Inhibition of Bone Morphogenic Protein 4 Restores Endothelial Function in db/db Diabetic Mice

Yang Zhang,* Jian Liu,* Xiao Yu Tian, Wing Tak Wong, Yangchao Chen, Li Wang, Jiangyun Luo, Wai San Cheang, Chi Wai Lau, Kin Ming Kwan, Nanping Wang, Xiaoqiang Yao, Yu Huang

Objective—Bone morphogenic protein 4 (BMP4) is involved in the development of endothelial dysfunction in hypertension. This study investigated whether the inhibition of BMP4 signaling improves endothelial function in db/db diabetic mice.

Approach and Results—Male db/db mice were treated with noggin via osmotic pump infusion (1 µg/[h·kg−1]) for 2 weeks. Adenovirus BMP4-short hairpin RNA was introduced via tail vein injection at a dosage of 10⁷ pfu/mouse and its effects were examined 7 days after. Vasoreactivity was studied on wire and pressure myograph. Both noggin treatment and adenovirus BMP4-short hairpin RNA transduction improved endothelium-dependent relaxations in aortae and flow-mediated dilatation in mesenteric arteries of db/db mice. Ex vivo treatment with BMP4 inhibitors and adenovirus BMP4-short hairpin RNA rescued the impaired endothelium-dependent relaxations in db/db mouse aortae and reduced reactive oxygen species overproduction determined by dihydroethidium staining, CM-H₂DCFDA fluorescence imaging, and chemiluminescence assay in db/db mouse aortae, and also in ex vivo cultured C57BL/6 mouse aortae or primary mouse aortic endothelial cells treated with high glucose. Likewise, activin receptor–like kinase 3 silencing by short hairpin RNA lentivirus improved endothelium-dependent relaxations in db/db mouse aortae accompanied by reactive oxygen species inhibition in endothelial cells. In addition, noggin reduced BMP4 upregulation in high-glucose–treated endothelial cells and in C57BL/6 mouse aortae and in aortae from db/db mice.

Conclusions—Inhibition of BMP4/activin receptor–like kinase 3/reactive oxygen species signaling improved endothelial function in diabetic mice through limiting oxidative stress in endothelium. Inhibiting BMP4 cascade can become another potential therapeutic strategy against diabetic vascular dysfunction. (Arterioscler Thromb Vasc Biol. 2014;34:152-159.)

Key Words: bone morphogenetic protein 4, mouse ■ diabetes mellitus ■ endothelial dysfunction ■ reactive oxygen species

Bone morphogenic protein 4 (BMP4), a member of BMPs belonging to the transforming growth factors-β superfamily, plays an essential role in embryonic development, cartilage formation, and bone mineralization. In the cardiovascular system, mutations or anomalies of BMP-mediated signals are related to many vascular diseases, including pulmonary hypertension, cardiovascular calcification, stroke, and type 2 diabetes mellitus. As a proinflammatory cytokine, BMP4 can be induced under the oscillatory shear stress stimulation. BMP4 is upregulated in vasculature of atherosclerosis and hypertension. BMP4 impairs endothelium-dependent vasodilatation. Noggin is a negative regulator in bone development and neuronal differentiation, which binds to and inactivates BMP4. Noggin improves endothelial function in hypertension, inhibits BMP4-induced hypertension in mice, and counteracts the proatherogenic effects of BMP4 in endothelial cells. BMP4 is also upregulated by saturated free fatty acid or in obese mice. However, the involvement of BMP4 in diabetic vascular dysfunction is less clear. BMPs bind to 2 types of serine threonine kinase receptor: type I and type II. BMP type I receptors can be divided into 2 subgroups: activin receptor–like kinase 3 (ALK3 or BMPR1a) and ALK6 (BMPR1b) group and the ALK1 and ALK2 group. BMP4 interacts with ALK3/6 with much higher affinity to form a binding complex and to trigger the downstream signaling. The activation of BMP4 signaling leads to Smad-dependent and Smad-independent pathways, including the activation of NADPH oxidases, which results in elevated production of reactive oxygen species (ROS), p38 mitogen-activated protein kinases activation, and cyclooxygenase-2 upregulation.
In type 2 diabetes mellitus, endothelial dysfunction is both the trigger and the hallmark of cardiovascular complications. Oxidative stress is one of the major factors that cause endothelial dysfunction in hyperglycemic conditions and insulin resistance. Vascular expression of BMPs and Smad activation is enhanced by hyperglycemic stimulation in type 2 diabetic mice. However, the role of BMP4 to mediate endothelial enhanced by hyperglycemic stimulation in type 2 diabetic mice. NO bioavailability in blood vessels.

