Bone Morphogenic Protein-4 Impairs Endothelial Function Through Oxidative Stress–Dependent Cyclooxygenase-2 Upregulation: Implications on Hypertension

Wing Tak Wong,* Xiao Yu Tian,* Yangchao Chen, Fung Ping Leung, Limei Liu, Hung Kay Lee, Chi Fai Ng, Aimin Xu, Xiaoqiang Yao, Paul M. Vanhoutte, George L. Tipoe, Yu Huang

Rationale: Bone morphogenic protein (BMP)4 can stimulate superoxide production and exert proinflammatory effects on the endothelium. The underlying mechanisms of how BMP4 mediates endothelial dysfunction and hypertension remain elusive.

Objective: To elucidate the cellular pathways by which BMP4-induced endothelial dysfunction is mediated through oxidative stress–dependent upregulation of cyclooxygenase (COX)-2.

Methods and Results: Impaired endothelium-dependent relaxations, exaggerated endothelium-dependent contractions, and reactive oxygen species (ROS) production were observed in BMP4-treated mouse aortae, which were prevented by the BMP4 antagonist noggin. Pharmacological inhibition with thromboxane prostanoid receptor antagonist or COX-2 but not COX-1 inhibitor prevented BMP4-induced endothelial dysfunction, which was further confirmed with the use of COX-1−/− or COX-2−/− mice. Noggin and knockdown of BMP receptor 1A abolished endothelium-dependent contractions and COX-2 upregulation in BMP4-treated aortae. Apocynin and tempol treatment were effective in restoring endothelium-dependent relaxations, preventing endothelium-dependent contractions and eliminating ROS overproduction and COX-2 overexpression in BMP4-treated aortae. BMP4 increased p38 mitogen-activated protein kinase (MAPK) activity through a ROS-sensitive mechanism and p38 MAPK inhibitor prevented BMP4-induced endothelial dysfunction. COX-2 inhibition blocked the effect of BMP4 without affecting BMP4-induced ROS overproduction and COX-2 upregulation. Importantly, renal arteries from hypertensive rats and humans showed higher levels of COX-2 and BMP4 accompanied by endothelial dysfunction.

Conclusions: We show for the first time that ROS serve as a pathological link between BMP4 stimulation and the downstream COX-2 upregulation in endothelial cells, leading to endothelial dysfunction through ROS-dependent p38 MAPK activation. This BMP4/ROS/COX-2 cascade is important in the maintenance of endothelial dysfunction in hypertension. (Circ Res. 2010;107:984-991.)

Key Words: bone morphogenic protein 4 ■ cyclooxygenase-2 ■ reactive oxygen species ■ endothelial dysfunction ■ endothelium-dependent contractions

Bone morphogenetic protein (BMP)4 belongs to the BMP family, 6 of which (BMP2 to BMP7) fall into the transforming growth factor-β superfamily. BMP4 was originally discovered to participate in embryonic development and bone and cartilage formation.1–3 BMP4 is upregulated in calcified atherosclerotic plaques4,5 and reduces endothelium-dependent relaxations (EDRs).6 BMP4 is a mechanosensitive and proinflammatory gene; disturbed flow increases BMP4 production in cultured endothelial cells.7 BMP4 promotes the expression of intracellular adhesion molecules and monocyte adhesion in a reactive oxygen species (ROS)-dependent manner.7–9 Overexpression of BMP4 in endothelial cells enhances pulmonary vascular remodeling in pulmonary hypertension.10 The infusion of

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BMP4 induces hypertension in mice partly through stimulating the expression and activity of vascular NADPH oxidases and the subsequent overproduction of ROS, which disturbs endothelial function.11 However, the exact underlying mechanisms of BMP4-induced oxidative stress and endothelial dysfunction remain elusive. COX-2 could be involved because ROS triggers the release of prostanoids via the action of COX-2.12 COX-2 is upregulated in atherosclerotic lesions13 and catalyzes the production of the majority of vascular prostanoids in human atherosclerotic areas.14 COX-2 inhibition improves endothelial function in patients with hypertension and coronary heart disease.15,16 COX-2 can be also expressed constitutively in rat and human vascular endothelial cells,17,18 and its expression is upregulated with aging in hamster aortae,19 suggesting that COX-2 plays an important role in both the physiological and pathological regulation of vascular function, depending on the level of its expression and activity.

