Microsomal Prostaglandin Synthase-1–Derived Prostaglandin E2 Protects Against Angiotensin II–Induced Hypertension via Inhibition of Oxidative Stress

Zhanjun Jia, Xiaohua Guo, Hui Zhang, Mong-Heng Wang, Zheng Dong, Tianxin Yang

Abstract—Prostaglandin (PG) E2 has an established role in the control of vascular homeostasis, especially in the face of pressor stimuli.11–13 In response to Ang II, vascular cells exhibit enhanced production of PGE2 that dilates blood vessels, thereby mitigating the vasoconstriction induced by Ang II.14 Both in vivo and in vitro evidence exists to suggest the existence of mPGES-1 in the vascular cells, raising a possibility that this PGES may be involved in the regulation of vascular tone and reactivity. In support of this notion, we have shown recently that mice lacking mPGES-1 exhibit enhanced sensitivity to chronic Ang II infusion, as well as chronic salt loading.15 The present study was undertaken to further explore the protective role and mechanism of vascular mPGES-1 in the setting of Ang II infusion.

Methods

Animals
mPGES-1 mutant mice were originally generated by Trebino et al.4 This mouse colony was propagated at the University of Utah and maintained on a mixed DBA/1lacJ×C57BL/6 background. The

PGE2 has an established role in the control of vascular homeostasis, especially in the face of pressor stimuli.11–13 In response to Ang II, vascular cells exhibit enhanced production of PGE2 that dilates blood vessels, thereby mitigating the vasoconstriction induced by Ang II.14 Both in vivo and in vitro evidence exists to suggest the existence of mPGES-1 in the vascular cells, raising a possibility that this PGES may be involved in the regulation of vascular tone and reactivity. In support of this notion, we have shown recently that mice lacking mPGES-1 exhibit enhanced sensitivity to chronic Ang II infusion, as well as chronic salt loading.15 The present study was undertaken to further explore the protective role and mechanism of vascular mPGES-1 in the setting of Ang II infusion.
sibling matings were performed for ≥10 generations. Homozygous mPGES-1−/− males and females were both fertile and crossed to generate homozygous mPGES-1−/− offspring. Nonlittermate mPGES-1−/− mice on the same genetic background were used as control mice. mPGES-1−/− mice were born at the expected mendelian ratio and did not exhibit gross developmental or morphological abnormalities. Under baseline conditions, adult mPGES-1−/− mice did not exhibit major cardiovascular or metabolic phenotypes, except a modest reduction of mean arterial pressure (MAP), as described previously. Genotypes were identified by PCR.

Telemetric Recordings of MAP and Chronic Infusion Experiments

The telemetric device was implanted into male mPGES-1−/− and mPGES-1+/− mice (3- to 4-month-old) through catheterization of the carotid artery, as described previously. Daily MAP was recorded as mean values of 4-hour recordings from 9 AM to 1 PM. After collection of baseline MAP for 3 days, mPGES-1−/− mice were implanted with an osmotic minipump driving infusion of vehicle or Ang II at 0.35, 0.70, or 2.00 mg/kg per day. The minipump was placed under the skin of the flank region. mPGES-1−/− mice were implanted with 2 osmotic minipumps, one for Ang II infusion at 0.35 mg/kg per day (Sigma) and another one for Tempol infusion at 50 mg/kg per day (Alexis Biochemicals); on day 7, the Tempol minipump was removed without interruption of Ang II infusion, and MAP was monitored for additional 7 days. Before sacrifice, animals were placed in metabolic cages for 24-hour urine collection. A separate group of mPGES-1−/− mice was implanted with an osmotic minipump driving Ang II infusion at 0.35 mg/kg per day. Three days after Ang II infusion, these mice were given apocynin at 50 mg/kg per day via gavage (suspended in saline).

Measurement of Renal Hemodynamic Response to Ang II

Mice were anesthetized with isoflurane, and body temperature was maintained at 37°C using an operating table with the heating system. Catheters were placed in jugular vein for fluid infusion and in femoral artery for direct blood pressure measurement. A flow probe (Transonic) was placed around the left renal artery for measurement of total renal blood flow (RBF). Five-percent BSA in saline was infused at 0.5 mL/h to maintain constant plasma oncotic pressure during surgery and for the duration of an experiment. After a 30-minute stabilization period after the surgery, Ang II was infused at 150 nmol/kg per minute (at the same infusion rate of 0.5 mL/h) via the jugular vein over a period of 40 minutes. Parameters of MAP and RBF were collected every 2 minutes for the first 10-minute period and then every 10 minutes for the following 30-minute period. Renal vascular resistance was calculated by MAP divided by RBF.