In the present study, we hypothesize that inhibition of BMP4 protects against endothelial dysfunction in db/db diabetic mice through limiting ROS production in endothelial cells and thus increases nitric oxide (NO) bioavailability in blood vessels.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results

Inhibition of BMP4 In Vivo Improves Endothelial Function of db/db Mice
Acetylcholine-induced endothelium-dependent relaxations (EDRs) in db/db mouse aortae were diminished compared with those in db/m+ mouse aortae. Noggin infusion to db/db mice restored the impaired EDRs in both aortae (Figure 1A and 1B) and mesenteric arteries (Figure 1C). By contrast, sodium nitroprusside (SNP)–induced endothelium-independent relaxations were unchanged in the 3 groups (Figure IIA in the online-only Data Supplement). Noggin treatment reduced systolic blood pressure from 120.9±3.1 to 101.7±4.0 mm Hg (P<0.05; Figure 1A in the online-only Data Supplement) without affecting insulin sensitivity or fasting glucose (Figure 1B and 1C in the online-only Data Supplement) in db/db mice. In addition, noggin did not affect insulin-induced relaxations of mesenteric arteries from db/db mice (Figure IIB in the online-only Data Supplement).

BMP4 mRNA silencing by short hairpin RNA (shRNA) adenoviral transduction via tail vein injection suppressed BMP4 content in db/db mouse aortae (Figure IIIA in the online-only Data Supplement). En face immunofluorescence staining showed a 70% decrease in the expression of BMP4 in endothelial cells of db/db mouse aortae (Figure IIIIB and IIIIC in the online-only Data Supplement). In vivo treatment with BMP4-shRNA adenovirus improved acetylcholine-induced EDRs in db/db mouse aortae compared with treatment with scramble control (Figure 1D), whereas endothelium-independent relaxations were unaffected (Figure IID in the online-only Data Supplement). In addition, flow-mediated dilatation in the second-order mesenteric arteries was evoked by creating a pressure difference of 20 mm Hg that equals an initial shear stress of ≈15 dynes/cm². Flow-mediated dilatation was augmented in these resistance arteries of db/db mice with intravenous delivery of adenovirus BMP4-shRNA to suppress BMP4 expression (Figure 1E).

Ex Vivo Inhibition of BMP4 Restores EDRs
Apart from in vivo inhibition of BMP4, ex vivo suppression of BMP4 in tissue culture by 3 BMP4 inhibitors, chordin (200 ng/mL), follistatin (400 ng/mL), and noggin (100 ng/mL), also restored the impaired EDRs in isolated db/db mouse aortae (Figure 2A) without affecting SNP-induced relaxations (Figure IIE in the online-only Data Supplement). Moreover, 48-hour treatment with high glucose (30 mmol/L) attenuated EDRs in aortae from C57BL/6 mice, which was reversed by cotreatment with noggin and chordin, as well as ROS scavenger tiron plus diethyldithiocarbamate (Figure 2C). By contrast, relaxations to SNP were comparable in different groups (Figure IIF in the online-only Data Supplement).

Ex vivo 48-hour exposure to adenovirus BMP4-shRNA augmented EDRs in db/db mouse aortae (Figure 2B). Ex vivo adenovirus BMP4-shRNA transduction also restored EDRs that were impaired by high glucose in C57BL/6 mouse aortae (Figure 2D). Neither scramble nor BMP4-shRNA adenovirus affected EDRs in C57BL/6 mouse aortae (Figure IIH in the online-only Data Supplement).

Inhibition of BMP4 Reduces ROS Generation in Endothelial Cells
The ROS levels measured by dihydroethidium (DHE) fluorescence were elevated in aortae from db/db mice compared with those from db/m+. Noggin infusion reduced the ROS production in db/db mouse aortae (Figure 3A and 3B). Ex vivo treatment with noggin, chordin, or follistatin all reduced ROS accumulation in db/db mouse aortae (Figure 3E and 3F). Figure 3G and 3H shows that the elevated ROS generation in endothelial cells was reversed by treatment with the 3 BMP4 inhibitors. In vivo BMP4-shRNA adenovirus transduction reduced DHE fluorescence intensity in db/db mouse aortic endothelial cells (Figure 3C and 3D). The similar results were also obtained in both cross-sections and endothelial cells in situ in db/db mouse aortic rings, which were treated with BMP4-shRNA adenovirus ex vivo (Figure 3I; Figure IV in the online-only Data Supplement). Finally, both BMP4-shRNA adenovirus transduction and noggin treatment reversed high-glucose–stimulated increase in the content of superoxide anions in C57BL/6 mouse aortae (Figure 3J).