The present study hypothesizes that upregulated COX-2 in endothelial cells plays a critical role in BMP4-induced endothelial dysfunction. Mouse, rat, and human arteries were used to investigate whether or not BMP4 impairs EDRs and facilitates endothelium-dependent contractions (EDCs) and whether oxidative stress serves as a link between BMP4 stimulation and downstream COX-2 upregulation. Finally, the possible clinical relevance of this BMP4/COX-2 pathway in hypertension was examined.

Methods

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

Animals

C57BL/6J mice, spontaneously hypertensive rats (SHR), and Wistar–Kyoto (WKY) rats were supplied by Chinese University of Hong Kong. C57BL/6J mice, spontaneously hypertensive rats (SHR), and Wistar–Kyoto (WKY) rats were supplied by University of Hong Kong. All of the experiments were conducted under our institutional guidelines for the humane treatment of laboratory animals.

Human Renal Arteries Specimens

The present study was approved by the Joint Chinese University of Hong Kong–New Territories East Cluster Clinical Research Ethics Committee. Human renal arteries were harvested from nephrectomy specimens from normotensive and hypertensive patients after obtaining informed consent. The mean age of patients was 62.5 years (range, 44 to 70 years). The indications for surgery included tumor (5 patients) and poorly functioning kidney (1 patient) in each group. History of hypertension was defined as having persistent elevated blood pressure of ≥140 mm Hg, or diastolic pressure of ≥90 mm Hg and requiring medical therapy.

Blood Vessel Preparation

Adult male mice and rats were euthanized by CO2 suffocation, and mouse aorta or rat intralobar renal arteries were removed and placed in ice-cold Krebs solution (mmol/L): 119 NaCl, 4.7 KCl, 2.5 CaCl2, 1 MgCl2, 25 NaHCO3, 1.2 KH2PO4, and 11 d-glucose. Arteries were cleaned of adventitial adipose tissue and cut into ring segments of 2 mm in length. As described previously,20,21 mouse aortic rings were incubated for 12 hours in DMEM (Gibco, Grand Island, NY) culture media with 10% FBS (Gibco), 100 IU of penicillin, and 100 µg/mL streptomycin and were placed in a CO2 incubator with 95% O2 plus 5% CO2 with and without BMP4. After 12 hours of incubation, rings were suspended in a myograph (Danish Myo Technology, Aarhus, Denmark) for recording of changes in isometric tension. Briefly, 2 steel wires (40 µm in diameter) were inserted through the lumen of the vessel, and each wire was fixed to the jaws built in the myograph. The organ chamber was filled with 5 mL of Krebs solution and gassed by 95% O2/5% CO2 at 37°C (pH≈7.4). Each ring was stretched to 3 mN, an optimal tension, and then allowed to stabilize for 90 minutes before the start of each experiment.

Functional Studies

Some arterial rings were exposed to BMP4 in control solution or in the presence of one of the following inhibitors: noggin (BMP4 antagonist, 100 ng/mL), apocynin (NADPH oxidase inhibitor, 100 µmol/L), tempol (SOD mimetic, 100 µmol/L), or SB202190 (p38 mitogen-activated protein kinase [MAPK] inhibitor; 10 µmol/L). After incubation with BMP4 for 12 hours, rings were suspended in a myograph and subjected to 30-minute exposure to 1 × 10−6 mol/L L-NAME (thromboxane prostanoid receptor antagonist; 100 mol/L), celecoxib (3 mol/L), or L-NAME-1 (100 µmol/L). The concentration of these individual inhibitors is known to be specific against the respective target.19,22 The first series of experiments examined the alterations in EDRs. Rings were contracted with phenylephrine (1 µmol/L) to establish a stable tension and acetylcholine (ACh) was then added cumulatively (1 nmol/L to 1000 mol/L). ACh-induced relaxations were abolished by 100 µmol/L, Nω-nitro-l-arginine methyl ester (L-NAME) (NOS inhibitor), or by endothelial removal. The second set of experiments examined EDCs. Aortic rings were first treated for 30 minutes with 100 µmol/L L-NAME to eliminate the interference of endothermally derived nitric oxide (NO), a procedure commonly adopted to unmask ACh-induced EDCs.19,23 and then contractions were elicited by ACh (0.1 to 30 mol/L). EDC was expressed as active tension by dividing the peak contraction (mN) by 2 × vessel length in millimeters. Intralobar renal arteries were dissected from SHR and WKY rats and subjected to 12-hour organ culture in DMEM in control and in the presence of 100 ng/mL noggin or 3 µmol/L celecoxib. Both EDRs and EDCs were studied and compared in different treatment groups. Human arteries were treated using the same protocol as for rat renal arteries.