Specific Methods

Please see the data supplement, available online at http://hyper.ahajournals.org.

Statistical Analysis

All of the values are presented as means±SEs. Repeated-measures ANOVA was used to analyze data from the time course studies (Figures 1 and 6) with unpaired Student t test to identify differences at a single time point. For the end point studies of urinary excretion of PGE_2, 8-isoprostane, and gene expression, the unpaired Student t test was used for comparisons between mPGES-1−/− and mPGES-1+/− mice and the paired Student t test for comparisons within mPGES-1−/− and mPGES-1+/− mice. Differences were considered to be significant when the P value was <0.05.

Results

Role of Reactive Oxygen Species in Ang II–Induced Hypertension in mPGES-1−/− Mice

mPGES-1+/− mice were infused for 7 days with various doses of Ang II via an osmotic minipump, and daily MAP was monitored by telemetry; MAP was unchanged, with a dose range from 0.35 to 0.70 mg/kg per day, whereas a significant hypertension developed when the dose was raised to 2.0 mg/kg per day (Figure 1A). In contrast, the nonpressor dose of 0.35 mg/kg per day seen in mPGES-1+/− mice induced a marked hypertensive response in mPGES-1−/− mice (93±3 versus 120±3 mm Hg; P<0.05; Figure 1B). To study the role of reactive oxygen species (ROS) in this hypertensive response, we tested effectiveness of Tempol in this model. Strikingly, Ang II–induced hypertension in mPGES-1−/− mice was completely normalized by coadministration with Tempol via a separate osmotic minipump. When the Tempol minipump was removed on day 7, Ang II–induced hypertension was gradually restored (Figure 1B). To assess the involvement of NADPH oxidase, the effect of
an NADPH oxidase inhibitor apocynin on Ang II–induced hypertension in mPGES-1 knockout mice was tested. Apocynin treatment produced a similar blood pressure–lowering effect as Tempol (Figure 1C).

Urinary PGE₂ excretion, as assessed by enzyme immunoassay, was significantly increased in mPGES-1−/− mice in response to Ang II infusion; there was a tendency for a further increase in response to the combined Ang II and Tempol treatment, but this did not reach a statistical significance. In contrast, mPGES-1−/− mice had a reduced baseline level of urinary PGE₂ excretion that was unresponsive to the treatment with Ang II alone or in combination with Tempol (Figure 2A). After a 7-day Ang II infusion, mPGES-1−/− mice did not exhibit significant changes in urinary excretion of 8-isoprostane, nitrate/nitrite (NO₂⁻), or cGMP (Figure 2B through 2D). In contrast, in response to the Ang II treatment, mPGES-1−/− mice exhibited a significant increase in urinary 8-isoprostane excretion and a parallel decrease in urinary excretion of NO₂⁻ and cGMP that were all corrected by Tempol treatment (Figure 2B through 2D). After a 7-day Ang II infusion, urine flow was significantly increased (+/+: from 2.0 ± 0.2 to 2.8 ± 0.15 mL/24 hours, P < 0.05; −/−: from 1.4 ± 0.3 to 2.5 ± 0.5 mL/24 hours, P < 0.05) in parallel to urinary sodium excretion (+/+: from 0.256 ± 0.012 to 0.308 ± 0.011 mmol/24 hours, P < 0.05; −/−: from 0.236 ± 0.023 to 0.295 ± 0.031 mmol/24 hours, P < 0.05) in both mPGES-1−/− and mPGES-1−/− mice, and there was no difference between the 2 strains.

