Diabetes mellitus is frequently complicated by cardiovascular disease, such as vascular calcification. Hyperglycemia and diabetes activate vascular BMP activity, which is instrumental in promoting vascular calcification and medial calcification (also referred to as media sclerosis or Mo¨nckeberg disease). Medial calcification, in particular, is considered to be a characteristic of diabetes and vascular calcification13–15 and may play a role in ocular angiogenesis in diabetic retinopathy.16 The BMPs belong to the transforming growth factor (TGF)-β superfamily and elicit their response via the so-called type I and II receptors. BMP-2 and -4 interact with the activin-like kinase receptor (ALK)2, ALK3, and ALK6, which are type I receptors that form complexes with the BMP type II receptor (BMPRII).17 In canonical BMP signaling, the receptors phosphorylate specific regulated (R)-SMAD proteins, which translocate into the nucleus and regulate gene transcription.17 SMAD1/5/8 mediate BMP-signaling, whereas SMAD2/3 mediate TGF-β signaling. In previous work, we identified a BMP-triggered pathway that modulates expression of vascular endothelial growth factor (VEGF) in endothelial cells (ECs).18,19 In this pathway, BMP-4 interacts with ALK2 to induce expression of ALK1,20 a related type I receptor that is essential for normal angiogenesis.21

Bone morphogenetic protein (BMP)-2 and -4 are inflammatory mediators in vascular endothelium responsive to disturbed flow, increased oxidative stress, and inflammation.11,12 Increased BMP activity enhances atherogenesis and vascular calcification13–15 and may play a role in ocular angiogenesis in diabetic retinopathy.16

Diabetes mellitus is associated with severe cardiovascular complications, including vascular calcification and accelerated atherosclerosis, leading to increased morbidity and mortality in diabetic patients.1–3 Vascular calcification is frequently seen in 1 of 2 forms, atherosclerotic lesion calcification and medial calcification (also referred to as media sclerosis or Mo¨nckeberg disease). Medial calcification, in particular, is considered to be a characteristic of diabetes and occurs along the elastic lamellae. Both types of calcification involve activation of osteogenic cell differentiation.5,6 Diabetes also causes endothelial dysfunction,7–9 which promotes diabetic vascular disease. Even though a number of signaling pathways have been implicated in diabetic vasculopathy,5,7,10 the links between hyperglycemia and vascular disease are still incompletely understood.

Bone morphogenetic protein (BMP)-2 and -4 are inflammatory mediators in vascular endothelium responsive to disturbed flow, increased oxidative stress, and inflammation.11,12 Increased BMP activity enhances atherogenesis and vascular calcification13–15 and may play a role in ocular angiogenesis in diabetic retinopathy.16

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which stimulates ALK1,22 induces expression of matrix Gla protein (MGP), a BMP inhibitor known to limit vascular calcification.13,14 and VEGF. Thus, expression of ALK2, ALK1, MGP, and VEGF appears to be closely linked.

Reports from other investigators suggest that vascular BMPs are responsive to hyperglycemia. Bovine vascular smooth muscle cells (SMCs) secrete more BMP-2 when treated with high glucose,24 vascular expression of BMP-2, BMP-7, and BMPRII increases in early autoimmune diabetes in mice,25 and aortic BMP-4 increases as diabetes progresses in db/db mice.26 There is also evidence that diabetes stimulates osteogenic differentiation in aortic myofibroblasts by augmenting the BMP-2/Msx2-Wnt pathway in fat-fed low-density lipoprotein receptor–null mice.10,27 However, the overall effect of hyperglycemia on vascular BMP activity and its association to vascular disease is still poorly understood.

In this study, we demonstrate that high glucose strongly promotes BMP activity in endothelial cells. We further show high aortic BMP activity in 3 diabetic animal models representative of type 1 and 2 diabetes, in which BMP-4, ALK2, ALK1, and MGP were preferentially detected in proximity to the endothelium. The high BMP activity was associated with a remarkable increase in aortic expression of osteogenic markers and calcification. Increased BMP inhibition, as mediated by a MGP transgene, limited these changes.

