

VSMC migration from media to intima and subsequent proliferation are the hallmarks of pathogenesis of restenosis after angioplasty. However, the mechanism that regulates the VSMC migration response to injury is not fully understood. In this study, we show that PGE2, derived from COX-2, not COX-1, promotes vascular neointima formation followed by mechanical injury. Both EP3α and EP3β isoforms are identified to mediate PGE2–induced VSMC migration via multiple approaches. Deletion of EP3 receptor influences polarization and directional migration of VSMCs by decreasing small GTPase activity and restricts vascular neointimal hyperplasia, whereas overexpression of EP3α and EP3β augments neointima formation. Activation of the EP3α or EP3β receptors directly fine-tunes RhoA activity in VSMCs through inhibitory Gαi signaling and regulates other small GTPase activity through the liberated Gβγ subunits. Thus, COX-2–derived PGE2 facilitates neointimal hyperplasia response to injury through EP3α/β–mediated cAMP/protein kinase A and phosphatidylinositol 3-kinase pathways, indicating EP3 receptor may be a promising target for prevention of in-stent restenosis.
Cyclooxygenase-2–Derived Prostaglandin E₂ Promotes Injury-Induced Vascular Neointimal Hyperplasia Through the E-prostanoid 3 Receptor

Jian Zhang, Fangfang Zou, Juan Tang, Qianqian Zhang, Yanjun Gong, Qingsong Wang, Yujun Shen, Lixia Xiong, Richard M. Breyer, Michael Lazarus, Colin D. Funk and Ying Yu

_Circ Res._ 2013;113:104-114; originally published online April 17, 2013;
doi: 10.1161/CIRCRESAHA.113.301033

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Supplemental Material

Supplemental Methods

Animals
COX-1>COX-2 and COX-2 null mice used for the experiments were initially produced on a mixed C57BL/6 x Sv129 genetic background (50%:50%) and maintained on this hybrid C57BL/6/ Sv129 background for over 20 generations. EP3\(^{\text{floxed/floxed}}\) mice were maintained on a mixed C57BL/6 x Sv129 genetic background\(^{22}\) and crossed with FVB EIIa Cre to generate EP3 KO mice. All animals were maintained and used in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences, Chinese Academy of Sciences.

Reagents
AH-6809, L-161,982, Pertussis Toxin, arachidonic acid (AA), PGE\(_2\), Misoprostol, H-89, Y-27632 (hydrochloride), LY294002, Wortmannin, and SB216763 were obtained from Cayman Chemical Company (Cayman Chemical, Ann Arbor, MI). LPS and L-798,106 were purchased from Sigma Chemical Company (Sigma-Aldrich, St. Louis, MO). SC-51322, HIMO (1L-6-Hydroxymethyl-chiro-inositol-2-[(R)-2-O-methyl-3-O-octadecylcarbonate]), and forskolin were obtained from Enzo (Enzo, Plymouth Meeting, PA, USA).

Cell culture
8- to 12-week-old mice were sacrificed by CO2 overexposure. Aortas were isolated from the arch to the iliac bifurcation, and cut into 4mm long pieces then covered by an autoclaved glass cover slip. Smooth muscle cells were cultured in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum at 37 °C, 5 % CO2. Cells at passages 3 to 5 were used in all experiments. Each experiment was repeated at least 3 times with different cell preparations.

Femoral artery wire injury model
The femoral artery wire injury model was described previously elsewhere\(^{17}\). In brief, 8-12 week old male mice were anesthetized with isoflurane during surgery. The sham-injured left femoral arteries served as negative controls. Right femoral arteries were exposed by blunt dissection and monitored under a surgical microscope. After the distal artery was encircled with a 5-0 nylon suture, a vascular clamp was placed near the inguinal ligament, and a guide wire 0.38 mm in diameter (Cook Inc, Bloomington, IN) was inserted into the arterial lumen through an arteriotomy made in the distal perforating branch. The guide wire was left in place for 3 minutes to denude the artery. Then, the wire was removed, and the silk suture was released to restore blood flow. The skin incision was closed with a 6-0 silk suture. Mice were allowed to recover and carotid arteries were harvested 4 weeks after the injury and fixed in 4% formalin overnight. Sections were stained with hematoxylin and eosin. Intimal area is the area encircled by internal elastic lamina minus lumen area. Medial area is calculated as the area encircled by the external elastic lamina minus intima area. The intima-to-media ratio was calculated as the intimal area divided by the medial area. Restenosis index was calculated as intimal area divided by the area encircled by internal elastic lamina.
**Immunofluorescence staining**