In human umbilical vein endothelial cells and mouse aortic endothelial cells, intracellular ROS were measured using CM-H₂DCFDA (DCF) dye. High-glucose treatment increased DCF signal, which was reversed by noggin (100 ng/mL), chordin (200 ng/mL), and follistatin (400 ng/mL), as well as ROS scavenger tempol (100 μmol/L) and putative nicotinamide adenine dinucleotide phosphate oxidase inhibitor diphenylene iodonium (0.1 μmol/L; Figure V and VIIIB in the online-only Data Supplement).

Nonstandard Abbreviations and Acronyms

<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>ALK3</td>
<td>activin receptor–like kinase 3</td>
</tr>
<tr>
<td>BMP4</td>
<td>bone morphogenetic protein 4</td>
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<tr>
<td>DHE</td>
<td>dihydroethidium</td>
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<tr>
<td>EDR</td>
<td>endothelium-dependent relaxation</td>
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<td>NO</td>
<td>nitric oxide</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>shRNA</td>
<td>short hairpin RNA</td>
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<td>SNP</td>
<td>sodium nitroprusside</td>
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BMP4 and Endothelial Dysfunction in Diabetes

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Silencing ALK3 Improves Endothelial Function in db/db Mice

To illustrate the role of BMP4 receptor ALK3 in endothelial dysfunction in diabetic mice, ALK3 was silenced using 2 ALK3 shRNAs (shRNA-1 and shRNA-3) via lentiviral transfection and confirmed by Western blotting data (Figure 4B). ALK3 silencing inhibited the ability of BMP4 to impair EDRs in C57BL/6 mouse aortae (Figure 4A) and reduced

![Image](A.png)

**Figure 1.** Inhibition of bone morphogenetic protein 4 (BMP4) in vivo improved endothelial function in db/db mice. A. Representative traces of acetylcholine (ACh)-induced endothelium-dependent relaxations (EDRs) in aortae from db/m+, db/db, db/db infused with noggin. Concentration–response curves of ACh in (B) aortae and (C) main mesenteric arteries. Results are mean±SEM (n=6), *P<0.05 vs db/m+; #P<0.05 vs db/db. Transfection with BMP4-shRNA adenovirus in db/db mice (D) enhanced EDRs in aortae, and (E) increased flow-mediated dilatations in second-order mesenteric arteries compared with those of Ad-scramble control. Results are mean±SEM (n=5), *P<0.05 vs Ad-scramble. Phe indicates phenylephrine; and shRNA, short hairpin RNA.

![Image](B.png)

**Figure 2.** The ex vivo effect of bone morphogenic protein 4 (BMP4) inhibition on acetylcholine (ACh)-induced endothelium-dependent relaxations (EDRs) in mouse aortae. Improved EDRs in db/db mouse aortae after (A) 24-hour exposure to BMP4 antagonists, noggin (100 ng/mL), chordin (200 ng/mL), and follistatin (400 ng/mL) and (B) 48-hour after transfection with BMP4-shRNA adenovirus (Ad-BMP4-shRNA). The impaired EDRs in C57BL/6 mouse aortae after 48-hour exposure to high glucose (HG, 30 mmol/L) was reversed by cotreatment with noggin, chordin, or reactive oxygen species scavenger tiron (1 mmol/L) plus diethyldithiocarbamate (100 μmol/L); and by Ad-BMP4 shRNA transduction (D). Results are means±SEM (n=5–6). A, *P<0.05 vs control; (B) *P<0.05 vs Ad-BMP4 shRNA; (C) *P<0.05 vs normal glucose (NG; 5 mmol/L); #P<0.05 vs HG; (D) *P<0.05 vs NG, #P<0.05 vs HG+Ad-scramble shRNA.
Figure 3. Inhibition of bone morphogenic protein 4 (BMP4) reduced reactive oxygen species (ROS) overgeneration in the endothelium. Representative images and summarized data showing (A and B) ROS production estimated by dihydroethidium (DHE) staining in sections of aortae from db/m+ and db/db mice, *P<0.05; (C and D) in vivo BMP4-short hairpin RNA (shRNA) adenovirus transduction suppressed ROS level in endothelium en face of db/db mouse aortae, *P<0.05 vs Ad-scramble; (E and F) 24-hour exposure to 3 BMP4 inhibitors (ie, noggin, chordin, and follistatin) reduced ROS generation in db/db mouse aortae, *P<0.05 vs control; (G and H) 24-hour exposure to noggin, chordin, and follistatin reduced ROS generation in db/db mouse aortae, *P<0.05 vs control. I, Relative quantitative fluorescence intensity reflecting ROS level in the vascular wall and en face endothelial cells of db/db mouse aortae after 48-hour exposure to Ad-scramble or Ad-BMP4 shRNA, *P<0.05 vs Ad-scramble. J, Relative chemiluminescence units showing the superoxide anion level in C57BL/6 mouse aortae after 48-hour high glucose (HG) exposure, *P<0.05 vs normal glucose (NG), #P<0.05 vs HG control. Results are mean±SEM (n=5–6). Red, DHE fluorescence (excitation: 515 nm) in the nucleus; green, autofluorescence of elastin underneath the endothelium (excitation, 488 nm). RLU indicates relative luminescence units.
the BMP4-stimulated ROS production in vitro (Figure VIIIA and VIIB in the online-only Data Supplement). Both ALK3 shRNAs improved EDRs of db/db mouse aortae compared with the scramble (Figure 4C), whereas SNP-induced relaxations were again unaffected (Figure IIG in the online-only Data Supplement). In addition, ROS production measured by en face DHE fluorescence in the aortic endothelium of db/db mice was inhibited by ALK3 shRNAs (Figure 4D; Figure VIIIC in the online-only Data Supplement).