Methods

The generation of rats transgenic for human islet amyloid polypeptide (HIP rats) has been previously described.28 The Ins2Akita+/− mice (homozygous for a mutation in one allele of the insulin-2 gene)30 and the db/db mice (homozygous for the spontaneous mutation Leprtb),31 both on C57BL/6J background, were obtained from The Jackson Laboratory (Bar Harbor, ME). MGP<sup>wt</sup> mice, generated in our laboratory on a C57BL/6J background,31 were crossed with Ins2Akita+/− mice to generate MGP<sup>wt</sup>;Ins2Akita+/− mice. Heterozygous MGP<sup>wt</sup> mice were used because the phenotype was apparent in MGP<sup>wt</sup> mice and a low birth rate made it difficult to obtain hemizygous MGP<sup>wt</sup> mice.31 Genotyping was performed by PCR as previously described.31 The rats were housed individually and fed Rodent Diet 8604 (50% carbohydrate, 24% protein, and 4% fat; Harlan Teklad, Madison, WI) ad libitum; the mice were fed standard chow (Diet 8604, Harlan Teklad) also ad libitum. All animals were subjected to the standard 12-hour light/12-hour dark cycle. The studies were reviewed by the Institutional Review Board and conducted in accordance with the animal care guidelines set by the University of California, Los Angeles.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org and provides details on analytic procedures, cell culture and small interfering (si)RNA transfection, RNA analysis, immunoblotting, immunostaining, alkaline phosphatase (ALP) assay, quantification of calcium deposition, proliferation and tube-formation assays, ELISA, determination of media thickness, histochemical staining, and statistical analysis.

Results

Hyperglycemia Induces the Expression of BMP Ligands, Inhibitors, and Receptors in Human Aortic Endothelial Cells

We hypothesized that hyperglycemia promotes expression of BMP-related proteins in human aortic endothelial cells (HAECs). To test our hypothesis, we treated HAECs for 24 hours with increasing concentrations of glucose (5.5 to 27.5 mmol/L; 5.5 mmol/L is the normal media concentration) or D-mannitol (5.5 to 27.5 mmol/L) as a control. The results showed that the ligands BMP-2 and -4; the inhibitors MGP and Noggin; and the receptors ALK1, ALK3, ALK6, and BMPRII dose-dependently increased, as determined by real-time PCR and immunoblotting (Figure 1). Because the MGP antibodies worked poorly for immunoblotting, immunohistochemistry was performed for MGP (Figure 1). In addition, VEGF, which can be regulated through a BMP-triggered pathway,18,19 was 2-fold induced after treatment with 27.5 mmol/L glucose (Figure 1). Human aortic smooth muscle cells (HASMCs) that were treated similarly with glucose or D-mannitol for 24 hours did not show induction of the BMP-related proteins (data not shown). The results suggested that aortic EC respond readily to hyperglycemia with an increase in BMP ligands, inhibitors and receptors.

Increased BMP Levels and BMP Activity in Media From HAECs Treated With High Glucose

To determine whether the increase in BMP expression resulted in higher secretion of BMP-2 and -4, we determined BMP-2 and -4 by ELISA in the media from HAECs treated with high glucose for 24 hours. The results showed a dose-dependent increase in both BMP-2 and -4 (Figure 2A). We then examined the effect of BMP-2 and -4 in four assays sensitive to BMP activity, BMP-stimulated ALP activity (early osteogenic marker) and calcium accumulation (late osteogenic marker) in calcifying vascular cells (CVCs), and proliferation and tube formation in bovine aortic endothelial cells (BAECs). We prepared conditioned media from glucose-treated (5.5 to 22 mmol/L) HAECs that had been transfected with scrambled control siRNA or siRNA for

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BMP-2 or -4 to specifically deplete the respective BMP in the media. CVCs were incubated with the conditioned media from the glucose-treated HAECs, and AP activity and calcium accumulation were measured after 2 and 8 days, respectively. The results showed that both ALP activity and calcium accumulation progressively increased with media from control HAECs and that the effect was abolished by Noggin, an inhibitor of both BMP-2 and -4 (Figure 2B). Depletion of BMP-2, but not BMP-4, abolished the effect (Figure 2B, left), suggesting that BMP-2 is a stronger stimulator of osteogenesis than BMP-4. CVCs treated directly with increasing concentrations of glucose did not show increases in ALP activity or calcium accumulation compared with controls (data not shown).

