Cell Selective Cardiovascular Biology of Microsomal Prostaglandin E Synthase-1

Lihong Chen, MD, PhD; Guangrui Yang, MD, PhD; Xiufeng Xu, PhD; Gregory Grant, PhD; John A. Lawson, BS; Mohammad Bohlooly-Y, PhD; Garret A. FitzGerald, MD, FRS

Background—Global deletion of microsomal prostaglandin E synthase 1 (mPGES-1) in mice attenuates the response to vascular injury without a predisposition to thrombogenesis or hypertension. However, enzyme deletion results in cell-specific differential use by prostaglandin synthases of the accumulated prostaglandin H₂ substrate. Here, we generated mice deficient in mPGES-1 in vascular smooth muscle cells, endothelial cells, and myeloid cells further to elucidate the cardiovascular function of this enzyme.

Methods and Results—Vascular smooth muscle cell and endothelial cell mPGES-1 deletion did not alter blood pressure at baseline or in response to a high-salt diet. The propensity to evoked macrovascular and microvascular thrombogenesis was also unaltered. However, both vascular smooth muscle cell and endothelial cell mPGES-1–deficient mice exhibited a markedly exaggerated neointimal hyperplastic response to wire injury of the femoral artery in comparison to their littermate controls. The hyperplasia was associated with increased proliferating cell nuclear antigen and tenascin-C expression. In contrast, the response to injury was markedly suppressed by myeloid cell depletion of mPGES-1 with decreased hyperplasia, leukocyte infiltration, and expression of proliferating cell nuclear antigen and tenascin-C. Conditioned medium derived from mPGES-1–deficient macrophages less potently induced vascular smooth muscle cell proliferation and migration than that from wild-type macrophages.

Conclusions—Deletion of mPGES-1 in the vasculature and myeloid cells differentially modulates the response to vascular injury, implicating macrophage mPGES-1 as a cardiovascular drug target. (Circulation. 2013;127:233-243.)

Key Words: endothelial cell  mPGES-1  macrophage  vascular injury  vascular smooth muscle cell

Prostaglandin E₂ (PGE₂) mediates pain and inflammation and has been implicated in the regulation of blood pressure, thrombosis, and atherogenesis. It is generated from a prostaglandin substrate, prostaglandin H₂, itself formed by the metabolism of arachidonic acid by PGG/H synthase enzymes, commonly known as cyclooxygenases. Three distinct forms of PGE synthases have been identified, 1 cytosolic and 2 microsomal, designated cPGES, mPGES-1, and mPGES-2, respectively. Based on excretion of the major urinary metabolite of PGE₂, 9,15-dioxy-11α-hydroxy-2,3,4,5-tetranor-prostane-1,20-dioic acid, mPGES-1 is the dominant contributor to biosynthesis of PGE₂ under physiological conditions in humans and rodents.

Clinical Perspective on p 243

mPGES-1 attracted attention as a drug target following the association of nonsteroidal anti-inflammatory drugs selective for inhibition of cyclooxygenase 2 (COX-2) with cardiovascular adverse effects. Some but not all studies suggested that the impact of mPGES-1 deletion was indistinguishable from that resulting from deletion or inhibition of COX-2 in animal models of analgesia. Furthermore, we reported that, unlike COX-2 deletion or inhibition, global deletion of mPGES-1 did not render mice more susceptible to hypertensive or thrombogenic stimuli. This differential impact on cardiovascular biology was consistent with enhanced biosynthesis of cardioprotective prostacyclin (PGI₂) in mPGES-1 knockout mice in contrast to its suppression consequent to COX-2 disruption. Studies of mice lacking the 1 prostanoid receptor for PGI₂ reveal accelerated atherogenesis and an enhanced response to vascular injury, whereas atherogenesis and the proliferative response to vascular injury are restrained in mPGES-1 knockout mice coincident with enhanced biosynthesis of PGI₂, as reflected by augmented urinary 2,3-dinor 6-keto prostaglandin F₁α.

Although these results raise the possibility that mPGES-1 inhibitors might not only have a desirable cardiovascular risk profile, but also possess cardiovascular efficacy, some
fundamental concerns remain. Specifically, the consequences of mPGES-1 deletion might differ among cell types, reflective of prostaglandin H₂ substrate diversion to differing prostaglandin synthases with consequent formation of products with contrasting biological effects. Furthermore, in some settings, an inflammatory response may have a salutary effect; 1 example is cardiac remodeling after myocardial infarction which is impaired in mPGES-1 global knockout mice. To elucidate the cell-specific biology of mPGES-1, we generated mice lacking the enzyme selectively in vascular and myeloid cells and examined their response to vascular injury.

Methods

Animals

The mPGES-1-flox mice (described in online-only Data Supplement Methods) were crossed with SM22Cre, Tie2Cre, or LysMCre mice (Jackson laboratory) to yield mice with mPGES-1 deletion in vascular smooth muscle cells (VSMCs), endothelial cells (ECs), or myeloid cells, respectively (termed SM22Cre/flox, Tie2CRE/flox, and Mac-mPGES-1-KO). All mice at 3 to 4 months of age were maintained under 12:12 hours light/dark cycle (lights on at 7 AM and lights off at 7 pm) before and throughout experiments. All procedures were in accordance with the guidelines approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Wire-Mediated Vascular Injury

Wire injury of the femoral artery was performed as previously described. In brief, after anesthesia, the right femoral arteries were exposed by blunt dissection. The distal femoral artery was incircled with a 6-0 silk suture for temporary cessation of the blood flow and then removed, and the silk suture was denuded and dilated the artery and then removed, and the wire was left in place for 3 minutes to into the arterial lumen through a transverse arteriotomy made in the exposed carotid artery from a distance of 5 cm for 120 minutes until thrombosis occurred, after which the mice still showing blood flow were assigned as a value of 120 minutes. Stable occlusion was defined as a blood flow at 0 mL/min for 5 minutes.