For immunohistochemistry, tissue sections were incubated with primary antibodies against α-smooth muscle actin (Sigma-Aldrich), followed by incubation with horseradish peroxidase–conjugated secondary antibody (ProteinTech Group, Inc, Chicago, IL). After three washings with PBS, samples underwent DAB staining, hematoxylin restaining, dehydration, detected with horseradish peroxidase-conjugated antibody and cover glass mounting. For immunocytochemistry, the VSMCs grown on slides and serum-starved overnight were “scratched” with a P200 Gilson pipette tip and challenged with PGE$_2$ (10 μmol/L). 6 Hours after “wounding”, VSMCs were fixed in cold methanol, washed with PBS, blocked in TBST containing 1% BSA and stained with primary antibodies overnight at 4°C, stained with secondary antibody for 2 hours at room temperature and photographed using fluorescent microscopy. Primary antibodies were used at the dilutions indicated: anti-α-tubulin (Cell Signaling Technology, Danvers, MA), 1:1000; anti-F-actin (Cell Signaling Technology), 1:1000 and γ-tubulin (Sigma-Aldrich), 1:2000 to identify the Microtubule Organizing Centers (MTOCs) reorientation. The MTOCs that localized between the nucleus and the leading edge were determined as front-polarized cells. The percentage of front-polarized cells was calculated to assess cell polarity and migration during wound healing process. After incubation with Alexa Fluor-conjugated secondary antibodies (1:1000; Invitrogen, Carlsbad, CA) staining was performed with DAPI. The samples were mounted (ProLong® Gold Antifade Reagent; Invitrogen) on glass slides and were visualized using an inverted fluorescent microscope (Carl Zeiss, Oberkochen, Germany). Subsequent image processing was performed using Photoshop CS2.

**RNA Extraction and Real-Time PCR**

Total RNA from vascular tissues and primary VSMCs was extracted by use of Trizol reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer’s instructions. Briefly, total RNA was reverse-transcribed to cDNA by use of Reverse Transcription Reagent kits (Takara) according to the manufacturer’s protocol. Real-time PCR was performed with use of SYBR Green mix (TaKaRa Biotechnology, Co., Ltd. Dalian, P. R. China). Each sample was analyzed in triplicate and was normalized to the level of actin mRNA. The PCR protocol was 5 min at 95°C for 1 cycle followed by 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 5 minutes. A dissociation curve was obtained for each PCR product. The primer sequences are summarized in Supplemental Table I. PCR products were validated by electrophoresis on 2% agarose.

**Western blot**

VSMCs were incubated with different inhibitors before PGE$_2$ stimulation. The proteins were extracted in lysis buffer containing protease inhibitors. Total cell protein was determined by the BCA method using Pierce BCA Protein Assay Kit (Pierce, Rockford, IL). VSMCs were resolved by SDS-PAGE with 8% Tris acetate or 8% Tris-glycine gels. The proteins were transferred electrophoretically onto ImmobilonP membranes (Millipore, Bedford, MA), which were probed using antibodies against COX-1 (1:1000; Cayman), COX-2 (1:1000; Cayman), Gai (1:1000; SantaCruz), HA-tag (1:1000), P(Ser9)-GSK3β (1:1000), GSK3β (1:1000), P(ser473)-Akt (1:1000) and Akt (1:1000) and horseradish peroxidase–conjugated secondary antibodies (ProteinTech). Equivalent protein loading was evaluated with anti-Actin (1:2000; Sigma-Aldrich) antibody. An ECL detection kit (Super Signal (R) West Pico Chemiluminescent Substrate) was used to detect protein, which was quantified by image J 1.44p.
**PG extraction and analysis**

Mouse urine was collected for 24 h in metabolic cages before and 3 days after wire injury. Urinary prostanoid metabolites were extracted and quantitated utilizing liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) analyses. In brief, to 100 µl of urine was added 31 µl acetonitrile (ACN) containing internal standard. The sample was mixed well and allowed to equilibrate at room temperature for 15 min, at which time 262 µl of an aqueous solution of methoxyamine hydrochloride (1g/ml) was added and the sample again was allowed to stand for 30 min. The sample was then diluted to 1 ml with water and applied to a StrataX SPE cartridge that was conditioned with 1ml ACN. The StrataX SPE cartridge was washed with 1 ml 5% ACN in water, dried by the application of vacuum for 15 min, and eluted with 2 ml solvent (ethyl acetate: ACN, 1:1). The eluant was dried under a gentle stream of nitrogen, dissolved in 100 µl 10% ACN in water, and passed through small centrifugal filters with a 0.2-µm nylon membrane filtered before analysis by LC/MS/MS. The urinary PG metabolites were normalized against creatinine.