The aortas freshly isolated from the vehicle or Ang II–treated mPGES-1−/+ and mPGES-1−/− mice were analyzed for NADPH oxidase activity using a luminescence assay. The Ang II infusion induced a 2-fold increase in aortic NADPH oxidase activity in mPGES-1−/− mice, and this increase was much greater (3.5-fold) in mPGES-1−/− mice (Figure 3A). The aortic samples were also subjected to real-time RT-PCR analysis of various subunits of NADPH oxidase. In mPGES-1−/− mice, the Ang II infusion induced a moderate increase in aortic expression of only Nox1 and p22phox but not p47phox, gp91phox, or Nox4 (Figure 3B through 3F). In contrast, aortic p47phox, gp91phox, and Nox1 in mPGES-1−/− mice became highly responsive to Ang II (Figure 3B through 3D), corresponding with the elevated enzyme activity levels in these samples. However, different patterns were observed for p22phox and Nox4. In mPGES-1−/− mice, despite the elevated baseline levels of both subunits, p22phox was unresponsive to Ang II (Figure 3E), whereas Nox4 was significantly decreased by this treatment (Figure 3F). To test whether antioxidant expression was affected in mPGES-1−/− mice, the aortic samples were analyzed for expression of the 3 isoforms of superoxide dismutase (SOD1 through SOD3). Ang II treatment induced a parallel reduction of SOD1 and SOD2 in the aortas of mPGES-1−/− mice that was not different from that of mPGES−/− mice (Figure S1, available online at http://hyper.ahajournals.org). In contrast, this treatment unregulated the aortic expression of SOD3 in mPGES-1−/− mice, whereas mPGES-1−/− mice had an elevated baseline expression that was not responsive to Ang II.

**Regulation of Vascular PGES by Ang II**

The aortas were isolated from vehicle or Ang II–treated mice and subjected to analysis of mPGES-1 expression by
immunohistochemistry and real-time RT-PCR. Immunohistochemistry showed a significantly enhanced immunoreactivity of mPGES-1 predominantly in the smooth muscle cells of the aortas of Ang II–treated mPGES-1+/− mice (Figure 4A). This labeling was remarkably diminished in the aortas of Ang II–treated mPGES-1−/− mice, thereby confirming specificity of the antibody. In line with this finding, mPGES-1 mRNA expression in the aortas of mPGES-1−/− mice, as assessed by real-time RT-PCR, increased 10-fold in response to Ang II treatment. Similar degrees of stimulation were also observed for mPGES-2 and cPGES (Figure 4B).

Primary mouse smooth muscle cells from mPGES-1+/+ and mPGES-1−/− mice were exposed for 24 hours to 100 nmol/L Ang II and then subjected to analyses of PGE2 concentrations and mRNA expression of various PGES isoforms. In +/+ mouse smooth muscle cells, Ang II treatment induced a 2-fold increase in medium PGE2 concentrations, accompanied by a parallel increase in mRNA expression of mPGES-1 and mPGES-2 and cPGES (Figure 5). In contrast, the stimulation of PGE2 release was completely abolished in the −/− mouse smooth muscle cells. In these cells, the baseline expression of mPGES-2 was elevated and there was no further elevation after Ang II treatment, whereas the baseline level of cPGES was not affected, nor was the response to Ang II (Figure 5).

Effect of PGE2 on Ang II–Induced Oxidative Stress in Cultured Smooth Muscle Cells

To evaluate the antioxidant properties of PGE2 in vitro, ROS levels in cultured vascular cells exposed to Ang II in the presence or absence of PGE2 were determined using dichlorodihydrofluorescein diacetate and dihydroethidine. The human smooth muscle cells were exposed for 30 minutes to 100 nmol/L Ang II in the presence or absence of 0.5 μmol/L of PGE2. Ang II treatment induced an ∼2-fold increase in ROS production that was almost normalized by PGE2 (Figure S2A). Similarly, this treatment enhanced dihydroethidine staining that was sensitive to PGE2 (Figure S2B). Considering that NAPD oxidase may serve as a major source of ROS production induced by Ang II, we evaluated this enzyme at the levels of activity, as well as gene expression. An exposure of human smooth muscle cells to 100 nmol/L Ang II for 24 hours induced a marked increase in NAPD oxidase activity that was almost completely normalized by 0.5 μmol/L of PGE2 (Figure S3A). In agreement with these data, Ang II treatment induced a 2-fold increase in p47phox, a 10-fold increase in gp91phox, and a 6-fold increase in Nox1, and all of these increases were abolished in the presence of PGE2 (Figure S3B through S3D). Of note, PGE2 treatment lowered p47phox mRNA far below the control level.