**Noggin Reduces BMP4 Upregulation in db/db Mice**

Compared with db/m+ mouse aortae, db/db mouse aortae expressed higher levels of BMP4 and this increase was reversed by noggin infusion (Figure 5A). The immunohistochemical images of the walls of aortae show that BMP4 expression upregulated in both endothelial and vascular smooth muscle cells was inhibited by ex vivo treatment with noggin in db/db mouse aortae (Figure 5B). Likewise, immunofluorescence staining shows that noggin reduced the BMP4 expression in endothelial cells en face of db/db mouse aortae (Figure 5C and 5D). In addition, high glucose increased the BMP4 expression and noggin inhibited this effect in ex vivo–cultured C57BL/6 mouse aortae (Figure 5E). Furthermore, high glucose increased the BMP4 expression in mouse aortic endothelial cells in a time-dependent manner (Figure IXA in the online-only Data Supplement) and noggin reversed this effect of high glucose (Figure 5F).

**Discussion**

The present study demonstrates that BMP4 mediates endothelial dysfunction in db/db mice. The inhibition of BMP4 reduces oxidative stress, increases NO bioavailability, and improves endothelial function in diabetic mice and in cultured endothelial cells under hyperglycemic stimulation. BMP4 receptor 1a (ALK3) plays a crucial role in mediating BMP4 to induce oxidative stress and endothelial dysfunction.

BMP4 is involved in the development of endothelial dysfunction in hypertension,13,19 atherosclerosis,22,23 and vascular calcification.24 BMP4 is also an upstream regulator in the process of diabetic nephropathy induced by streptozotocin in BMP4-overexpressing mice.25 Previous studies showed that high glucose or free fatty acids upregulate BMP4 in endothelial cells,10,17 indicating that BMP4 may be important in endothelial dysfunction associated with diabetes mellitus and hyperglycemia. However, the role of BMP4 in regulating endothelial function of type 2 diabetes mellitus is largely unclear. The present study shows that functional inhibition of the BMP4 signaling by either BMP4 antagonists or RNAi or silencing of ALK3 restores the impaired endothelial function in db/db mice.

The present results show that noggin infusion augmented endothelial function in both conduit and resistance arteries from db/db mice. Improved endothelial function in resistance vessels may contribute to the blood pressure reduction after noggin infusion. However, noggin infusion did not alter insulin tolerance or insulin-induced relaxations in db/db mouse aortae, suggesting that the improvement of endothelial function by BMP4 inhibition is unlikely to be attributed to the change of insulin sensitivity. More importantly, suppression of BMP4 by inhibitors or adenovirus shRNA directly improved endothelial function in isolated db/db mouse aortae and C57BL/6 mouse aortae treated with high glucose in ex vivo culture, indicating that noggin or BMP4 silencing produces a direct beneficial effect on the arteries, thus suggesting a positive role of BMP4 in vascular dysfunction in diabetic mice.

BMP4 induces oxidative stress and subsequent upregulation of proinflammatory cytokines and adhesion molecules, in response to oscillatory shear stress, or stimulation of cardiovascular risk factors such as free fatty acid and hyperglycemia.10,17,26 BMP4-induced ROS production is mediated through the activation of Nox1, Nox2, and Nox4.17,19,26–28 Here, we showed that ROS production in the aortic endothelium of
db/db mice was inhibited by BMP4 inhibitors, BMP4 silencing, and ROS scavengers, as well as in the endothelial cells exposed to high glucose.