Intravitral Microscopy

Anesthetized adult male mice were kept at 37°C by using a thermal pad. A jugular vein catheter was inserted for subsequent intravenous injections of antibodies and drugs. A small incision was performed on the scrotum along the midline, and the cremaster muscle was exposed, pined, and continuously rinsed with phosphate-buffered saline. Arteriolar injury was induced in the cremaster muscle by a nitrogen dye blue laser (440 nm, FRET/FRAP/Photoablation system). Digital sequence images of thrombus formation were obtained by using an Olympus BX61WI microscope. For quantification of microthrombosis, fluorescence intensity was measured and corrected by the background brightness for each image as previously described.

Cell Culture

Primary mouse ECs and VSMCs from thoracic aorta were isolated as previously described with modifications. In brief, after removal of the connective tissues, mouse aortas were cut into rings of 2 mm in length. For EC culture, the aortic segments were placed on Matrigel-coated culture dishes and incubated in Dulbecco’s modified Eagle’s medium containing 20% fetal bovine serum, 100 mg/mL EC growth supplements, 90 mg/mL heparin, 1% penicillin-streptomycin, and 100 μM Fungizone. The vessel rings were removed after outgrowth by the cells was observed. The cells were passaged routinely thereafter, and their purity was identified by staining for platelet endothelial cell adhesion molecule-1 and by 1,1′-dioctadecyl-3,3,3′,3′-tetramethylinodocarbocyanine perchlorate uptake experiments as previously described. The aortic segments were cultured in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum, 1% penicillin-streptomycin, and 1% glutamine in regular dishes without Matrigel for VSMC culture. Cells were treated with or without 10 ng/mL interleukin 1β (IL-1β) (Sigma-Aldrich, MO) for 24 hours, and the culture medium was collected for assessment of prostanoid formation by liquid chromatography-tandem mass spectrometry. Whole cell lysates were prepared in radioimmunoprecipitation assay lysis buffer for Western blot analysis or in RLT buffer (QiAGEN) for RNA extraction and quantitative reverse transcriptase polymerase chain reaction analysis. All experiments were performed on cells at passage 3–5.

Thioglycollate-elicited macrophages were harvested from Mac-mPGES-1-WT and Mac-mPGES-1-KO mice as previously described. Cells were or not exposed to 5 μg/mL lipopolysaccharide (LPS) (Sigma-Aldrich, MO) at 24 hours, and the culture medium was collected for assessment of prostaglandin formation by liquid chromatography-tandem mass spectrometry. Whole cell lysates were prepared for Western blot and quantitative reverse transcriptase polymerase chain reaction analysis.

Cell Proliferation and Migration

Macrophages from Mac-mPGES-1-WT and Mac-mPGES-1-KO mice were treated with LPS for 6 hours, the culture medium was harvest after centrifugation (1000g, 10 minutes), then filtered through 0.22-μm-pore membranes (Millipore, Schwabach, Germany), and stored at −20°C in aliquots. This medium was termed conditioned medium. Basically, 1 mL of conditioned medium was collected from 1 × 10⁶ macrophages.

VSMCs from mPGES-1-flox mice were plated in fibronectin-coated 96-well plates, and conditioned medium from Mac-mPGES-1-WT or Mac-mPGES-1-KO macrophages were added (10%) with
or without 10 μg/mL platelet-derived growth factor-BB or PGE2 (50 nmol/L) (Sigma-Aldrich, MO). Forty-eight hours later, the culture medium was collected for evaluation of VSMC proliferation with the CellTiter 96 AQueous One Solution cell proliferation assay (Promega). Cell migration was assessed by a scratch-induced wound-healing assay. VSMCs from mPGES-1-flox mice were plated in fibronectin-coated 6-well plates. Confluent cells were scratched with use of P200 Gilson pipette tips followed by conditioned medium, platelet-derived growth factor-BB, and PGE2 treatment. Images were taken before and 18 hours after the scratching by using phase-contrast microscopy. The width between the wound edges in each well was measured by the use of a standard template placed on the image. Data were expressed as closure rate (%) based on initial wound width.

### Statistical Analysis

All significance testing was performed with 2-sample t tests with or without the Welch correction depending on whether the variances were significantly different, or nonparametric 2-sample Mann-Whitney tests when there were <4 replicates per condition. Paired tests were performed when appropriate. None of the nonparametric cases called for paired tests. A total of 69 2-sample tests were performed, with a significance threshold of 0.05. A total of 34 of the 69 P values are significant at the 0.05 level. We therefore would conservatively expect 0.05 × 69 = 3.45 false positives from 69 tests if all null hypotheses were true. Thus, we (conservatively) expect ≈30 of our rejected null hypotheses to be correctly rejected. With an expectation of 30 true positives, we can recalculate the expected number of falsely rejected null hypotheses to conservatively be 0.05 × 39 = 1.95. So, we can conservatively expect 2 false positives from the 34 rejected null hypotheses. This number is safely conservative because many of the observed P values are very small. The overall conclusions of this article are robust to such a small number of false positives, in particular, among the hypotheses with marginal P values.