For PG analysis in culture, cells were incubated in fresh medium containing arachidonic acid (AA, 30µmol/L; Cayman) as substrate for 10 minutes, and the medium was collected for PG production analysis and normalized to total protein.

**Rho GTPase Activity Assay**

Smooth muscle cells were grown to confluence on 15-cm² dishes and equal numbers of serum-starved cells were incubated with PGE₂ (10µmol/L) for 5 minutes at 37°C or vehicle in DMEM-F12. The cells were washed with cold PBS and then harvested in ice-cold lysis buffer. Cdc42/Rac1/RhoA activation assay kits were purchased from Cytoskeleton (Cell Biolabs, San Diego, CA) and used to detect active Cdc42, Rac1, and RhoA according to the manufacturer’s protocol.

**VSMC migration assay**

VSMC migration was assessed by wound healing and transwell assay. For the scratch wound healing, Cells were serum starved for 24 hours and confluent monolayers were scratched with a P200 Gilson pipette tip. Cellular progress was photographed at indicated timepoints after scratching and quantified by wound area at fixed location using Image Pro software (Media Cybernetics, Bethesda, MD). 6 fields at fixed location were selected per well to calculate cell migration. The data were collected from 3-4 independent experiments.

For transwell migration assays, 24-well cell culture inserts 6.5 mm in diameter with polycarbonate membrane filters containing 8-µm pores (Corning Inc, Corning, NY) were used. Cells were pretreated with PGE₂ (10µmol/L), then harvested and added into the insert. Culture medium containing 20% fetal bovine serum was added to the lower chamber. After 3 hours, the filter was fixed with cold 4% paraformaldehyde for 5 minutes, then non-migrating cells were removed from upper filter surfaces, the cells on the underside were stained by a 3-step stain set (Rechard-Allan scientific). 5-7 randomly selected fields of migrated cells were photographed and counted per well. Each experiment was duplicated. Data were collected from 3-4 independent experiments.

**Lentivirus generation and Transduction**

Lentivirus was produced using pGLV-EF1α-EGFP vector and cDNA encoding full-length mouse EP3α, EP3β or EP3γ cloned into the vector, and generated in the 293T viral packaging cell line. Femoral arteries were transduced locally with 10⁹ IU per mouse in the presence of 10µg/mL
DEAE-dextran after wire injury. The transduction efficiency was estimated by fluorescence using an EGFP-antibody (Amalgam).

**Overexpression of mEP3 Variants**
The mouse EP3 variant cDNAs were subcloned into pcDNA3.1 and a HA tag was added at the extracellular N terminus. Correct subclones were confirmed by sequencing. VSMCs were transiently transfected with each pcDNA3/EP3 variant or pcDNA3 empty vector using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer protocol.

**RNA Interference**
VSMCs were grown to about 80% confluence for siRNA transfection by RNAiFect Transfection Reagent (Qiagen, Crawley, UK) according to the manufacturer’s instructions with either a siRNA pool (GenePharma, Shanghai, P. R. China) containing three pairs of specific sequences for EP3 (EP3-1, EP3-2 and EP3-3, 100 nmol/L), EP3 splice variant targeting sequences or a scrambled siRNA (as a negative control; 100 nmol/L). The mouse EP3 siRNAs were designed to target 21-nucleotide sequences (Supplement Table II). Control siRNAs included scrambled derivatives of the EP3 siRNA sequences, an unrelated siRNA labeled by FAM, and a positive control GAPDH siRNA. In brief, knockdown efficiency was assessed by qRT-PCR. 4–8 x 10^4 cells per well were seeded in a 24-well plate overnight. 1 μg siRNA and RNAiFect Transfection Reagent were mixed and incubated for 10–15 min at room temperature to allow formation of transfection complexes before delivery on the cells.