ROS Detection by Electron Paramagnetic Resonance Spin Trapping

To measure ROS released from arterial tissues, electron paramagnetic resonance (EPR) was performed with 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine hydrochloride (TEMPONE-H, Alexis) and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO, Alexis) as spin trap agents. All EPR samples were placed in 100-µL glass tubes and
suspended in Krebs solution. To inhibit reactions catalyzed by transition metals, DTPA (0.2 mmol/L) was added. X-band EPR spectra were measured at room temperature using an EMX EPR spectrometer (Bruker). The EPR settings were as follows: field center, 3480 G; field sweep, 100 G; microwave frequency, 9.746 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 0.3 G; conversion time, 1024 ms; time constant, 640 ms.

**Constructs, Lentivirus Production, and Transduction**

We have designed 2 short hairpin (sh)RNAs targeting mouse BMP receptor 1a: shRNA1 (5'-GCT GGT AAA TTC AAC AGT GAC ACA AAT G-3') and shRNA2 (5'-TCT CTC TAT GAC TTC CTG AAA TGT GCC A-3'); and 1 shRNA targeting firefly luciferase as a control: 5'-TGC GCT GCT GGT GCC AAC CCT ATT CT-3'. DNA fragments containing shRNAs sequence were synthesized and cloned into lentiviral RNA interference vector pLUNIG after annealing as described previously. The VSV-G–pseudotyped lentiviruses were produced by cotransfecting 293T cells with the transfer vector and 3 packaging vectors (pMDL/pRRE, pRSV-REV, and pCMV-VSVG) as described previously. Subsequent purification was performed using ultracentrifugation. Mouse blood vessels were cultured in 24-well plates and were transduced with lentivirus and 8 µg/mL polybrene (Sigma).

**Statistical Analysis**

Results represent means±SEM from different animals or humans. Concentration–response curves were analyzed by nonlinear regression curve fitting using GraphPad Prism software (Version 4.0, San Diego, Calif). The protein expression was quantified by densitometer (FluorChem, Alpha Innotech, San Leandro, Calif) and normalized to GAPDH and then compared with control. Statistical significance was determined by 2-tailed Student’s t test or 1-way ANOVA followed by Bonferroni post hoc test when more than 2 treatments were compared. *P<0.05 indicates statistically significant difference.

**Results**

**BMP4 Impairs EDRs**

ACh-induced EDRs in mouse aorta were impaired in a concentration-dependent manner by 12 hours of exposure to BMP4 at 10, 20, and 80 ng/mL (Figure 1 A). BMP4 exposure also reduced EDRs in a time-dependent manner at 12, 18, and 24 hours (Figure 1B). The BMP4-treated aorta contracted in a concentration-dependent manner (12, 18, and 24 hours) on EDRs. BMP4 antagonist noggin at 100 ng/mL prevented BMP4-induced reduction of EDRs (Figure 1D). By contrast, endothelium-independent relaxations to sodium nitroprusside were unaffected by BMP4 (Figure 1E).

**BMP4-Induced Endothelial Dysfunction Is Mediated Through COX-2**

Thirty-minute treatment with COX-2 inhibitor celecoxib (3 µmol/L) or thromboxane prostanoid receptor antagonist S18886 (0.1 µmol/L) prevented the impaired EDRs in BMP4-treated aortae, whereas COX-1 inhibitor sc-560 (0.3 µmol/L) had no effect (Figure 2A). BMP4 failed to impair EDRs of aortae from COX-2-/- mice as compared with those from wild-type or COX-1-/- mice (Figure 2B and 2C). Celecoxib or S18886 also abolished BMP4-induced ACh-mediated EDCs in the presence of nitric oxide synthase inhibitor L-NAME (100 µmol/L), whereas sc-560 had less effect (Figure 2D). Similarly, the ability of BMP4 to enhance EDC response was also abolished in aortae from COX-2-/- mice, as compared with those from wild-type or COX-1-/- mice (Figure 2E and 2F).