### Results

#### Generation of the mPGES-1 Conditional (mPGES-1-flox) Mice

A 0.9-kb genomic DNA sequence that contains mPGES-1 exon 2 was inserted between an isolated loxP site and a loxP-flanked neomycin-resistant cassette, then placed between 4.0 kb 5' and 1.8 kb 3' homology sequences to obtain a mPGES-1 targeting construct (online-only Data Supplement Figure IA). The gene-targeting vector was linearized and electroporated into R1 mouse embryonic stem cells followed by G418 selection. Four hundred G418-resistant embryonic stem cell clones were picked up, and 13 clones underwent homologous recombination and were identified by polymerase chain reaction screening. Gene targeting of the candidate clones was confirmed by Southern blotting (online-only Data Supplement Figure IB). The targeted embryonic stem cells were injected into C57BL/6J blastocysts, and chimeric animals were mated with C57BL/6 mice to produce agouti heterozygous animals (F1). The mPGES-1 knock-out and conditional mice were prepared via breeding the F1 mice with Rosa26Cre and EIIaCre transgenic mice, respectively. To confirm that the mPGES-1 gene was functionally disrupted in knock-out mice, total RNA was extracted from tissues of 4-week littermates mPGES-1 null, heterozygous, and wild-type mice and was subjected to reverse transcription and polymerase chain reaction. Reverse transcriptase polymerase chain reaction indicates that exon 2 of the mPGES-1 is completely removed from the knock-out mice (online-only Data Supplement Figure IC).

### Conditional Deletion of mPGES-1 in VSMCs and ECs

VSMC or EC mPGES-1-deficient mice were generated by crossing mPGES-1-flox mice with SM22Cre or Tie2Cre mice, termed SM22Cre/flox or Tie2Cre/flox. Although Tie2 is also expressed in hematopoietic cells,20,21 we were unable to detect an impact on mPGES-1 expression in bone marrow cells of the Tie2Cre/flox mice (online-only Data Supplement Figure II). Aortic VSMCs and ECs were cultured from both SM22Cre/flox and Tie2Cre/flox mice. Real-time reverse transcriptase polymerase chain reaction revealed that mPGES-1 mRNA expression was absent in VSMCs, but not in ECs from SM22Cre/flox mice, and in ECs but not in VSMCs from Tie2Cre/flox mice under basal and IL-1β-stimulated conditions (Figure 1A and 1C). Although we generated a cellular population that was highly enriched in ECs (online-only Data Supplement Figure III), modest contamination with other cells in this preparation that express mPGES-1 likely accounts for the minor, but markedly depressed transcriptional response to IL-1β in the mutant ECs (Figure 1C). Western blot analysis verified a similar pattern of mPGES-1 protein expression in the 2 knock-out-derived cell lines (Figure 1A and 1C). COX-2 expression was unaltered in cells derived from either mutant mouse (Figure 1A and 1C). Correspondingly, IL-1β–stimulated PGE2 production was significantly suppressed in SM22Cre/flox VSMCs (Figure 1B) and in Tie2Cre/flox ECs (Figure 1D). This suppression occurred concomitant with augmented PGI2, as reflected by its hydrolysis product, 6-keto prostaglandin F1α, and increased prostaglandin D2, whereas thromboxane B2, the hydrolysis product of thromboxane A2, was unaltered (Figure 1B and 1D). Although mPGES-1 gene expression was completely suppressed in mutant VSMCs, a minor evocation of PGE2 by IL-1β in these cells likely reflects a contribution from other PGE synthases or nonenzymatic generation of the PGE2.

### Conditional Deletion of mPGES-1 in Myeloid Cells

Myeloid cell mPGES-1-deficient mice were generated by mating mPGES-1-flox mice with LysMCre mice. LysMCre mice express Cre recombinase in all myeloid cells, including macrophages, neutrophils, and some dendritic cells.22 Although there is constitutive expression of mPGES-1 in neutrophils, we failed to detect PGE2 generation by neutrophils with or without LPS stimulation.19 Similarly, only partial deletion (16%) was detected in CD11c+ splenic dendritic cells,22 so the dominant impact in the KOs is on macrophage mPGES-1 (Mac-mPGES-1-KO). The LysMCre mice (Mac-mPGES-1-WT) were used as controls. LPS-stimulated expression of mPGES-1 mRNA and protein was almost completely suppressed in Mac-mPGES-1-KO macrophages (Figure 2A and 2B). In contrast to what we observed with mPGES-1 deletion in vascular cells, LPS-stimulated COX-2 expression was also significantly suppressed in the Mac-mPGES-1-KO cells (Figure 2A and 2B), consistent with a PGE2-mediated positive feedback on COX-2 expression in macrophages.24 In addition, LPS-induced expression of the proinflammatory cytokine IL-1β was significantly suppressed (online-only Data Supplement Figure IV). As in mutant vascular cells, suppression of PGE2 production was accompanied by
increased production of PGI₂ (Figure 2C). By contrast, there was also a concomitant increase in thromboxane B₂, but prostaglandin D₂ was unaltered (Figure 2C).

### Vascular mPGES-1 Does Not Modulate Blood Pressure or Thrombogenesis

Neither VSMC nor endothelial cell mPGES-1 deletion altered baseline blood pressure and heart rate as measured telemetrically in KOs, in comparison with their littermate controls (Figure 3A). Systolic blood pressure rose significantly, but to a similar extent to mutants and controls after 3 weeks on a high-salt (8% NaCl) diet (Figure 3A). Heart rate was similarly unaltered (Figure 3B).