We used the same conditioned media for the angiogenesis assays. BAECs were incubated with the conditioned media from control HAECs and cell proliferation was determined by ³H-thymidine incorporation. The results showed that proliferation progressively increased with media from control HAECs and that the effect was abolished by Noggin, an inhibitor of both BMP-2 and -4 (Figure 2B). Depletion of BMP-2, but not BMP-4, abolished the effect (Figure 2B, left), suggesting that BMP-2 is a stronger stimulator of osteogenesis than BMP-4. CVCs treated directly with increasing concentrations of glucose did not show increases in ALP activity or calcium accumulation compared with controls (data not shown).

We previously found that BMP-4 stimulates expression of MGP and VEGF by increasing expression of ALK1 and ALK2 receptors, and that ALK1 is induced through ALK2, but not the other way around. We compared the time course and coordination of BMP-4, ALK1, ALK2, MGP and VEGF to those of BMP-2, ALK3, ALK6, BMPRII and Noggin. First, we performed time course experiments by treating HAECs with 16.5 mmol/L glucose for up to 24 hours.
Samples were collected every 4 hours, and gene expression was determined by real-time PCR. This revealed that glucose progressively stimulated expression of BMP-4, ALK1, ALK2, MGP, and VEGF for up to 24 hours (Figure 3A). However, expression of BMP-2, ALK3, ALK6, BMPRII, and Noggin exhibited a different time course with a rapid increase between 16 and 20 hours (Figure 3A).

We next used siRNA techniques to selectively deplete BMP components and examine the effect of glucose stimulation on the expression of ALK1, ALK2 (both induced by BMP-2, ALK6, Noggin and VEGF did not (Figure 3B). Depletion of the same factors, except for ALK1, also abolished the glucose-induced ALK2 expression (Figure 3B). Depletion of MGP increased expression of ALK1 and ALK2, consistent with its role as a BMP inhibitor.32 Although Noggin is also a BMP inhibitor, its depletion did not alter the expression of ALK1 and ALK2, suggesting differences in BMP affinity or cell-specific function between Noggin and MGP. Glucose-induced expression of BMP-4 and ALK3, however, was unaffected by depletion of any of the genes except for BMP-4 and ALK3, respectively. Thus, the induction of ALK2, ALK1, MGP, and VEGF is coordinated in glucose-treated HAECs, where it is stimulated by BMP-4, but not BMP-2. Furthermore, our results suggest that ALK3 mediates the induction of ALK2, which in turn mediates the induction of ALK1. BMPRII signals together with ALK1, ALK2, ALK3, and ALK6,17,19 and the depletion of BMPRII abolished the glucose-induced ALK1 and ALK2 expression (Figure 3B). Depletion of the same factors, except for ALK1, also abolished the glucose-induced ALK2 expression (Figure 3B). Depletion of MGP increased expression of ALK1 and ALK2, consistent with its role as a BMP inhibitor.32 Although Noggin is also a BMP inhibitor, its depletion did not alter the expression of ALK1 and ALK2, suggesting differences in BMP affinity or cell-specific function between Noggin and MGP. Glucose-induced expression of BMP-4 and ALK3, however, was unaffected by depletion of any of the genes except for BMP-4 and ALK3, respectively. Thus, the induction of ALK2, ALK1, MGP, and VEGF is coordinated in glucose-treated HAECs, where it is stimulated by BMP-4, but not BMP-2. Furthermore, our results suggest that ALK3 mediates the induction of ALK2, which in turn mediates the induction of ALK1. BMPRII signals together with ALK1, ALK2, ALK3, and ALK6,17,19 and the depletion of BMPRII may affect signaling through all these receptors.

Diabetes Mellitus Induces Aortic Expression of BMP Ligands, Inhibitors, and Receptors in Ins2Akita/+ and db/db Mice

To examine whether hyperglycemia activates vascular expression of BMP ligands, inhibitors and receptors in vivo, we took advantage of the Ins2Akita/+ and db/db diabetic mouse models. Heterozygous Ins2Akita/+ mice become spontaneously diabetic because of a mutation in one allele of the insulin-2 gene with a progressive decrease in insulin levels,29,30 whereas homozygous db/db mice develop obesity and diabetes because of a mutation in the leptin receptor.3 To determine aortic BMP expression, we prepared aortas from heterozygous Ins2Akita/+ mice, homozygous db/db mice and wild-type (wt) littermates aged 20 weeks, when serum glucose was significantly increased in both types of mice (Online Table I). Aortic expression of BMP ligands, inhibitors and receptors was determined. The results showed that expression of BMP-2, BMP-4, MGP, Noggin, ALK1, ALK2, ALK3, ALK6, BMPRII, and VEGF was significantly increased in both Ins2Akita/+ mice and db/db mice, as determined by real-time PCR (Figure 4A and 4B), immunoblotting (Figure 4C and 4D), and immunofluorescence for MGP (Figure 4E).