The expression of BMP4 is higher in db/db mouse aortae compared with that from db/m+, similar to the previous studies showing high-glucose–induced BMP4 upregulation in several cell types.\textsuperscript{10,29,30} We also observed that noggin reduced BMP4 expression both in vivo and in vitro. The inhibitory effect of noggin on BMP4 expression is likely to be attributable to the suppression of NADPH oxidase activity.\textsuperscript{22}

**Figure 5.** Noggin inhibited high-glucose (HG)–induced bone morphogenic protein 4 (BMP4) upregulation. A, Western blotting of BMP4 expression in aortae from db/m+, db/db, and noggin-infused db/db mice. B, Immunohistochemical staining of BMP4 expression in mouse aortae after 24-hour incubation in tissue culture. C, Representative images and D) summarized data showing en face immunofluorescence staining of BMP4 expression in endothelial cells of intact mouse aortae. Noggin inhibited BMP4 overexpression in (E) aortae from C57BL/6 mice and (F) mouse aortic endothelial cells (MAECs) induced by 48-hour HG exposure. Results are mean±SEM (n=5–6), *P<0.05. NG indicates normal glucose; and VE-cadherin, vascular endothelial-cadherin.
Knockdown of ALK3 improved EDs in isolated db/db mouse aortae, indicating active interaction between endogenous BMP4 and ALK3 in arteries of diabetic mice. BMP4 binds to the BMP receptors21-32 (ALK2, ALK3, and ALK6) and forms the ligand–receptor complex33 to exert its cellular actions. BMP4-activated ALK3 signaling mediates endothelial dysfunction induced by BMP4 and promotes remodeling of pulmonary vasculature during hypoxia.33 ALK3 is also involved in the development of obesity34 and cardiovascular disease.35 The present results suggest that ALK3 plays a more significant role to mediate BMP4-induced harmful effect on endothelial cells as ALK3 silencing reverses BMP4-stimulated ROS production in cultured endothelial cells. Despite a complete inhibition of BMP4-stimulated ROS elevation in endothelium en face of intact mouse aorta, ALK3 silencing only causes a partial reversal of BMP4-induced impairment of acetylcholine-induced relaxations in mouse aorta. Therefore, it is possible that other ALK isoforms may also play a contributory role in BMP4-impaired endothelial function in mouse arteries, which warrants further examination.

In summary, we elucidate the critical role of endothelial BMP4 and BMP4-induced oxidative stress in endothelial dysfunction of diabetic mice. Together with the existing evidence for the beneficial effect of BMP4 inhibition in atherosclerosis and hypertension, the present results add additional lines of new evidence supporting the importance of BMP4 signaling in diabetic vasculopathy.

Sources of Funding
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Disclosures
None.

References
Bone morphogenic protein 4 (BMP4), a proinflammatory mediator that can be induced by oscillatory flow, participates in the development of vascular dysfunction, hypertension, and atherosclerosis. The present study provides novel evidence that BMP4 is upregulated in arteries from diabetic mice, which is likely to account for increased oxidative stress and the impaired endothelial function in diabetes mellitus. BMP4 antagonism and silencing genes encoding either BMP4-binding receptor (activin receptor–like kinase 3) or BMP4 effectively normalize overproduction of reactive oxygen species and restore endothelial function in diabetic mice, thus revealing a critical pathological role of BMP4 signaling in diabetic vasculopathy.
Inhibition of Bone Morphogenic Protein 4 Restores Endothelial Function in db/db Diabetic Mice

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Material and Methods

Animals
This study was approved by the Animal Experimentation Ethics Committee, Chinese University of Hong Kong (CUHK). Male C57BL/6, db/m, db/db mice of 12-14 weeks old were supplied by the CUHK Laboratory Animal Centre. Mice were housed at constant temperature (22 ± 2°C) and humidity (55 ± 5%), with a 12-h light/dark cycle and free access to food and water. All experiments were conducted under the institutional guidelines for the humane treatment of laboratory animals. For chronic treatment, vehicle or noggin (0.4 mg/kg, R&D Systems, 6057-NG-100, Minneapolis, MN, USA) were infused subcutaneously in db/db mice using osmotic pumps (mode11002, Alzet, Cupertino, CA, USA) for 2 weeks.