The time to complete common carotid artery occlusion in either gender after photochemical injury was unaltered by mPGES-1 deletion in either ECs or VSMCs (Figure 3C).

Similarly, the occlusive response to vascular injury in cremaster muscle arterioles was comparable in KOs and controls (Figure 3D).

### Contrasting Effects of mPGES-1 Deletion in Vascular and Myeloid Cells on the Hyperplastic Response to Vascular Injury

Wire injury of the femoral artery results in a hyperplastic neointimal remodeling response. This was exaggerated in mice lacking mPGES-1 in either VSMCs or ECs (Figure 4A) as reflected by the intima-to-media ratio (4.4±0.6 for SM22Cre/flox mice, 3.3±0.2 for Tie2Cre/flox mice, and 2.0±0.3 for control mice; *P<0.01 Figure 4B), and the percentage vascular stenosis (87.9±5.1% for SM22Cre/flox mice, 79.3±3.4% for Tie2Cre/flox mice, and 56.2±5.9% for control mice; *P<0.001 for SM22Cre/flox mice and *P<0.05 for Tie2Cre/flox mice.
Figure 2. Validation of mPGES-1 deletion in macrophages. Real-time RT-PCR (A) and Western blot (B) of mPGES-1 and COX-2 expression in peritoneal macrophages from Mac-mPGES-1-WT (WT) or Mac-mPGES-1-KO (KO) mice. For RT-PCR, all samples were normalized to 18S rRNA (n=3; \(P<0.05\), nonparametric Mann-Whitney tests). C, Prostanoid profile in peritoneal macrophages. PGE\(_2\), 6-keto-PGF\(_{1\alpha}\), PGD\(_2\), and TxB\(_2\), were determined by mass spectrometry in the culture medium (n=6; \(P<0.05\), unpaired \(t\) tests with the Welch correction; **\(P<0.01\), unpaired \(t\) test). 6-keto-PGF\(_{1\alpha}\) indicates 6-keto prostaglandin F\(_1\); COX-2, cyclooxygenase 2; LPS, lipopolysaccharide; mPGES-1, microsomal prostaglandin E synthase 1; PGD\(_2\), prostaglandin D\(_2\); PGE\(_2\), prostaglandin E\(_2\); RT-PCR indicates reverse transcriptase polymerase chain reaction; and TxB\(_2\), thromboxane B\(_2\).

Figure 4C). There were no statistically significant differences in these responses between the mutants. Increased immunohistochemical staining of \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA), proliferating cell nuclear antigen and tenascin-C in the mutant response to injury (Figure 4D) was consistent with VSMC proliferation and migration along the tenascin scaffold to form the neointima.26–28

By contrast, deletion of mPGES-1 in myeloid cells markedly reduced intimal thickening in response to injury (Figure 5A), reflected by a reduced intima-to-media ratio (1.5±0.2 versus 2.6±0.2; \(P<0.01\); Figure 5B) and decreased percentage stenosis (39.0%±4.7% versus 59.9%±7.3%; \(P<0.05\), Figure 5C). The expression of \(\alpha\)-SMA, proliferating cell nuclear antigen, and tenascin-C was similarly reduced in the lesions in the mutant mice in comparison with controls (Figure 5D). Leukocyte recruitment, as reflected by CD45+ cells, was clearly augmented by vascular mPGES-1 deletion and correspondingly restrained by gene deletion in myeloid cells (Figure 5E).

We evaluated the comparative effects of conditioned medium derived from mutant and control peritoneal macrophages on the proliferation and migration of cultured VSMCs from mPGES-1-flox mice to characterize further the apparent restraining effect of myeloid deletion of mPGES-1.

Figure 3. Impact of mPGES-1 deletion in VSMCs or ECs on cardiovascular function. A, VSMC or EC deletion of mPGES-1 had no effect on blood pressure. The 3-week high-salt diet (HSD) elevated systolic blood pressure (SBP) similarly irrespective of genotype (n=7–9; \(P<0.05\) versus baseline, paired \(t\) tests). B, Heart rate was indistinguishable among the groups. C, Neither VSMC nor EC mPGES-1 deletion altered the time to thrombotic carotid artery occlusion after photochemical injury for both males (n=8–10) and females (n=5–10). D, Comparison of thrombogenesis in microscopic cremaster arterioles of SM22Cre/flox (n=8), Tie2Cre/flox (n=8), and mPGES-1-flox control mice (n=9). Median fluorescence intensity was plotted versus time after laser-induced injury of the cremaster arteriole vessel wall. No difference was observed in either VSMC or EC mPGES-1–deficient mice in comparison with controls. EC indicates endothelial cell; mPGES-1, microsomal prostaglandin E synthase 1; and VSMC, vascular smooth muscle cells.
on the response to vascular injury. Conditioned media from mutant mice restrained VSMC proliferation under basal and platelet-derived growth factor-BB–stimulated conditions (Figure 6A), whereas adding back PGE2 completely rescued this impaired proliferation response (Figure 6D). Similarly, conditioned media from mutant macrophages were less effective than that from controls in inducing VSMC migration (Figure 6B and 6C), and adding back PGE2 again rescued the phenotype (Figure 6E and 6F), consistent with a deficiency of PGE2 accounting for the restraint of the response to vascular injury attributable to myeloid mPGES-1 deletion.