**BMP4-Induced COX-2 Upregulation Is Mediated Through BMP Receptor 1A**

BMP4 antagonist noggin prevented EDCs (Figure 3A) and COX-2 upregulation (Figure 3B) in BMP4-treated aortae. Knocking down BMP receptor (BMPR)1A by shRNA also abolished EDCs (Figure 3C). Knockdown of BMPR1A reduced BMPR1A expression and COX-2 upregulation in BMP4-treated mouse aortae (Figure 3D). BMP4-induced COX-2 upregulation in mouse aortae was mainly confined to endothelial cells as the removal of endothelium significantly reduced the COX-2 expression showed by Western blotting (Figure 3E). Immunostaining also showed a similar increase of COX-2 expression in the endothelium triggered by BMP4 in mouse aortae (Online Figure 1).

**BMP4 Upregulates COX-2 Through ROS and MAPK**

Cotreatment of NADPH oxidases inhibitor apocynin (100 µmol/L) or ROS scavenger tempol (100 µmol/L) significantly improved EDRs in BMP4-treated aortae (Figure 4A). Cotreatment with specific p38 MAPK inhibitor
SB202190 at 10 μmol/L also improved the reduced EDRs (Figure 4B). Likewise, apocynin, tempol, or SB202190 prevented EDCs in BMP4-treated aortae (Figure 4C and 4D). Western blotting demonstrated that COX-2 upregulation by BMP4 was reduced by apocynin, tempol, or SB202190, but not by extracellular signal-regulated kinase inhibitor PD98059 (10 μmol/L) or c-Jun N-terminal kinase inhibitor SP600125 (10 μmol/L) (Figure 4E and 4F). In primary culture of murine aortic endothelial cells, BMP4-induced COX-2 upregulation and p38 MAPK phosphorylation was abolished by noggin, tempol, apocynin, or SB202190, whereas the COX-1 expression remained unaffected (Figure 5A and 5B).

**BMP4 Increases ROS Production**

BMP4 significantly elevated ROS production in ACh-stimulated (10 μmol/L) aortae, as determined by EPR spectroscopy (Figure 6A and 6B). Noggin, apocynin, tempol, or removal of endothelium significantly inhibited the ROS production in BMP4-treated mouse aortae (Figure 6C). In aortae from COX-1−/− or COX-2−/− mice, BMP4 induced a similar increase of ROS production as compared with aortae from wild-type mice (Figure 6D).

**Association Between BMP4 and COX-2 in Hypertensive Rats and Human Subjects**

Noggin or celecoxib abolished the enhanced EDCs in SHR renal arteries as compared with WKY (Figure 7A and 7B). Noggin or celecoxib abolished the enhanced EDCs in SHR renal arteries as compared with WKY (Figure 7C and 7D). Noggin also prevented the increased expression of both BMP4 and COX-2 and inhibited the increased phosphorylation of p38 MAPK in SHR renal arteries (Figure 7E through 7G). In addition, SB202190 inhibited the increased COX-2 expression and p38 phosphorylation in SHR renal arteries (Figure 7H and 7I).

**Discussion**

The present study demonstrates that upregulated expression of COX-2 plays an essential role in BMP4-induced endothelial dysfunction.
We demonstrate for the first time that the impaired EDRs and exaggerated EDCs in the BMP4-treated mouse aortae can be abolished by a BMP4 antagonist, COX-2 inhibitor, and ROS scavengers and are absent in COX-2/H11002/H11002 mice. We also show that BMP4 upregulates COX-2 expression through a BMP4/BMPR1A/ROS/p38 MAPK signaling pathway. Furthermore, the present study provides novel evidence for a significant contribution of BMP4 and COX-2 to endothelial dysfunction in spontaneously hypertensive rats and its relevance to human hypertension. Collectively, the present findings clearly support that BMP4 is an upstream activator which triggers overexpression of COX-2 in endothelial cells as an important downstream target enzyme responsible for the initiation and maintenance of endothelial dysfunction. A possible pathophysiological significance of BMP4 in hypertension is thus revealed.