To examine the localization of the expression of the different BMP components in the vascular wall, we selected the Ins2Akita/+ mouse and compared it to littermate controls. We colocalized expression of BMP-2, BMP-4, ALK1, ALK2, ALK3, ALK6, BMPRII, Noggin, MGP, and VEGF with that of smooth muscle α-actin, a marker of the medial SMCs, using immunofluorescence. The results revealed that BMP-4, ALK2, ALK1, and MGP, which showed coordination in HAECs, were detected mainly in the endothelium (Figure 4F), whereas BMP-2, Noggin, ALK3, ALK6 and BMPRII were detected throughout the aortic wall (Online Figure I). VEGF was detected throughout the aortic wall, suggesting...
induction through multiple pathways. Together, the results suggest that diabetes induces vascular BMP expression, which may contribute to vascular disease.

Diabetes Mellitus Induces Aortic Expression of BMP Ligands, Inhibitors, and Receptors in HIP Rats

The so-called HIP rats progressively develop diabetes because of overexpression of the human islet amyloid polypeptide in the pancreatic β-cells, leading to a progressive apoptosis of β-cells, and is a model of type 2 diabetes.28 To determine whether aortic BMP expression is induced also in these animals, and how expression correlates with the development of diabetes, we prepared aortas from HIP rats and littermate controls aged 3, 6, and 12 months. We found that expression of BMP-2, BMP-4, MGP, Noggin, ALK1, ALK2, ALK3, ALK6, BMPRII, and VEGF progressively increased as the glucose levels increased, as determined by real-time PCR, immunoblotting and immunofluorescence for MGP (Figure 5A and 5B). The glucose levels were already significantly increased after 3 months compared with controls, and peaked after 12 months (Online Table II). In the case of BMP-4, ALK2, ALK1, and MGP, the expression was lower at 12 months than at 6 months, but still increased compared with control (Figure 5A and 5B). The results support that vascular induction of BMP components is part of the development of diabetes also in rats and occurs at relatively modest levels of hyperglycemia.

Increased BMP Activity in Diabetic Vascular Wall and Serum

Because aortic expression of BMPs, receptors and inhibitors was induced to varying degrees, we determined the overall BMP activity in tissue using immunoblotting for phosphorylated (p)SMAD1/5/8 and compared with pSMAD2/3, which show TGF-β activation, and total SMAD. Protein extracts were prepared from aortas of Ins2Akita+/+ and db/db mice aged 20 weeks, and from HIP rats aged 3, 6, and 12 month, and subjected to immunoblotting. The results showed that pSMAD1/5/8 levels were increased in both mouse models (Figure 6A), and in HIP rats where they increased with age (Figure 6B). Furthermore, we confirmed the levels of pSMAD1/5/8 in mice and HIP rats using immunofluorescence and immunohistochemistry, respectively, and found that the levels increased similarly throughout the vascular wall (Figure 6C and 6D). The results support that the vascular BMP activity is increased in diabetes. In addition, serum levels of BMP-2 and -4 increased progressively in the HIP rats, resulting in greater ability to induce BMP-dependent osteogenesis in CVCs (Online Figure II).

Diabetes and Increased BMP Activity Is Associated With Vascular Calcification

To determine whether the diabetic animals had vascular calcification, we compared expression of osteogenic markers and calcium accumulation in diabetic and control aortas. We
found strong induction of Cbfa1 and Osterix, essential transcription factors in bone, in both Ins2Akita/ and db/db mice aged 20 weeks, and in HIP rats aged 3, 6, and 12 months, as determined by immunoblotting (Figure 7A and 7B). Expression of osteopontin, a multifunctional glycoprotein in bone, was also increased (Figure 7A and 7B). Total aortic calcium was significantly increased in the mouse models, and at 12 and 18 months of age in HIP rats compared with controls (Figure 7C). The calcium mineral and cartilage-associated mucopolysaccharides were easily visualized in the mouse models using Von Kossa and Alizarin Red staining for mineral, and Alcian blue staining for mucopolysaccharides (Figure 7D). The calcium was observed along the elastic lamellae, resembling medial calcification. In the HIP rats, however, only small punctate calcifications were seen (data not shown) even though the thickness of the aortic media had increased significantly at 6 and 12 months of age compared with controls (Figure 8E). Together, the results support that the increased BMP activity in diabetic mice and rats is associated with increased vascular calcification.