Expression of EP Receptors in Wire-Injured Femoral Arteries

PGE2 exerts its biological roles via 4 EP receptors. EP2 and EP4 activation restrains, whereas EP1 and EP3 promote vasoconstriction and VSMC proliferation and migration in vitro.29–31 mRNA expression of these receptors was analyzed by real-time polymerase chain reaction in femoral arteries of mice 28 days after wire injury. Expression of EP1 and EP3 were unaltered in any of the mPGES-1 mutants (online-only Data Supplement Figure V), whereas the expression of EP4 was significantly increased in the myeloid...
mPGES-1–deficient arteries (online-only Data Supplement Figure V).

**Discussion**

Nonsteroidal anti-inflammatory drugs selective for inhibition of COX-2 increase the risk of thrombotic events, hypertension, and heart failure,\(^\text{32}\) and the distinct elements of this hazard are recapitulated by pharmacological inhibition of COX-2\(^\text{33}\) or its selective deletion in vascular cells and cardiomyocytes.\(^\text{34,35}\) By contrast, we have previously reported that global deletion of mPGES-1 does not predispose to thrombogenesis or hypertension and that, in contrast to its suppression by inhibition or disruption of COX-2, biosynthesis of PGI\(_2\) is augmented with mPGES-1 inhibition consequent to prostaglandin H\(_2\) substrate diversion to PGI synthase.\(^\text{11,36}\) Here, we report that selective deletion of mPGES-1 in either ECs or VSMCs similarly has no impact on the response to thrombogenic or hypertensive stimuli, confirming a reassuring cardiovascular profile in comparison with disruption of vascular COX-2.\(^\text{35}\)

Much as was the experience with the expression of COX-2,\(^\text{33}\) investigators have had variable success in detecting endothelial expression of mPGES-1 in cultured cells\(^\text{37-40}\) and ex vivo in mice.\(^\text{15,42}\) Here, we report that mPGES-1, just like COX-2, is detectable and inducible in ECs, but that deletion of the 2 enzymes in ECs has contrasting functional consequences.\(^\text{35}\) Although studies in mice faithfully recapitulated the discrete

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**Figure 5.** Myeloid cell mPGES-1 deletion reduces intimal hyperplasia. **A,** Representative sections of hematoxylin and eosin staining of sham-operated and wire-injured femoral arteries of Mac-mPGES-1-WT (WT) or Mac-mPGES-1-KO (KO) mice 28 days after wire injury (bar=40 μm). Intima to media ratio (I/M) (B) and stenosis (C) were significantly decreased in KO mice in comparison with WT controls (n=10; *P<0.05, **P<0.01, unpaired t tests). **D,** Representative immunohistochemical staining of α-smooth muscle actin (α-SMA), proliferating cell nuclear antigen (PCNA), and tenascin-C (TN-C) in femoral arteries (bar=40 μm). **E,** Representative immunohistochemical staining of CD45 in injured arteries from both vascular and macrophage mPGES-1 deletion mice (bar=40 μm). mPGES-1 indicates microsomal prostaglandin E synthase 1.
elements of cardiovascular hazard conferred by nonsteroidal anti-inflammatory drugs selective for inhibition of COX-2.\textsuperscript{43} Caution is warranted for now in extrapolating the comparative cardiovascular profile of mPGES-1 deletion in mice to predict the cardiovascular consequences of mPGES-1 inhibition in humans.

There is considerable interest in the development of mPGES-1 inhibitors to relieve pain and reduce inflammation,\textsuperscript{8,36,44,45} we have also noted suggestions of cardiovascular efficacy that might derive from this strategy. Thus, global deletion of mPGES-1 retards atherogenesis,\textsuperscript{4} restrains angiotensin II–induced aortic aneurysm formation.
in hyperlipidemic mice,\textsuperscript{12} and limits the vascular proliferative response to injury,\textsuperscript{13} effects that appear to derive substantially from augmented vascular formation of PGI\textsubscript{2}.

Although all cells have the capacity to generate prostaglandins and express COX enzymes, 1 or 2 products tend to predominate for each cell type because of the differential expression of downstream prostaglandin synthases. Thus, platelets make predominantly thromboxane A\textsubscript{2}, ECs and VSMCs make predominantly PGI\textsubscript{1} and PGE\textsubscript{2}, and macrophages make predominantly PGE\textsubscript{2} and thromboxane A\textsubscript{2}. Given that these products have contrasting effects on many aspects of cardiovascular function,\textsuperscript{11,13,14,46,47} we sought to determine whether selective deletion of mPGES-1 in vascular cells and myeloid cells might differ in a setting — the proliferative response to vascular injury — where both cell types contribute to the phenotype. Although mPGES-1 is constitutively expressed in neutrophils, they make no detectable PGE\textsubscript{2}.\textsuperscript{19}

Also, the efficiency of mPGES-1 deletion in dendritic cells is only \(\approx 16\%\); thus, the dominant consequence of enzyme suppression may have been augmented by a trend toward a reduction in EP2 expression, whereas EP1 and EP3, whose expression is unaltered may contribute to restraint of proliferation in mPGES-1.