**Coimmunoprecipitation**
VSMCs were transiently transfected with pcDNA3/EP3 variants or pcDNA3.0 empty vector using Lipofectamine 2000 transfection reagent and then serum-starved VSMCs were challenged with PGE2 for 5 min after 48h transfection. EP3 receptors were immunoprecipitated by incubating 1mg of whole cell lysates in immunoprecipitation buffer with 5 μl of HA-tag antibody or normal IgG (Cell Signaling Technology) control at 4 °C for 3 h, followed by incubation with protein A/G agarose (Invitrogen) at 4 °C overnight with gentle agitation. After extensive washing, the immune complexes were recovered by boiling in loading buffer, and the proteins were detected by Western blotting with HA-tag antibody or Gα antibody (Santa Cruz Biotechnology).

**Measurement of cAMP levels**
70% Confluent monolayer cells were serum-starved overnight and then incubated at 37°C for 10 min with 10μmol/L Misoprotol, 1μmol/L forskolin or vehicle in DMEM-F12, respectively. The cells were washed with cold PBS and then harvested in ice-cold lysis buffer at a concentration of 1 x 10^7 cells/mL on ice. The amount of intracellular cAMP was determined using a cAMP Parameter Assay Kit (R&D Systems, Minneapolis, MN), according to the manufacturer’s instructions.

**PKA activity assay**
PKA activity was determined using a PKA kinase activity kit (Enzo Life Sciences) in a 96-well plate based assay according to the manufacturer’s recommendations. Primary VSMCs were grown in six-well plates and incubated with inhibitors before stimulated by 10μmol/L Misoprotol and lysed in 100μl buffer on ice. ATP (10μl; 2g/L) was added to the lysates to initiate the reaction at 30°C for 30
min and then incubated with 40μL of Phospho-specific Substrate Antibody at room temperature for 60 minutes. After extensive wash, Anti-Rabbit IgG: HRP conjugate (40μL; 1ng/L) was added and incubated at room temperature for 30 minutes. The reaction was terminated by adding 20μl stop solution. tetrathemylbenzidine substrate (TMB) was used as a highly specific substrate and absorbance measured at 450 nm for assessment of PKA activity.

**Statistical Analysis**
Data analysis was performed by using Prism 5.0. Data are expressed as the means ± SEM. Comparisons between two groups were analyzed by Student's t test. Comparisons between three or more groups were assessed by 2-way analysis of variance (ANOVA). Values of P < 0.05 were considered significant.

**Supplemental Table I**
Primers for real-time PCR analysis

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**Supplemental Table II**
siRNA sequences

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Supplement Figure I. Inflammatory cell staining of injured femoral arteries from wild type, COX-2 KO and COX-1>COX-2 mice at day 28.

Injured artery crossections were immunostained for CD11b, a marker for monocytes (A), CD68, a marker for macrophages(C), SM -actin, a marker for smooth muscle cells (E). The box outlines the region enlarged to the below. Scale bar, 50μm. CD11b+(B) and CD68+(D) cells were quantitated in random field. Data are presented as mean ± SEM, *P<0.05 vs. WT, n = 8-10.
Supplementary Figure II. PG profile of VSMCs from COX-2 KO, COX-1>COX-2 and WT mice.
VSMCs were incubated with arachidonic acid (30 μmol/L) for 10 min. The supernatants were collected, PGD$_2$ (A), PGE$_2$ (B), PGF$_{2α}$ (C), 6-keto-PGF$_{1α}$ (stable hydrolyzed product of PGI$_2$, D) and TxB$_2$ (E) were analyzed by mass spectrometry. Data were normalized to the values of WT. Data were shown as mean ± SEM, *P < 0.05 vs. WT, n = 9.
Supplement Figure III. COX-2 deletion inhibited the migration of cultured VSMCs.

A, Representative phase-contrast images of VSMCs from COX-2 KO and WT mice were taken at 0, 6, and 12 h after scratching. B, The wound area was measured and quantitated as described in the Methods section. Data are normalized by the wound area at the 0 timepoint. * P < 0.05 vs. WT, n = 3.
Supplement Figure IV. Knockdown and overexpression of EP3 in VSMCs and in injured femoral arteries.