Basic parameters test
Systolic blood pressure was measured by a non-invasive tail-cuff method. Insulin tolerance test was examined by measuring blood glucose levels using a commercial glucometer (Ascensia ELITE, #21279, Bayer, Mishawaka, IN, USA) at 0, 15, 30, 60 and 120 min after an injection of insulin (0.5 U/kg, I6634, Sigma, St. Louis, MO, USA) to each mouse after 2-hour fasting.

Endothelial cell culture
Human umbilical vein endothelial cells (HUVEC, Lonza, Walkersville, MD, USA) were cultured in endothelial cell growth medium (EGM, CC3024, Lonza) with 10% FBS and antibiotics. Cells from passage 4-8 were used for experiments. Primary mouse aortic endothelial cells (MAECs) were isolated and cultured using a method described before. Passage 2 MAECs were confirmed by positive immunostaining with anti-eNOS (610296, BD Transduction Laboratory, San Jose, CA, USA) and anti-CD31 (sc-1506, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and negative staining with anti-α-smooth muscle actin (ab5694, Abcam, Cambridge, UK) and anti-vimentin (sc-6260, Santa Cruz). MAECs were used for experiments at passage 2-4 when they became 70% confluence.

Vasoreactivity study in wire myograph
After mice were euthanized by CO₂ suffocation, aortae and mesenteric arteries were dissected out and placed in an 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, and cut into ring segments, ~2 mm in length. Aortic rings were suspended in wire myograph (Danish Myo Technology, Denmark) and bathed in oxygenated Krebs solution at 37 °C. The basal tension was 3 mN for aortae and 1 mN for mesenteric arteries. After 1-h equilibration, rings were first contracted by 60 mmol/L KCl-containing Krebs solution. Endothelium-dependent relaxations (EDRs) induced by acetylcholine (ACh, 0.003-10 μmol/L, A6625, Sigma) were recorded in phenylephrine-contracted rings while endothelium-independent relaxations to sodium nitroprusside (SNP, 0.001-10 μmol/L, 71778, Sigma) were also examined in aortae in the presence of L-NAME (100 μmol/L, 72760, Fluka).
Flow-mediated dilatation in pressure myograph
The second-order mouse mesenteric artery (external diameter: 180-250 µm) was dissected free of surrounding adipose tissue and was cannulated between two glass cannulas in a chamber filled with 10 mL of oxygenated Krebs solution kept as previously described. The vessel diameter was monitored by a Zeiss Axiovert 40 microscope, model 11 P, with video camera, and the Myo-View software (Danish Myo Technology, Aarhus Denmark). Under no-flow condition, the artery segment was subjected to stepwise increment of 20 mmHg in intraluminal pressure from 20 to 80 mmHg at 5-minute intervals at 37°C and 3 µmol/L phenylephrine was added to induce vasoconstriction after the vessel’s diameter stabilized. Flow-mediated dilatation (FMD) was triggered by pressure change that equals ~15 dynes/cm² shear stress. At the end of the experiment, the bathing solution was changed to Ca²⁺-free Krebs solution containing EGTA (2 mmol/L), which led to maximum passive dilatation. FMD was calculated as percent of diameter changes: (flow-induced dilatation–Phe tone)/(passive dilatation–Phe tone).

Organ culture of aortic rings
Mouse aortic rings (~2 mm in length) were dissected in sterile PBS and incubated in a Dulbecco’s Modified Eagle’s Media (DMEM, 11885, Gibco, Gaithersberg, MD, USA) supplemented with 10% fetal bovine serum (FBS, 10270106, Gibco), plus 100 IU/mL penicillin and 100 µg/mL streptomycin (15140122, Gibco). High glucose (HG, 30 mmol/L) medium was prepared with 25 mmol/L mannitol plus 5 mmol/L glucose as a normal glucose (NG) osmotic control. For ex vivo treatment, aortic rings were treated with the following agents, singularly or in combination in DMEM for 24 hours and they agents included BMP4 (20 ng/ml, 314-BP, R&D Systems), noggin (100 ng/ml, 6057-NG, R&D system, chordin (200 ng/ml,1808-NR, R&D Systems), follistatin (400 ng/ml, 4889-FN, R&D Systems), tiron (1 mmol/L, D7389, Sigma) and DETCA (100 µmol/L, Sigma). After the incubation period, rings were transferred to a chamber filled with fresh Krebs solution and suspended on myograph for measurement of isometric force.