Selective deletion of mPGES-1 in both ECs and VSMCs enhanced the neointimal proliferative response to vascular injury. Given that deletion of the I prostanooid receptor (or selective inhibition of COX-2) has a similar effect,\textsuperscript{14,48} this seems likely to reflect suppression of PGE\textsubscript{2} rather than augmented PGI\textsubscript{1} observed in the vasculature of these mice. Similarly, it seems unlikely that the minor increment in prostaglandin D\textsubscript{2} mediates this phenotype. Although there is no direct information addressing the role of prostaglandin D\textsubscript{2} in modulating the response to vascular injury, indirect information is consistent with it playing an anti-inflammatory role in the cardiovascular system. Thus, deletion of 1 of its 2 receptors, the D prostanooid receptor 1 modestly accelerates atherogenesis and aneurysm formation in hyperlipidemic mice.\textsuperscript{46} PGE\textsubscript{2}, like PGI\textsubscript{1}, acts to restrain VSMC contraction, migration, and proliferation and does so via the adenylate cyclase coupled receptors, EP2 and EP4.\textsuperscript{29,30} Failure of their activation owing to suppression of vascular PGE\textsubscript{2} formation would therefore afford an explanation for this observation. Indeed, the consequences of ligand suppression may have been augmented by a trend toward a reduction in EP2 expression, whereas EP1 and EP3, whose activation promotes vascular migration and proliferation in vitro,\textsuperscript{31,46} are not changed in the blood vessels of vascular mutant mice. By contrast, deletion of macrophage mPGES-1 restrains the proliferative response to vascular injury. Together with a reduction in the overall proliferative response and expression of tenasin C, which affords a scaffold along which VSMC migration occurs,\textsuperscript{37} elaboration of macrophage inflammatory cytokines and leukocyte infiltration were reduced by selective mPGES-1 deletion. The decrease of leukocyte infiltration in Mac-mPGES-1 KOs contrasts with the increase in leukocyte recruitment to the heart after myocardial infarction when bone marrow–derived mPGES-1 is ablated.\textsuperscript{30} Differences in the composition of mPGES-1–deficient cells (myeloid cells versus whole bone marrow–derived leukocytes), the site of tissue remodeling and the attendant microenvironment for myeloid cell development (mPGES-1 intact versus mPGES-1 null) may have contributed to this discrepancy.

Attenuation of the proliferative response to vascular injury in Mac mPGES-1 KOs again likely reflects predominantly suppression of PGE\textsubscript{2} rather than substrate rediversion; augmented thromboxane A\textsubscript{2} would exaggerate rather than limit the response to vascular injury.\textsuperscript{14} Again, aside from suppression of ligand formation, a shift in expression among the EPs may have contributed to the phenotype. Thus, a relative increase in EP4 expression, leaving EP1 and EP3 expression unaltered may contribute to restraint of proliferation in the Mac-mPGES-1 KOs. Consistent with these observations, conditioned media from mPGES-1–depleted macrophages was less likely than that from enzyme-replete macrophages to stimulate VSMC migration and proliferation.

The salutary effects of macrophage deletion of mPGES-1 are consistent with other lines of evidence implicating macrophages in promoting the proliferative response to vascular injury. After arterial endothelial injury, leukocytes, including macrophages, are recruited to the site and promote VSMC proliferation and neointimal growth.\textsuperscript{51} The number of inflammatory cells in the vessel wall relates positively to intimal thickening,\textsuperscript{52} whereas blockade of macrophage recruitment can attenuate it.\textsuperscript{53,54} Furthermore, systemic inactivation of macrophages by intravenous administration of liposomal clodronate significantly reduces neointimal thickening after balloon injury.\textsuperscript{55} Limited neointimal formation is observed in macrophage antigen-1 (Mac-1) knockout mice\textsuperscript{56} or with pharmacological blockade of Mac-1.\textsuperscript{54} Moreover, hyperplasia of vein grafts is suppressed by macrophage depletion.\textsuperscript{57}

In conclusion, unlike global\textsuperscript{11} or vascular\textsuperscript{35} deletion of COX-2, global\textsuperscript{19} and now vascular deletion of mPGES-1 does not predispose mice to evoked thrombosis or hypertension. However, some have reported a predisposition to hypertension in global knockouts of mPGES-1, which may reflect differences in genetic background.\textsuperscript{58–60} Restraint of atherogenesis, aneurysm formation, and the proliferative response to vascular injury in the globally deleted mice has suggested potential cardiovascular efficacies for mPGES-1 inhibitors, albeit that the inflammation-dependent remodeling response after myocardial infarction is impaired, leading to a delayed decline in left ventricular function. Here, we illustrate the contrasting effects of mPGES-1 deletion in vascular cells and macrophages on the vascular remodeling response to injury. Although our mouse models do not simulate precisely percutaneous angioplasty on an atherosclerotic background in humans, these results prompt consideration of selectively targeting macrophage mPGES-1 as an adjunct to treat pathological vascular remodeling. However, the extent to which complete gene deletion in mice will simulate the likely variable degree of mPGES-1 blockade attained by small molecule inhibitors in humans remains to be determined.

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Disclosures

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References


**CLINICAL PERSPECTIVE**

Nonsteroidal anti-inflammatory drugs selective for inhibition of cyclooxygenase 2 (COX-2) were developed to conserve the analgesic and anti-inflammatory efficacy of older nonsteroidal anti-inflammatory drugs that inhibited cyclooxygenase 1 and COX-2, while reducing the likelihood of gastrointestinal adverse effects. Although this objective was broadly attained, COX-2 inhibitors, which turned out to include some older nonsteroidal anti-inflammatory drugs like diclofenac, also caused thrombotic events, hypertension, and cardiac failure attributable to suppression of COX-2-derived PGI₂. The microsomal prostaglandin E synthase-1 (mPGES-1) enzyme is downstream of COX-2 and accounts for most of the prostaglandin E₂ that is formed in humans. Some, but not all studies in mice lacking mPGES-1 suggest analgesic and anti-inflammatory efficacy similar to nonsteroidal anti-inflammatory drugs. Most, but not all studies suggest that unlike COX-2 disruption or inhibition, global mPGES-1 deletion does not predispose to hypertension or thrombogenesis in mice. Here, we show that this reassuring cardiovascular phenotype extends to mice lacking endothelial or vascular smooth muscle cell mPGES-1, consistent with augmented prostacyclin because of the redirection of the mPGES-1 substrate to prostacyclin synthase. However, although global mPGES-1 deletion attenuates the response to vascular injury, again reflective of increased prostacyclin, selective enzyme deletion in vascular versus myeloid cells has contrasting effects on this phenotype. This appears in both cases to reflect suppression of prostaglandin E₂ rather than substrate redirection and implicates macrophage prostaglandin E₂ in an exaggerated response to vascular injury. Contrasting cellular roles of mPGES-1 may complicate systemic enzyme inhibitors, whereas locally delivered inhibitors targeted at the macrophage merit consideration as an adjunct to treat pathological vascular remodeling, such as restenosis after angioplasty.
Cell Selective Cardiovascular Biology of Microsomal Prostaglandin E Synthase-1
Lihong Chen, Guangrui Yang, Xiufeng Xu, Gregory Grant, John A. Lawson, Mohammad Bohlooly-Y and Garret A. FitzGerald