A, EP3 knockdown by a common siRNA pool significantly retarded VSMC migration (right panel). Knockdown efficiency was analyzed by RT-PCR (left panel). Data are presented as mean ± SEM, *P < 0.05 vs. scrambled, n = 3. B, mRNA levels of each EP3 variant were analyzed by RT-PCR in the presence or absence of LPS. C, EP3α/β knockdown by specific siRNAs significantly retarded VSMC migration (right panel). Knockdown efficiency was analyzed by RT-PCR (left panel). *P < 0.05 vs. scrambled control, n = 3. D, Relative mRNA levels of EP3(common), EP3α, EP3β or EP3γ variants were analyzed by RT-PCR in VSMCs from EP3 KO mice transfected with EP3α, EP3β, EP3γ or control vector constructs, respectively. The data were normalized to the levels of β-actin. *P < 0.05 vs. untransfected control, n = 3. E, Femoral arteries were transduced with lentivirus encoding EP3α, EP3β, or EP3γ after injury in EP3 KO mice. Transduction efficiency was detected by immunofluorescence using a GFP-specific antibody (red), Nuclei were stained with DAPI (blue). The white dotted line represents the internal elastic lamina. Scale bar, 50μm.
Supplement Figure V. mRNA expression of all PG receptors in VSMCs from EP3 KO and wild type mice. Relative mRNA level of PG receptors was analyzed by RT-PCR with or without LPS stimulation in VSMCs from wild type mice (A) and EP3 KO mice (B). The data were normalized to the levels of β-actin. The results were repeated 3 times.
Supplement Figure VI. Urinary excretion of PGDM, PGEM, PGFM, PGIM and TxM in EP3 KO mice and wild type mice after wire injury.

24 Hour urine samples from EP3 KO and WT male mice were collected. Urinary excretion of PGDM(A), PGEM(B), PGFM(C), PGIM(D) and TxM(E) were measured. Data are normalized to the values of WT before injury. Data are presented as mean ± SEM, *P < 0.05 vs before injury group, n = 8-10.
Supplement Figure VII. MTOC reorientation in VSMCs response to wound.
Cells with front polarized MTOCs at wounding edges in response to PGE$_2$ were counted at 6 h after wounding or without wound. The average of 5-6 random selected fields were photographed per slide. Data are presented as mean ± SEM, *P < 0.05 vs. No wound, n = 6. The experiments were repeated 3 times.
Supplement Figure VIII. The effect of H-89 on Rac1 and Cdc42 activity in EP3α and EP3β re-expressed VSMCs.

VSMCs from EP3 KO mice were transfected with and control vectors, respectively. Cells were pretreated with PKA inhibitor H-89 (10 μmol/L) and stimulated with PGE2 (10 μmol/L). Cells lysates was subjected to pull down assay followed by Western blots using antibodies against Rac1, Cdc42 and HA-tag.
Supplement Figure IX. EP3α/β mediated activation of PI3K-Akt-GSK3β axis upon PGE2 stimulation. 

**A**, PGE2-stimulated GSK3β phosphorylation in VSMCs. **B**, Dynamic change of GSK3β phosphorylation in VSMCs in response to treatment with the EP3 antagonist L-798,106 (10 μmol/L) and Gi antagonist PTX (0.5 mg/L). Left, representative western blots of phosphorylation of GSK3β and Akt of VSMCs at 6 h post wounding. Right, Quantification of GSK3β phosphorylation normalized to total GSK3β. Data are presented as mean ± SEM, *P < 0.05, n = 3. The results were repeated 3 times. **C** Effect of Akt inhibitor (HIMO, 10 μmol/L) and GSK3β inhibitor (SB216763, 20 μmol/L) phosphorylation of GSK3β in EP3 and EP3β re-expressed VSMCs isolated from EP3 KO mice. **D** Effect of PKA inhibitor (H89, 10 μmol/L) on phosphorylation of GSK3β in EP3 and EP3β re-expressed VSMCs isolated from EP3 KO mice.
Supplement Figure X. RhoA activity alteration in response to PI3K/ Akt/ GSK3β inhibition in EP3α and EP3β re-expressed VSMCs.

VSMCs isolated from EP3 KO mice were transfected with EP3α, EP3β, or control vector constructs, respectively. Cells were pretreated with PI3K inhibitor, Wortmannin (100 nmol/L), AKT inhibitor, HIMO (10 μmol/L), and GSK3β inhibitor, SB216763 (20 μmol/L) and stimulated with PGE₂ (10 μmol/L). Cells lysates was subjected to pull down assay using HA-tag antibody followed by Western blots using RhoA and HA-tag antibodies.