Because high phosphate levels are known to enhance vascular calcification, we determined serum phosphate in all animals. The phosphate levels did not differ significantly between animals (Online Tables I and II).

Enhanced BMP Inhibition Limits Diabetic Vascular Disease in the Ins2Akita/+ Mice

To determine whether enhanced BMP inhibition would limit diabetic vascular disease, we generated MGPtg/wt;Ins2Akita/+ mice with a transgene for MGP, which efficiently inhibits BMP-2 and -4. We compared them with the wild type, MGPtg/wt, and Ins2Akita/+ mice at age 20 weeks. The MGP transgene did not affect serum glucose or phosphate levels (Online Table III). We compared aortic expression of BMPs,

Figure 4. Aortic expression of BMP components in Ins2Akita/+ and db/db mice. Aortas were obtained from Ins2Akita/+ and db/db mice aged 20 weeks. A and B, Expression of the indicated genes was determined by real-time PCR and compared with wild-type (n=4 animals in each group). C through E, Protein levels were determined by immunoblotting or immunofluorescence (for MGP). F, Aortas were obtained from Ins2Akita/+ mice aged 20 weeks and used for immunofluorescence. BMP-4, MGP, ALK1, and ALK2 (green) were detected on the luminal side (up) by the endothelium and did not colocalize with smooth muscle α-actin (red). Asterisks indicate statistically significant differences compared with wild type: *P<0.05, **P<0.01, ***P<0.001 (Tukey test).
receptors and inhibitors, BMP activity, osteogenic expression, and calcification. The results revealed increased aortic expression of MGP, as determined by real-time PCR and immunofluorescence, ~3-fold in the MGPtg/wt mice and the Ins2Akita+/+ mice, and 8-fold in the MGPtg/wt;Ins2Akita+/+ mice as compared with wild type (Figure 8A and 8C). Immunofluorescence also revealed that the increased MGP was predominantly found in proximity to the endothelium, suggesting that the endothelium is important in limiting calcification. The expression of ALK1, ALK2, and VEGF was significantly decreased in the MGPtg/wt;Ins2Akita+/+ mice compared with Ins2Akita+/+ mice, as determined by real-time PCR and immunoblotting (Figure 8A and 8B). The high MGP level also led to less aortic BMP activity, as determined by immunoblotting and immunofluorescence for pSMAD1/5/8 (Figure 8D and 8E), demonstrating that more BMP inhibition shifted the balance in overall vascular BMP activity. We also detected less expression of Cbfa1, osterix, and osteopontin when MGP was high, as determined by immunoblotting (Figure 8F). Finally, there was less aortic calcium mineral and cartilage-associated mucopolysaccharides, as determined by total aortic calcium (Figure 8G) and histochemical staining (Figure 8H). Thus, increased BMP inhibition as mediated by enhanced MGP expression decreased total BMP activity and prevented vascular osteogenesis and calcification caused by diabetes.

Discussion

In this study, we demonstrate that hyperglycemia and diabetes are strong activators of the BMP signaling system in endothelial cells in vitro and the aortic wall in vivo. High vascular BMP activity was found in 3 diabetic animal models and was associated with a remarkable increase in expression of osteogenic markers and medial calcification. This was largely prevented by enhancing BMP inhibition using a MGP transgene, which caused a decrease in total BMP activity.