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SUPPLEMENTAL MATERIAL

Cell Selective Cardiovascular Biology of Microsomal Prostaglandin E Synthase-1

Chen, et al  Cell specific role of mPGES-1
Lihong Chen\textsuperscript{1,2,*}, MD, PhD; Guangrui Yang\textsuperscript{1,2,*}, MD, PhD; Xiufeng Xu\textsuperscript{3}, PhD; Gregory Grant\textsuperscript{1,2}, PhD; John A. Lawson\textsuperscript{1,2}, BS; Mohammad Bohlooly-Y\textsuperscript{3}, PhD and Garret A. FitzGerald\textsuperscript{1,2}, MD, FRS

\textsuperscript{1}, Institute for Translational Medicine and Therapeutics,

\textsuperscript{2}, Department of Pharmacology,

University of Pennsylvania, Philadelphia, PA, 19104

\textsuperscript{3}, RAD-Transgenics, Discovery Sciences, Astra Zeneca R&D, Mölndal, 43183 Malmö, Sweden

* These authors contributed equally to this work.

Corresponding Author:

Garret A. FitzGerald

Institute for Translational Medicine and Therapeutics, Department of Pharmacology, University of Pennsylvania, 10-122 Translational Research Center, 3400 Civic Center Blvd, Philadelphia, PA, 19104

Fax: 215-573-9135, Tel: 215-898-1184,

E-mail: garret@upenn.edu

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Supplemental Methods

1. Targeted disruption of mPGES-1

To construct a mPGES-1 gene targeting vector, 4.0 kb, 0.9 kb and 1.8 kb genomic DNA fragments were prepared from a BAC clone which contains about 200 kb 129/Sv genomic sequence including the mPGES-1 gene (ResGen, Invitrogen Corporation) for 5’ homology, deletion and 3’ homology sequences respectively. The fragments were cloned into a plasmid containing loxP-loxP-PGKneo-loxP sequence, applying a standard cloning technique. In brief, a 0.9 kb fragment containing exon 2 of mPGES-1 was inserted between an isolated loxP site and loxP-flanked PGK-Neo cassette and then placed between the two homology sequences. The targeting vector was linearized and electroporated into R1 mouse embryonic stem (ES) cells and ES cell clones were selected with G418 (300 µg/ml). The candidate ES cell clones underwent homologous recombination were screened by PCR and confirmed by Southern blotting according to a standard protocol. The targeted ES cells were injected into C57BL/6J blastocysts and the injected embryos were implanted into the uteruses of pseudopregnant B6CBA mice. Chimeric males were mated with C57BL/6 females to produce agouti heterozygous animals (F1). The heterozygous F1 males were bred with Rosa26Cre transgenic mice on a C57BL/6 background to delete the whole loxP floxed region, including the exon 2 and the neo cassette. mPGES-1 null mice were then obtained by intercrossing. Offspring were genotyped by PCR using primers 822, 823 and 824 (822, forward: 5’- AAGGTTGATGGTGCACGTCTA-3’; 823, reverse: 5’-TTTGTTTGGCCTCACTCTACA-3’; 824, forward: 5’-CTCAGGCCAGGCTCATCTACC-3’) that detected wild type alleles (0.279 kb fragment) and null alleles (0.470 kb fragment), respectively. For generating mPGES-1 conditional mice, the heterozygous F1 mice were bred with EIIaCre transgenic mice on a C57BL/6 background to
delete the loxP flanked PGK-Neo cassette only, keeping the loxP flanked 0.9 kb deletion fragment in the genome. The genotyping for conditional mice was achieved by PCR using primers 822 and 823 to identify the wild type (0.279 kb fragment) and floxed alleles (0.399 kb fragment), respectively.

Three primers located in different exons, 854, 855 and 856 (854, forward: 5'-CCAGGTGCTCCCGCCCTTTCT-3', 855, reverse: 5'-CGCTCCACATCTGGGTCACTC-3'; and 856, reverse: 5'-GTAGGCCACGGTGTTGACCAC-3') were used for RT-PCR analysis. A 0.171 kb fragment can be amplified by RT-PCR from wild type, but not from KO mice with primers 854 and 855. Applying primers 854 and 856, 0.332 kb or 0.239 kb fragments can be obtained by RT-PCR from wild type and KO mice, respectively.