Renal Hemodynamic Response to Ang II

Under anesthesia, in mPGES-1+/+ mice, IV infusion of Ang II at 150 nmol/kg per minute over a period of 40 minutes induced a rapid and transient pressor response as evidenced by increases of MAP from 88±3 to 117±11 mm Hg at 2 minutes that returned to 104±4 mm Hg at 4 minutes and to 101±2 mm Hg at 20 minutes. In contrast, the pressor response was markedly potentiated in mPGES-1−/− mice, as evidenced by more sustained elevation of MAP (118±10 mm Hg at 2 minutes, 123±8 mm Hg at 4 minutes, and 108±8 mm Hg at 20 minutes). In parallel with the changes in
MAP, during Ang II infusion in mPGES-1−/− mice, RBF was reduced by 58% at 2 minutes and by 23% at 4 minutes and back to baseline levels at 10 minutes. In contrast, the reduction of RBF in mPGES-1−/− mice was much greater at all of the time points (reduction by 75% at 2 minutes and by 61% at 4 minutes). The increases in renal vascular resistance in response to Ang II infusion were 3 times higher in mPGES-1−/− mice than that in mPGES-1+/+ mice (at 4 minutes; Figure 6).

Figure 4. Expression of PGES in the aortas. A, Immunohistochemistry showing increased mPGES-1 antibody labeling in the aortas of mPGES-1−/− mice after a 7-day Ang II infusion (0.35 mg/kg per day). The labeling was remarkably reduced in the mPGES-1−/− aortas and was almost indistinguishable from a negative control with omission of primary antibody. Shown are representatives of 3 experiments. B, Real-time RT-PCR evaluation of mPGES-1, mPGES-2, and cPGES in the aortas of mPGES-1−/− mice infused with vehicle or Ang II. Results are means ± SEMs. n=6 to 10 in each group.

Figure 5. Effects of Ang II on PGE₂ release (A) and mRNA expression of mPGES-1 (B), mPGES-2 (C), and cPGES (D) in primary mouse smooth muscle cells derived from mPGES-1+/+ and mPGES-1−/− mice. The cells were exposed for 24 hours to vehicle or 100 nmol/L Ang II. Medium PGE₂ concentration was determined by ELISA and mRNA expression of PGES isoforms by real-time RT-PCR and normalized by GAPDH. Results are means ± SEMs. n=4 in each group.
vascular oxidative stress via an NADPH oxidase–independent mechanism. In a previous study, we discovered a novel role of mPGES-1–derived PGE2 in modulating Ang II–induced hypertensive response. In extension of this work, the present study demonstrated that the vasculoprotection of PGE2 is mediated by a second antioxidant, apocynin, produced a similar blood pressure–lowering effect. Apocynin is widely used as an inhibitor of NADPH oxidase but was recently found to inhibit vascular oxidative stress via an NADPH oxidase–independent mechanism. Together, the similar results obtained with structurally distinct antioxidants have established an essential role for ROS in mediating the enhanced hypertensive response to Ang II in mPGES-1−/− mice, although the results of apocynin may not necessarily support involvement of NADPH oxidase.

However, several other lines of evidence do support involvement of NADPH oxidase. In mPGES-1−/− mice, a 7-day of II infusion increased NADPH oxidase activity in the aortas associated with selective induction of Nox1 and p22phox but not p47phox, gp91phox, or Nox4, a similar pattern seen in the rat kidney after Ang II treatment. As compared with the mPGES-1−/− controls, Ang II infusion induced a much greater increase in aortic NADPH oxidase activity in mPGES-1−/− mice. Among various subunits of NADPH oxidase analyzed, p47phox, gp91phox, and Nox1 in the mPGES-1−/−–deficient aortas exhibited a robust stimulation in response to Ang II treatment, likely accounting for the increased activity of NADPH oxidase. A distinct pattern of regulation was observed for p22phox and Nox4. In the mPGES-1−/−–deficient aortas, these 2 subunits of NADPH oxidase both had elevated baseline expression, neither was upregulated by Ang II, and Nox4 expression was even downregulated. These findings indicate that p22phox and Nox4 may not contribute to an Ang II–induced increase in aortic NADPH oxidase activity in mPGES-1−/− mice. It is somewhat puzzling that the elevation of baseline expression of these 2 subunits does not correlate with the unchanged baseline level of NADPH oxidase activity in mPGES-1−/− mice. The functional implication of the distinct regulation of vascular p22phox and Nox4 in mPGES-1−/− mice needs to be explored in future studies.