Our results are consistent with previous reports on increased vascular expression of BMP-2, -7, and the BMPRII in early autoimmune diabetes in mice,25 and increased aortic expression of BMP-4 as diabetes progresses in db/db mice.26 An increase in endothelial BMP-2 in the adventitia may also
be instrumental in promoting osteogenic differentiation in aortic myofibroblasts by augmenting the BMP-2/Msx2-Wnt pathway as has been reported for diabetic low-density lipoprotein receptor–null mice by Al-Aly et al.\textsuperscript{27} In contrast, we did not detect increased expression of BMP components in HASMCs when treated with high glucose, even though previous studies show increased BMP-2 secretion from bovine SMCs treated with high glucose for 48 hours.\textsuperscript{24} In addition, CVCs did not show enhanced ALP activity or calcium accumulation even if treated with high glucose for up to 10 days. The differences may be attributable to variations in cell systems or timing of treatment.

Our results point to a role for the endothelium in preventing vascular calcification. The enhanced MGP expression in MPG\textsuperscript{tg/wt} and MPG\textsuperscript{tg/wt,Ins2\textsuperscript{Akita/+}} mice was mainly found in the endothelium even though calcification was prevented in the media. Endothelial BMP-4 has been shown to be important in mediating atherogenic stimuli such as disturbed flow and increased oxidative stress,\textsuperscript{11,12} and it is possible that it is also an initiating factor for medial calcification. However, the exact mechanisms of endothelial-medial crosstalk responsible for such effects will require further studies. Other investigators have found that BMP-2, which in our experiments is expressed in both the endothelium and the media, is also an important inflammatory cytokine in the endothelium,\textsuperscript{12} which also would be inhibited by MGP. However, it is possible that Noggin is more important for inhibiting BMP-2 based on similar distributions of BMP-2 and Noggin in the vascular wall. The findings that depletion of BMP-4 abolished the proangiogenic effects of conditioned media from glucose-treated HAECs, whereas depletion of BMP-2 abolished the procalcific effects further support that BMP-2 and 4 have distinct vascular roles despite their molecular similarities.

There appears to be a hierarchy in how BMP/TGF-\(\beta\) receptors are used in vascular cells. The finding that ALK3 is responsible for induction of ALK2 adds to our previous observations that ALK2 and ALK1 are responsible for induction of ALK1 and ALK5, respectively.\textsuperscript{19,20} Thus, the BMP/TGF-\(\beta\) receptors appears to be used in the order ALK3, ALK2, ALK1 and ALK5, where each receptor may correlate to a specific stage in vascular growth and development.

Aortic calcium increased in all animals as diabetes developed. It occurred later in the HIP rats, suggesting that rats are more resistant to vascular calcification. The calcification pattern in Ins2\textsuperscript{Akita/+} and db/db mice was distinct, following the elastic lamellae and resembling that of medial calcification considered characteristic for diabetes. Indeed, our studies showed that both Ins2\textsuperscript{Akita/+} and db/db mice are new models of diabetic vascular calcification and would be suitable for testing interventions aimed at diabetic vascular disease. We did not detect any significant development of atherosclerotic lesions during the study period. An advantage with the animal models used in this study is that they develop diabetes spontaneously, thus avoiding toxic effects of streptozotocin or other agents used to induce diabetes, which might interfere with organ or stem cell function and make the results difficult to interpret.

Considering that the vascular endothelium altogether is a very large organ, it is interesting to speculate whether it also provides feedback for the pancreatic islet cells. Goulley et al\textsuperscript{34}
recently reported that BMP-4 improves insulin sensitivity in pancreatic islet cells. Autocrine BMP-4 maintained healthy insulin secretion and could be replaced by BMP-4 infusion. Thus, BMP-4 derived from the endothelium may have a physiological role in modulating insulin secretion in the pancreas, and persistently high levels of BMP-4 in vascular tissues or serum may be early signs of diabetes.

In summary, BMP signaling is strongly activated in the diabetic vessel wall and associated with vascular calcification. Lowering BMP activity by means of an MGP transgene limited the calcification, suggesting that vascular BMP inhibition may be a new strategy for treating diabetic vascular disease.

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Disclosures
None.

References
Suppression of vascular BMP activity by an MGP transgene limits diabetic vascular disease. Thus, BMP activity appears to be an important determinant of diabetic vascular disease, and suppression of vascular BMP activity may be a new strategy to treat vascular complications.

What New Information Does This Article Contribute?

- Enhanced MGP expression is predominantly detected in the vascular endothelium, but BMP activity is inhibited throughout the vascular wall.