2. Immunohistochemistry

After conventional deparaffining and hydration, sections were treated with 3% H₂O₂ for 10min to eliminate endogenous activity of peroxidase. Then sections were heated in a target retrieval solution (DakoCytomation, Carpinteria, CA) for 30min. After cooling at room temperature and washing with PBS, sections were incubated with primary antibodies against α-SMA, PCNA (Abcam, Cambridge, MA), TN-C (Chemicon International Inc., Temecula, CA) or CD45 (Abcam, Cambridge, MA) for 60min followed by incubation with antibody enhancer and Polymer-horseradish peroxidase–conjugated secondary antibodies and chromogen AEC staining (Golden Bridge International, Mukilteo, WA). After several washes with distilled water, sections were counterstained with hematoxylin and mounted routinely.

3. Endothelial cell characterization

The identification of mouse endothelial cells was confirmed by staining for platelet endothelial cell adhesion molecule (PECAM-1) and by Dil-Ac-LDL uptake as previously described¹. Briefly,
the cells were passaged onto sterile glass coverslips and grown overnight. For PECAM-1 staining, the cells were incubated with or without mouse anti-PECAM-1 monoclonal antibody (1:100) for 1 hour at room temperature after being fixed for 10 minutes in acetone. Then they were washed with PBS and incubated with rhodamine-conjugated secondary antibody (1:100) for 1 hour. After extensive washing, the cells were visualized with a fluorescence microscope. For the acetylated LDL uptake experiments, the cells were incubated with 10μg/mL DiI-Ac-LDL (Biomedical Technologies Inc., Stoughton, MA) in DMEM for 1 hour at 37°C. Then the medium was removed and the cells were washed with PBS and the DiI-Ac-LDL was visualized with a fluorescence microscope.
Supplemental Figure 1

Wild-type allele

Targeting vector

Targeted allele

Conditional allele

Deleted allele

Supplemental Figure 1
Supplemental Figure 1
Supplemental Figure 2

A

mPGES-1

β-actin

B

Relative Protein Level

flox  Tie2Cre/flox
Supplemental Figure 3

A

**Fluorescence**

![PECAM-1](image)

**Phase**

![Phase Image](image)

No primary antibody

B

**Fluorescence**

![Uptake of Dil-Ac-LDL](image)

**Phase**

![Phase Image](image)

Uptake of Dil-Ac-LDL
Supplemental Figure 5
Supplemental Figure Legends

Supplemental Figure 1. Targeted disruption of the mPGES-1 gene. A, The native 5’ region of the mPGES-1 gene and targeting vector (top), the targeted and conditional allele (middle) and the disrupted mPGES-1 gene (bottom) are shown. A region containing exon 2 was flanked by loxP sites and was deleted after breeding with Cre transgenic mice. The open rectangles with numbers indicate the exons. The filled rectangle shows the untranslated region (UTR). Horizontal bars indicate the probe used for Southern blotting. DTA, diphtheria toxin A fragment gene cassette. S, SpeI. The triangle indicates loxP site. The arrow indicates the oligos used for genotyping. B, Southern blot analysis of the targeted ES clones. Genomic DNA was digested with SpeI and probed with a 3’ flanking probe shown in (A). Expected sizes of DNA fragments of the wild-type and mutant alleles are indicated in (A). Lane 1 and 2, markers; Lane 3-13, targeted clones. Lane 14-15, wild type ES clones. C, mPGES-1 transcript (top) and RT-PCR analysis (bottom). Tissues were obtained from mPGES-1 +/-, +/- and -/- littermates. The arrows indicate the oligos used for RT-PCR.

Supplemental Figure 2. Western blot analysis of mPGES-1 expression in bone marrow cells of the Tie2Cre mice. Total protein of bone marrow (BM) cells was extracted from mPGES-1-flox and Tie2Cre/flox mice. Vas Deferens (VD) was used as a positive control for mPGES-1 expression and β-actin staining was performed as a protein loading control. 40µg protein of each sample was loaded. A) Representative blots. B) Quantitative analysis of mPGES-1 protein expression (normalized to β-actin) (n=3).

Supplemental Figure 3. Characterization of mouse ECs. A, The ECs isolated from the mouse aorta were examined for the presence of the endothelium-specific marker PECAM-1. Fluorescence and phase images show that the positive PECAM-1 staining was observed in
almost all of the cells. Images with no primary antibody were used as background controls. B, Ecs were identified by the Dil-Ac-LDL uptake experiments. Fluorescence and phase images show that almost all cells are able to uptake Dil-Ac-LDL after 1 hour incubation.

**Supplemental Figure 4. Inflammatory cytokine expression in mPGES-1 deleted macrophages.** Real-time RT-PCR of IL-1β expression in peritoneal macrophages. Thioglycollate-elicited macrophages harvested from Mac-mPGES-1-WT (WT) or Mac-mPGES-KO (KO) mice were treated with or without 5μg/ml LPS for 24h and whole cell lysates were prepared for quantitative RT-PCR analysis. All samples were normalized to 18s rRNA (n=3; *P=0.05, nonparametric Mann-Whitney test).

**Supplemental Figure 5. Expression of EP receptors in wire-injured femoral arteries.** A, Real-time RT-PCR analysis of the relative expressions of EP1, EP2, EP3 and EP4 in injured arteries from SM22Cre/flox, Tie2Cre/flox mice and mPGES-1-flox mice 28 days after wire injury(n=4-5). B, Real-time RT-PCR analysis of the relative expressions of EP1, EP2, EP3 and EP4 in injured arteries from Mac-mPGES-1-WT or Mac-mPGES-1-KO mice 28 days after wire injury (n=5; *P<0.05 , unpaired t-test with welch’s correction).
Supplemental References