Oxidative stress is typically a consequence of imbalance between ROS production and antioxidant capacity. Other than the increased ROS production, reduced antioxidant capacity is another important determinant of oxidative stress. We found that Ang II treatment induced a parallel, remarkable reduction of SOD1 and SOD2 in the aortas of mPGES-1−/− mice that was not different from that of mPGES−/− mice. These findings suggest that the downregulation of these 2 SOD isoforms may be required for Ang II–induced oxidative stress but does not likely contribute to differences in the oxidative responses between the 2 strains. However, unlike SOD1 and SOD2, aortic SOD3 expression was stimulated by Ang II treatment in mPGES-1−/− mice, and this stimulation was not observed for p22phox and Nox4 in mPGES-1−/− mice needs to be explored in future studies.

Discussion

Ang II infusion induces an immediate vasoconstrictive response that is typically followed by a gradual recovery despite continued infusion of the pressor agent. The buffering effect on Ang II–induced vasoconstriction is at least in part mediated by vasodilatory PGs, including PGE2. In a previous study, we discovered a novel role of mPGES-1–derived PGE2 in modulating Ang II–induced hypertensive response. In extension of this work, the present study demonstrated that the vasculoprotection of PGE2 is mediated by the suppression of oxidative stress.

Ang II–induced hypertension in mPGES-1−/− mice was completely prevented by Tempol treatment and was fully restored on termination of the antioxidant. Treatment with a second antioxidant, apocynin, produced a similar blood pressure–lowering effect. Apocynin is widely used as an inhibitor of NADPH oxidase but was recently found to inhibit vascular oxidative stress via an NADPH oxidase–independent mechanism. Together, the similar results obtained with structurally distinct antioxidants have established an essential role for ROS in mediating the enhanced hypertensive response to Ang II in mPGES-1−/− mice, although the results of
along with expression of p47phox, gp91phox, and Nox1, was induced by Ang II and inhibited by PGE2. Together, these findings reinforce the concept that PGE2 may exert a vasculoprotective action via targeting specific subunits of NADPH oxidase in vascular cells. In line with this notion, PGE1 in the range of 40 to 50 μmol/L inhibited the activation of NADPH oxidase in a cell-free system, supporting the antioxidant activity of PGE1. However, in the same study, PGE1 failed to inhibit the superoxide production by stimulated neutrophils in a whole-cell system. In our previous study, we showed that PGE2 processes antioxidant activity that likely mediates the antifibrotic effect of this PG in cultured renal epithelial cells. The effect of PGE2 was mimicked by cAMP elevation agents, such as forskolin, but was unaffected by blockade of the production of endogenous cAMP. It remains elusive whether elevation of intracellular cAMP is responsible for the antioxidant activity of PGE2.

Increasing evidence indicates that the vascular and renal actions of PGE2 may be mediated by NO, raising a possibility that the NO-cGMP pathway may be affected in mPGES-1−/− mice. We found that Ang II–induced hypertension in mPGES-1−/− mice was associated with a significant reduction of urinary NOx excretion that was restored by Tempol treatment. Similar results were obtained with measurements of urinary cGMP, a reliable index of urinary NO levels. The results suggest that Ang II–induced hypertension in mPGES-1−/− mice is associated with reduced NO bioavailability that is likely attributable to oxidative stress. Therefore, PGE2 may influence NO bioavailability via inhibition of oxidative stress, although a direct stimulatory effect of PGE2 on NO production cannot be ruled out.