Earlier works by others demonstrated the involvement of BMP4 in atherosclerosis and hypertension and in mediating inflammatory responses of endothelial cells induced by shear stress via a ROS-dependent mechanism involving Nox1-based NADPH oxidase.\textsuperscript{7,8} In mouse, the infusion of BMP4 caused hypertension and impaired ACh-induced aortic relaxations through stimulation of NADPH oxidase,\textsuperscript{11} suggesting that BMP4 could serve as a potential novel predictor of vascular dysfunction. Indeed, the present results show that BMP4 directly impaired EDRs in blood vessels from 3 different species. The data are in line with BMP4-induced endothelial dysfunction in rat carotid arteries.\textsuperscript{6} The harmful effect of BMP4 on endothelial cells is confirmed by the use of noggin. Like other members of the transforming growth factor-\textbeta superfamily, BMPs exert their cellular actions via membrane receptor complex (BMPR1a, -1b, and -2).\textsuperscript{26,27} Existing evidence suggests that BMPR1a plays an important role in the formation of blood vessels and of the atrioventricular valves.\textsuperscript{28,29} The present study used shRNA to knock down BMPR1a in the mouse aortae and shows that the detrimental vascular action of BMP4 is mediated through the BMP receptor.

The present study also demonstrates that BMP4 facilitates EDCs, which is a pathophysiological response seen in hypertension. Similar to the previous demonstration of the pivotal role of COX-2 in the appearance of EDCs in hamster aortae,\textsuperscript{19} BMP4-induced EDC is abolished by COX-2 inhibitor and thromboxane prostanoid receptor antagonist, suggesting that COX-2–dependent arachidonic acid metabolites act as endothelium-derived contracting factors in BMP4-treated arteries. By contrast, COX-1 mediated EDCs in the aortae of aged mice and spontaneously hypertensive rats,\textsuperscript{22,30} suggesting a significant difference in the role of COX isoforms, depending on the pathological initiators and species. If the vascular effect of BMP4 depends on the activation of COX-2, then this enzyme could be the target responding to BMP4-induced oxidative stress. The critical role of COX-2 in BMP4-induced
endothelial dysfunction is supported by the observations that treatment with celecoxib prevented the BMP4-induced endothelial dysfunction. More importantly, BMP4 lost its ability to impair endothelial function in COX-2/H11002/H11002 mice. ROS produced by Nox1-based NADPH oxidase is the major downstream target of BMP4 to mediate inflammatory vascular responses. In the present study, BMP4 induces ROS production in cultured endothelial cells and in isolated rat arteries, which is in line with previous reports. Moreover, the use of EPR spectroscopy spin trap to assess the ROS production in mouse aortae in situ permitted the confirmation of the endothelial origin of ROS production induced by BMP4 both in the presence and absence of ACh stimulation. The ROS production was not reduced by a selective COX-2 inhibitor and was present in the aortae of COX-2/H11002/H11002 mice. ROS removal by scavengers annulled BMP4-induced COX-2 overexpression. Taken together, these findings imply that ROS induced by BMP4 is responsible for BMP4-mediated upregulation of COX-2, which is constitutively expressed in mouse endothelial cells. By contrast, a specific COX-1 inhibitor did not influence the vascular effect of BMP4. Finally, BMP4 did not alter the expression of COX-1 in mouse aortae and cultured mouse aortic endothelial cells. Collectively, our study provides evidence showing that COX-2 is most likely to serve as the link between BMP4 and endothelial dysfunction.

The present study indicates that p38 MAPK activation participates in the regulation of COX-2 expression because a MAPK inhibitor attenuated COX-2 upregulation and improved endothelial function in BMP4-treated mouse aortae. ROS enhances the activity of p38 MAPK in vascular tissues. BMP4 can activate MAPK (p38 and p44/42) in endothelial cells and myocytes. The present findings thus suggest that MAPK activation is functionally coupled to COX-2 upregulation, COX-2–dependent EDCs, and impaired EDRs in response to BMP4. In addition, ROS scavengers prevented the BMP4-induced COX-2 upregulation and phosphorylation of p38 MAPK in cultured mouse endothelial cells, thus confirming that endothelial cells are the primary site of action for BMP4 and also supporting an essential role of ROS-dependent p38 MAPK activation in the harmful action of BMP4.