ROS determination
Vascular ROS production was measured by dihydroethidium (DHE, D1168, Invitrogen, USA, excitation: 515 nm; emission: 565-605 nm) fluorescence. Frozen sections of the aortae were prepared in 10-µm thickness using a cryostat (Leica CM1100, Germany) and incubated in Krebs solution containing 5 µmol/L DHE for 15 min at 37 °C, and then washed twice in Krebs solution. ROS measurement in en face endothelium of mouse aortae was performed as described. For determination of intracellular ROS in vitro, HUVECs or MAECs were incubated with CM-H₂DCFDA (DCF, 5 µmol/L, C6827, Invitrogen, excitation: 488 nm; emission filter: 505-525 nm) in extracellular medium (in mmol/L: 121 NaCl, 5 NaHCO₃, 10 Na-HEPES, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 2 CaCl₂, 10 glucose; pH=7.4) for 30 minutes. Fluorescent images were captured by confocal system (FV1000, Olympus, Tokyo, Japan) and analyzed by FLUOVIEW software (Version 3.05a, Olympus, Japan). In addition, the amount of vascular superoxide anion was also determined using the lucigenin-enhanced chemiluminescence method.
Lentiviral RNA interference on mouse aortae

Lentivirus particles of ALK3 shRNA and scramble were prepared as previous described. Mouse aortic rings were transfected with lentivirus (10⁶ pfu) in the presence of 8 μg/mL polybrene (107689, Sigma) for 4 hours in FBS-free DMEM, and then transferred to DMEM with 10% FBS for both functional and molecular examination.

Adenoviral RNA interference transfection in vivo and ex vivo

The shRNA (5’-CCCTAGTCAACTCTGTTAATT-3’, Vector Biolabs, USA) targeting mouse BMP4 mRNA was sub-cloned into pAdTrack shuttle vector (16405, Addgene, USA) with the human U6 promoter. The sequence had been validated for 85% knockdown of BMP4 mRNA in Hepa 1-6 cells. The constructed shuttle vector was recombined with the adenoviral backbone plasmid pAdeasy-1 (16400, Addgene, USA) and the correct adenoviral construct was transfected to HEK293 cells for the generation of corresponding recombinant adenovirus. Virus were stepwise amplified and purified with a PEG precipitation method, and its measured titer was 3×10⁹ pfu/mL. For in vivo studies, adenovirus was delivered by tail vein injection at 10⁸ pfu per mouse and experiments were carried out 7 days after injection. For ex vivo incubation, aortic rings were exposed to 10⁵-10⁶ pfu adenovirus for 36 hours before being collected for functional study.

Western blotting

Tissues were homogenized in an ice-cold RIPA lysis buffer containing a cocktail of protease inhibitors (Complete Protease Inhibitor Cocktail and PhosSTOP Phosphatase Inhibitor Cocktail, Roche, USA). Cell lysates were centrifuged at 20,000 g for 20 min at 4°C. Extracted protein concentration was determined using the Lowry method (Bio-Rad, Hercules, USA). Sample buffer containing 5% β-mercaptoethanol was added to the sample, and then denatured by boiling for 10 min. Equal amount of protein samples together with the prestained protein molecular weight marker were electrophoresed on a 10% SDS-polyacrylamide gel. The resolved proteins were electrophoretically transferred onto an Immune-Blot polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA) at 100 V for 90 min on ice. The membranes were blocked by 2% BSA in 0.05% Tween-20 phosphate-buffered saline (PBST), and incubated overnight at 4°C with primary antibodies including ALK3 (sc-20736, Santa Cruz, CA, USA), BMP4 (ab39973 Abcam, UK) and GAPDH (AM4300, Ambion, USA). The membrane was washed for three times in PBST before adding the corresponding secondary antibodies conjugated to horseradish peroxidase (HRP) (Dako Cytomation, USA). The membranes were developed with an enhanced chemiluminescence (ECL) detection system (RPN2108, Amersham Pharmacia, USA), and finally exposed to X-ray films. GAPDH was selected as housekeeping proteins for checking equal loading of each sample. Summarized data represented the means of 4-5 separate experiments.

En face immunofluorescence staining

The aortic segments were fixed by 4% paraformaldehyde, and permeabilized by 0.01% Triton X-100 in PBS for 15 minutes. Then the rings were blocked by 5 % normal donkey serum in PBS for 1 hour, and incubated with rabbit anti-BMP4 (1:100 dilution, ab39973
Abcam, UK) and goat anti-VE cadherin (1:100 dilution, sc-6458, Santa Cruz, USA) antibodies for 24 hours at 4 °C, followed by incubation with fluorescent second antibody, AlexaFluor 488 anti-rabbit and AlexaFluor 546 anti-goat (1:500 dilution, A-21202 and A-11056, Molecular Probes, USA), for 2 hours at room temperature. The specimens were then cut open and imaged by a confocal system as described.