PGE2 may exert diverse roles in the regulation of vascular tone, depending on the vascular beds. In most vascular beds, PGE2 serves as a physiologically important vasodilator. However, PGE2 is a potent vasoconstrictor in the tail artery and mesenteric arteries. The vascular effects of PGE2 are also species dependent. For example, in isolated rabbit afferent arterioles, PGE2 elicited vasodilation, whereas in the blood-perfused juxtamedullary nephron preparation, it caused afferent arteriolar vasoconstriction in the rat. Using mPGES-1–deficient mice, we for the first time demonstrated that the net effect of endogenous PGE2 is vasodilatory, at least in the setting of Ang II infusion. In agreement with this finding, a large body of evidence suggests that defective PG synthesis or signaling may be involved in the pathogenesis of hypertension. In particular, the lack of ability of PGE2 and PGH2 to buffer the vasoconstrictive action of Ang II or thromboxane A2 has been demonstrated in the renal vasculature of spontaneously hypertensive rats. Along this line, the spontaneously hypertensive rat kidney exhibited a reduction of PGE2-simulated adenylyl cyclase activity, suggesting a deficiency in the G protein coupled to PGE2 receptors.

The receptor subtypes involved in mediating the antioxidant activity of PGE2 remain elusive. In general, EP2 and EP4 signal by intracellular cAMP and mediate smooth muscle relaxation, whereas EP2 and EP4 elevate intracellular calcium and elicit smooth muscle contractile responses. Deletion of either EP2 or EP4 significantly attenuates the vasodilator response induced by PGE2. Mice lacking EP2 develop salt-sensitive hypertension. Recently, activation of EP2 receptors was reported to mediate endothelium-dependent stimulation of endothelial NO synthase activity via dephosphorylation at Thr495. It is possible that the cAMP elevating EP receptors, eg, EP2 and EP4, may coordinately stimulate NO release and inhibit ROS production in the vasculature, thereby shifting the NO-ROS balance to favor vasodilatation. Future studies are needed to determine whether EP2 or EP4 is coupled to redox signaling in the vasculature.

Perspectives

The present study examined the role and mechanism of vascular mPGES-1 in the setting of Ang II treatment. We have identified antioxidant activity of mPGES-1–derived PGE2 that likely confers protection against Ang II–induced vascular constriction. This information will help us understand the potential cardiovascular consequences of mPGES-1 inhibitors.

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Disclosures

None.

References

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Supplemental Data

Antioxidant Activity of mPGES-1-Derived PGE$_2$ Confers Protection Against Angiotensin II-Induced Hypertension

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Methods

Enzyme immunoassay.

Urine samples were centrifuged for 5 min at 10,000 rpm and diluted 1:1 with EIA Buffer. Concentrations of PGE$_2$, 8-isoprostane, and cGMP were determined by enzyme immunoassay according to manufacturer’s instructions (Cayman Chemicals).

Nitrate/nitrite assay.

The nitrate oxide/nitrite (NO$_x$) assay kit (Cayman Chemicals) was used according to the manufacturer’s instruction. Briefly, the centrifuged 80 µl of 1:10 diluted urine samples was added to a 96-well plate, followed by addition of 10 µl of each of the Enzyme Cofactor mixture and the Nitrate Reductase mixture. The plate was covered and incubated at room temperature for 1 h, followed by sequential additions of 50 µl of Griess Reagent R1 and the same amount of Griess Reagent R2. After 10 min incubation at room temperature, the color developed and the absorbance was read at 540 nm.

Real time RT-PCR

Total RNA isolation and reverse transcription were performed as previously described $^1$. Oligonucleotides were designed using Primer3 software (available at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Real time PCR amplification was performed using the SYBR Green Master Mix (Applied Biosystems) and the Prism 7500 Real time PCR Detection System (Applied
Biosystems). Cycling conditions were 95°C for 10 min followed by 40 repeats of 95°C for 15 s and 60°C for 1 min.

**Immunohistochemistry.**

Abdominal aortas were fixed with 3% paraformaldehyde and embedded in paraffin. Aorta sections (4-µm thickness) were incubated in 3% H2O2 for 10 min at room temperature to block endogenous peroxidase activity. The slides were boiled in antigen retrieval solution [Tris-HCl 1 mM, ethylenediaminetetraacetic acid (EDTA) 0.1 mM, pH = 8.0)] for 15 min at high power in a microwave oven. The sections were incubated overnight at 4 °C with primary antibodies at appropriate dilutions (anti-mPGES-1, Cayman). After washing with PBS, the secondary antibody was applied and the signals visualized using the ABC kit (Santa Cruz).