To further elucidate the pathophysiological significance of BMP4 and COX-2 in endothelial dysfunction, the present study examined the vascular protective effect of the BMP4 antagonist noggin or the COX-2 inhibitor celecoxib in spontaneously hypertensive rats. Treatment of SHR renal arteries with either noggin or celecoxib normalized relaxations to the level in WKY rat arteries and abolished EDCs. Although the infusion of BMP4 causes hypertension in healthy mice, it was unclear whether or not BMP4 is a biomarker or is
BMP4 and COX-2 are expressed in hypertensive human renal arteries, the present data may be relevant to cardiovascular disease. Indeed, the present findings in mouse, rat, and human arteries supports an important role of BMP4-dependent COX-2 in endothelial dysfunction in hypertension. Our results also suggest that the BMP4 signaling cascade could be a potential target for pharmacological intervention to prevent COX-2-dependent vascular dysfunction.

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Disclosures
None.

References
BMP4, COX-2, and Endothelial Dysfunction


Novelty and Significance

What Is Known?

- Oxidative stress contributes to the vascular complications of hypertension.
- Bone morphogenetic protein (BMP)4 activates NADPH oxidase and is associated with vascular inflammation.
- Infusion of BMP4 in mice induces hypertension and causes endothelial dysfunction

What New Information Does This Article Contribute?

- BMP4 impairs endothelium-dependent relaxations and induces endothelium-dependent contractions in the mouse aorta by activating BMP receptor 1A.
- BMP4-induced endothelial dysfunction is mediated by oxidative stress–dependent upregulation of cyclooxygenase (COX)-2.
- The expression of COX-2 and BMP4 were increased in renal arteries from hypertensive patients compared with those from normotensive subjects.

BMP4 is a proinflammatory gene induced by disturbed flow in endothelial cells. The limited data available indicate that BMP4 reduces endothelium-dependent relaxations and induces hyper-tension by activating NADPH oxidase. However, it is unknown whether or not COX-2, another mediator of vascular inflammation, contributes to BMP4-induced endothelial dysfunction in hypertension. The present study demonstrates that BMP4 ex-aggerates endothelium-dependent contractions in mouse aorta, whereas silencing of BMP receptor 1A prevents the harmful effects of BMP4. In mouse arteries and endothelial cells, BMP4 increases the production of reactive oxygen species, which in turn activate p38 MAPK and contribute to the BMP4-induced COX-2 upregulation and the altered vascular reactivity. BMP4-induced endothelial dysfunction is absent in COX-2–deficient mice, supporting a close association between the 2 proinflam-matory factors. Finally, we confirm the role of BMP4 and COX-2 in endothelial dysfunction in hypertensive rats and humans. The BMP4 antagonist noggin improves endothelial function and reduces BMP4 and COX-2 expression in renal arteries from hypertensive rats and patients. Thus, BMP4 and its downstream oxidative stress–dependent upregulation of the expression and activity of COX-2 are important in the development of hypertension. These findings suggest that strategies specifically targeting this signaling cascade could be potentially useful in treating vascular dysfunction related to human hypertension.
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SUPPLEMENTAL MATERIAL

DETAILED METHODS

Animals
C57BL/6J mice, spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats were supplied by CUHK Laboratory Animal Center, while COX-1-/- or COX-2-/- mice were supplied by University of Hong Kong. All of the experiments were conducted under our institutional guidelines for the humane treatment of laboratory animals.

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treatment groups. Human arteries were treated using the same protocol as for rat renal arteries.