**Statistical Analysis**

Results represent means ± SEM from separated mice. Concentration–response curves were analyzed by nonlinear regression curve fitting using GraphPad Prism software (San Diego, CA, USA), followed by Student’s t test. The protein expression was quantified by Quantity One (Bio-Rad, USA) and normalized to GAPDH. Comparisons among groups were made using ANOVA followed by an unpaired Student's t-test. P<0.05 was considered as statistically significant.

**References**


Supplemental Figure I. Arterial blood pressure (A) and insulin tolerance test (B) in db/db mice after 2-week noggin infusion. (C) Fasting glucose of db/m⁺, db/db, and noggin-infused db/db mice. *p<0.05 vs db/m⁺. Results are means ± SEM from 8 different mice.
Supplemental Figure II. Endothelium-independent relaxations to SNP in aortae (A) and to insulin in mesenteric arteries (B) from db/m+, db/db, noggin-treated db/db mice. (C) ACh-induced relaxations in main mesenteric arteries and (D) SNP-induced relaxations in aortae from db/db mice in vivo transfected with scramble and BMP4 shRNA adenovirus. (E, F, G) SNP-induced relaxations in C57BL/6 and db/db mouse aortae under different treatments. (H) ACh-induced relaxations in C57BL/6 mouse aortae after 48-hour exposure to Ad-scramble and Ad-BMP4 shRNA. Results are means ± SEM from 5 mice.
Supplemental Figure III. Western blotting (A) and en face immunofluorescence staining (B&C) showing the knockdown of BMP4 expression by Ad-BMP4 shRNA transduction in db/db mice in vivo. Ad-Scr: Ad-scramble, Ad-Bsh: Ad-BMP4 shRNA. Results are means ± SEM from 5 mice. *p<0.05 vs Ad-Scramble.
Supplemental Figure IV. Representative images showing that inhibitory effects of BMP4 shRNA adenovirus transduction on ROS production in (A) aortic vascular wall and (B) endothelial cells en face of db/db mouse aortae, as compared to Ad-Scramble control.
Supplemental Figure V. Representative images (A) and quantitative data (B) showing ROS generation detected by CM-H$_2$DCFDA in HUVECs under different treatments. Results are means ± SEM from 5-6 experiments. *p<0.001 vs NG, #p<0.001 vs HG.
Supplemental Figure VI. ROS production in endothelium en face of C57BL/6 mouse aortae (A&C) and in HUVECs (B&D) with different treatments. Results are means ± SEM from 5-6 experiments, *p<0.001, vs control, #p<0.001 vs BMP4.
Supplemental Figure VII. ROS production in primary cultured mouse aortic endothelial cells (MAECs) receiving various pharmacological treatments. Results are means ± SEM from 5-6 experiments. (A) *p<0.001 vs control, #p<0.001 vs BMP4. (B) *p<0.001 vs NG, #p<0.001 vs HG.
Supplemental Figure VIII. (A) Representative images (B) and quantitative data showing ROS production in mouse aortic endothelial cells (MAECs) detected by CM-H$_2$DCFDA. Results are means ± SEM from 4 experiments, *p<0.05 vs Control. #p<0.05 vs Scramble+BMP4. Scramble: Lenti-scramble shRNA, shRNA-1/-3: Lenti-ALK3 shRNA-1/-3. (C) ROS production in endothelium from lentivirus transfected db/db mouse aortae measured by en face DHE staining.
Supplemental Figure IX. (A) Time course for the protein expression of BMP4 in mouse aortic endothelial cells (MAECs) exposed to high glucose (HG, 30 mmol/L), *p<0.05 vs 0h, ***p<0.001 vs 0h. (B) Concentration-dependent effect of glucose on the BMP4 expression in MAECs with 5 mmol glucose + 25 mmol mannitol (Man) as osmotic control for 36 hours, *p<0.05 vs NG, ***p<0.001 vs NG.
**Supplemental Figure X.** ALK3 silencing by shRNA-1 or shRNA-3 inhibited high glucose (HG, 30 mmol/L)-stimulated BMP4 expression in mouse aortic endothelial cells (MAECs). NG: normal glucose (5 mmol/L), HG: high glucose (30 mmol/L), Sramble: Lenti-scramble shRNA, shRNA-1/-3: Lenti-ALK3 shRNA-1/-3. Results are means±SEM. *p<0.05 vs NG, #p<0.05 vs Scramble + HG.