**Cell culture experiments**

Normal primary cultures of human smooth muscle cells (HSMCs) derived from aortas (batch F-14579) were obtained from American Type Culture Collection (Manassas, VA) and maintained in Ham's F-12K medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 10 µg/ml insulin (Life Technologies, Paisley, UK), 1 µg/ml transferrin (Sigma), 10 ng/ml sodium selenite (Sigma), 20 µg/ml endothelial cell growth supplement (Sigma). Cells between 2 and 6 passages grown in 6-well plastic plates were used for the experiments. Primary cultures of mouse SMCs (mSMCs) were performed as previously described. Briefly, under anesthesia, the thoracic aortae from mPGES-1 +/+ and -/- mice were harvested, washed in PBS and placed in culture medium (DMEM high glucose with 10% FBS and 1% penicillin/streptomycin). The tunica adventitia and intima of the isolated aortae were dissected out by scraping, and the tunica media was chopped into
1-2 mm pieces. Then the pieces were transferred into 25 cm$^2$ culture flask precoated with 0.1% gelatin containing culture medium. Capped flasks were incubated at 37°C with 5% CO$_2$, and the media were changed every other day until the cells reached confluence. The third passage was used for the experiments. mSMCs were seeded into the 6-well plates, and after 90% confluence, the cells were fasted by serum free medium for 24 hours. Then Ang II at 100 nM was given to the mSMCs for 24 hours.

**Measurement of reactive oxygen species (ROS).**

The fluorogenic substrate 2',7'-dichlorofluorescein diacetate (DCFDA) is a cell-permeable dye that is oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) by H$_2$O$_2$ and can therefore be used to monitor intracellular generation of ROS. For measurement of ROS, cells were seeded to 96-well plate and after confluence, they were washed twice with PBS and incubated for 30 min with 50 µM DCFDA diluted in CS-C medium with 10% FCS and then for additional 30 min with 100 nM Ang II in the presence or absence of 0.5 µM PGE$_2$. At the end of the incubation period, relative fluorescence was measured using a fluorescence plate reader (FLUOstar OPTIMA) at excitation and emission wavelengths of 485 and 528 nm, respectively, three times at 90-s intervals. For dihydroethidium (DHE) staining, HSMCs cells were seeded to a 6-well plate. Upon confluence, they were fasted for 24 h, followed by incubation with Ang II (100nM) in the presence or absence of PGE2 (0.5 µM) for 30 min. Following this treatment, cells were incubated with DHE (10 µM) for additional 20 min. After washing with PBS, fluorescence was observed using fluorescent microscope.

**Measurement of NADPH oxidase activity.**
Under anesthesia, the thoracic aortae were harvested, dissected free of perivascular adventia attached but contained endothelia. The homogenates of the thoracic aortae and cultured SMCs were prepared in Krebs solution using a glass-to-glass homogenizer and sonicator, respectively. Activity of NADPH oxidase was measured by a luminescence assay with 5 µmol/L lucigenin as the electron acceptor and 100 µmol/L NADPH as the substrate. The reaction was started by addition of NADPH to the homogenates. Luminescence was measured every 1.8s for 3 min in a luminometer. Buffer blank was subtracted from each reading. Activity was expressed as arbitrary units/mg protein.

References


Figure S1. Real time RT-PCR determination of SOD1-3 in the aortae of mPGES-1 +/+ and -/- mice treated with vehicle or Ang II (0.35 mg/kg/day). N=7 in each group.
Figure S2. Evaluation of ROS production in cultured HSMCs. The confluent cells were treated for 30 min with vehicle, 100 nM Ang II in the presence or absence of 0.5 µM PGE$_2$. ROS production was quantified using 2',7'-dichlorofluorescein diacetate (DCFDA) (A) and also evaluated by DHE staining (B). In (a), n=12 in each group. The images in (b) are representatives of 2-3 experiments.
Figure S3. Evaluation of NADPH oxidase activity and subunit expression in cultured HSMCs. The confluent cells were treated for 24 h with vehicle, 100 nM Ang II in the presence or absence of 0.5 µM PGE2. The enzyme activity was determined using a luminescence assay (a) and expression of p47phox (b), gp91phox (c), and Nox1 (d) was analyzed by real time RT-PCR and normalized by GAPDH. N=4 in each group.