**Primary culture of murine aortic endothelial cells**

The method was modified from Kobayashi et al. as previously reported 5. Briefly, male mice were anaesthetized with an intraperitoneal injection of pentobarbital sodium. 0.5 mL PBS containing 1,000 U/ml of heparin was perfused into the circulation from the left ventricle. The abdominal aortae were rapidly removed and placed in 20% FBS-DMEM, cleaned off surrounding tissues and then cut open. The aortae were incubated with collagenase type II for 10 min at 37 °C. Endothelial cells were collected by centrifugation at 3000 rpm for 5 min. The pellet was gently re-suspended by pipette with 5 mL of 20% FBS-DMEM and cultured in a 25 cm² tissue culture flask. After 1-h incubation at 37 °C, the medium was removed, changed to EGM (endothelial cell growth medium supplemented with bovine brain extract, Lonza, Walkersville, MD). The identity of endothelial cells was confirmed by immunohistochemical staining against eNOS, while there was no positive staining of α-smooth muscle actin (αSMA) for detecting smooth muscle cell and prolyl-4-hydroxylase (P4H) for detecting fibroblast.

**ROS detection by EPR spin trapping**

To measure ROS released from arterial tissues, electron paramagnetic resonance (EPR) was performed with 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine hydrochloride (TEMPONE-H, Alexis) and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO, Alexis) as spin trap agents. All EPR samples were placed in 100 μL glass tubes and suspended in Krebs solution. In order to inhibit reactions catalyzed by transition metals, DTPA (0.2 mmol/L) was added. X-band EPR spectra were measured at room temperature using an EMX EPR spectrometer (Bruker). The EPR settings were as follows: field center, 3480 G; field sweep, 100 G; microwave frequency, 9.746 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 0.3 G; conversion time, 1024 msec; time constant, 640 msec.

**Constructs, lentivirus production and transduction**

We have designed two shRNAs (short hairpin RNA) targeting mouse BMP receptor 1a: shRNA1 (5'- GCT GTT AAA TTC AAC AGT GAC ACA AAT G -3') and shRNA2 (5'- TCT CTC TAT GAC TTC CTG AAA TGT GCC A -3'); and one shRNA targeting firefly luciferase: 5'-TGC GCT GCT GGT GCC AAC CCT ATT CT-3' as a control. DNA fragments containing shRNAs sequence were synthesized and cloned into lentiviral RNAi (RNA interference) vector pLUNIG after annealing as previously described 6, 7. The VSV-G-pseudotyped lentiviruses were produced by cotransfecting 293T cells with the transfer vector and three packaging vectors: pMDLg/pRRE, pRSV-REV, and pCMV-VSVG as previously described. Subsequent purification was performed using ultracentrifugation. Mouse blood vessels were cultured in 24-well plates and were transduced with lentivirus and 8 μg/ml polybrene (Sigma).

**Western blotting**

Isolated arteries were subjected to similar procedures as in functional studies, frozen in liquid nitrogen, and homogenized in an ice-cold RIPA lysis buffer that contained 1 μg/mL leupeptin, 5 μg/mL aprotinin, 100 μg/mL PMSF, 1 mmol/L sodium orthovanadate, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L sodium fluoride, and 2 μg/mL β-glycerophosphate. Tissue or cell lysates were centrifuged at 20,000 ×g for 20 min. The supernatants were collected and protein concentrations were analyzed using the Lowry method (Bio-rad, Hercules, CA, USA) 8. Protein samples were electrophoresed on a 10% SDS-polyacrylamide gel and transferred onto an immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Nonspecific binding sites were blocked by 5% non-fat milk in 0.05% Tween-20 phosphate-buffered saline (PBST), then incubated overnight at 4 °C with primary antibodies including COX-2 or COX-1 (Cayman, Ann Arbor, MI, USA), p38 MAPK, phospho-p38 MAPK (Cell Signaling Technology, Beverly, MA, USA), BMPR1A (Santa Cruz, CA, USA) or BMP4 (Sigma-Aldrich, St. Louis, MO, USA), followed by a HRP-conjugated swine anti-rabbit or anti-mouse IgG (DakoCytomation, Carpinteria, CA, USA), developed with an enhanced chemiluminescence detection system (ECL reagents, Amersham Pharmacia), and finally exposed to X-ray films. Equal protein loading
was verified with use of a housekeeping anti-GAPDH antibody.

**Immunohistochemistry**
Aortic rings were fixed in 4% paraformaldehyde at 4°C overnight, dehydrated, processed and embedded in paraffin. Cross sections at 5 μm were cut on microtome (Leica Microsystems, Germany). After rehydrated to water, sections were microwave boiled in 0.01 mol/L citrate buffer (pH 6.0) for 10 min for antigen retrieval, then incubated for 15 min with 3% H2O2 at room temperature to block endogenous peroxidase activity. After washed with phosphate buffer saline (PBS), sections were blocked in 5% normal donkey serum (Jackson Immunoresearch, West Grove, PA, USA) for 1 hour at room temperature. Primary antibody (goat polyclonal anti-COX-2, 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA; rabbit polyclonal anti-eNOS, 1:100, Abcam, Cambridge, UK) diluted in normal serum were incubated overnight at 4°C. The slides were washed with PBS three times (5 min each). Biotin-SP conjugated goat anti-rabbit secondary antibodies (1:500, Jackson Immunoresearch) diluted in PBS were added and incubated for 1 h at room temperature. Slides were washed with PBS three times (5 min each) and incubated for 30 min with streptavidin-HRP conjugate (1:500, Zymed laboratory, San Francisco, CA, USA) at room temperature, and washed. Positive staining was developed as brown precipitate by 3,3’-diaminobenzidine tetrachloride (DAB) chromogen substrate (Vector laboratory, Burlingame, CA, USA). Slides were rinsed with water and counterstained with hematoxylin. Pictures were taken under Leica DMRBE microscope with a SPOT-RT digital camera and SPOT Advanced software (Diagnostic Instruments, Sertling Heights, MI, USA.

**Drugs**
BMP4, acetylcholine, L-NAME, phenylephrine, noggin, tempol and sodium nitroprusside (SNP) were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA). SB202190, PD98059 and SP600125 were from Tocris (Avonmouth, UK). Apocynin were from Calbiochem, EMD Biosciences (La Jolla, CA, USA). S18886 (3-[(6-amino-(4-chlorobenzensulphonyl) -2-methyl-5,6,7,8- tetrahydronapht] -1-yl) propionic acid) and sc-560 were kind gifts from the Institut de Recherches Servier (Suresnes, France). Celecoxib was from Pfizer. Acetylcholine, L-NAME, phenylephrine, tempol were prepared in distilled water and the others in DMSO (Sigma-Aldrich).

**Statistical analysis**
Results represent means±SEM from different animals or humans. Concentration-response curves were analyzed by non-linear regression curve fitting using GraphPad Prism software (Version 4.0, San Diego, CA). The protein expression was quantified by densitometer (FluorChem, Alpha Innotech, San Leandro, CA) and normalized to GAPDH and then compared with control. Statistical significance was determined by two-tailed Student’s t-test or one-way ANOVA followed by the Bonferroni post-hoc test when more than two treatments were compared. P<0.05 indicates statistically significant difference.
DETAILED METHODS REFERENCES:


Online Figure I.

Representative pictures of immunohistochemistry showing COX-2 expression increased in the endothelium in mouse aortae after BMP4 treatment (20 ng/mL, 12h), while eNOS is used to label the presence of endothelium. In the negative control, slices were processed under the same procedure without the primary antibody. Arrows indicate the endothelial expressions of COX-2.
Online Figure II. (a) Effect of tempol (100 μmol/L, 12 h incubation) on acetylcholine (ACh)-induced endothelium-dependent relaxations (EDR) in renal arteries from SHR precontracted with phenylephrine (Phe). (b) Effect of tempol (100 μmol/L, 12 h incubation) on ACh-induced endothelium-dependent contraction (EDC) in renal arteries from SHR in the presence of L-NAME. Data are means±SEM of 6 rats. * p<0.05 vs control. Effect of tempol (100 μmol/L, 12 h incubation) on the expressions of BMP4 (c), COX-2 (d), p38 MAPK phosphorylation (e) in renal arteries from SHR compared to those from WKY. Data are means±SEM of 4 rats. * p<0.05 vs WKY. # p<0.05 vs SHR. ROS production measured by EPR spectroscopy in renal arteries from SHR as showed in the representative EPR spectra (f) and summarized data (g). Data are means±SEM of 4 rats. * p<0.05 vs WKY.