The BMPs, a family of multifunctional growth factors and morphogens, have been associated with atherosclerosis and vascular calcification. We demonstrate that high glucose levels enhance BMP activity in endothelial cells in culture, and that diabetes enhances vascular BMP activity in mice and rats with type 1 or 2 diabetes mellitus. Suppression of the BMP activity by high expression of MGP, an inhibitor of BMP-2 and -4, limits medial calcification and expression of bone-related genes in diabetic mice. This is the first study to show that an intervention that decreases BMP activity prevents diabetic vascular disease. The study also reveals that MGP expression is increased in diabetic patients, providing a potential target for future therapeutic interventions.
Activation of Vascular Bone Morphogenetic Protein Signaling in Diabetes Mellitus
Kristina I. Boström, Medet Jumabay, Aleksey Matveyenko, Susanne B. Nicholas and Yucheng Yao

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ONLINE SUPPLEMENT
Detailed Method Section

Animals

The generation of rats transgenic for human islet amyloid polypeptide (HIP rats) has been previously described.\(^1\) The Ins\(^2\)\(^{\text{Akita/+}}\) mice (heterozygous for a mutation in one allele of the insulin-2 gene)\(^2,3\) and the db/db mice (homozygous for the spontaneous mutation Lept\(^{db}\))\(^4\), both on C57BL/6J background, were obtained from the Jackson Laboratories (Bar Harbor, ME). MGP\(^{tg/wt}\) mice, generated in our laboratory on a C57BL/6J background\(^5\), were crossed with Ins\(^2\)\(^{\text{Akita/+}}\) mice to generate MGP\(^{tg/wt};\)Ins\(^2\)\(^{\text{Akita/+}}\) mice. We used the MGP\(^{tg/wt}\) mice since the phenotype was apparent in MGP\(^{tg/tg}\) mice, and a low birth rate of MGP\(^{tg/tg}\) mice made it difficult to obtain MGP\(^{tg/tg}\) mice.\(^5\) The rats were housed individually and fed Rodent Diet 8604 (50% carbohydrate, 24% protein, and 4% fat; Harlan Teklad, Madison, WI) ad libitum; the mice were fed standard chow (Diet 8604, Harlan Teklad, Laboratory, Madison, WI) also ad libitum. All animals were subjected to the standard 12-h light-dark cycle. The studies were reviewed by the Institutional Review Board and conducted in accordance with the animal care guidelines set by the University of California, Los Angeles.

Analytical Procedures

Plasma glucose was measured by the glucose oxidase method (Beckman Glucose Analyzer 2; Beckman Coulter, Fullerton, CA). Serum phosphate levels were measured using a QuantiChrom™ Phosphate Assay kit as per manufacturer's instructions (BioAssay Systems, Hayward, CA). Total calcium in lyophilized aortic tissue was determined as previously described.\(^6\)

Cell Culture and SiRNA Transfection

Bovine aortic endothelial cells (BAEC), human aortic endothelial cells (HAEC), human aortic smooth muscle cell (HASMC) and bovine calcifying vascular cells (CVC) were prepared and cultured as previously described.\(^7-11\) For treatment, cells were seeded at a confluency of 50-80%, and treatments were added to the media 20-24 hours later. Transient transfections of HAEC were performed with Lipofectamine™2000 (Invitrogen) using 60 nM siRNA as previously described.\(^7\) Briefly, the amount of siRNA was optimized as per the manufacturer's instructions. Three separate siRNAs (Silencer® predesigned siRNA, Ambion) and scrambled siRNA with the same nucleotide content were tested. When compared with unrelated control siRNA and scrambled siRNA, the specific siRNAs resulted in an 80-95% decrease in mRNA and protein levels as determined by real-time PCR and immunoblotting, respectively. The siRNA that provided the most efficient inhibition (90-95%) was used for experiments, and treatments were usually initiated 24 hours after the start of the transfection. Silencer® predesigned siRNAs were obtained for BMP-2, BMP-4, ALK1, ALK2, ALK3, ALK6, BMPRII, MGP, Noggin, and VEGF.

RNA Analysis

Real-time PCR analysis was performed as previously described,\(^12,13\) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as control gene.\(^12\) Primers and probes for human, rat and mouse BMP-2, BMP-4, MGP, Noggin, ALK1, ALK2, ALK3, ALK6, BMPRII, and VEGF were obtained from Applied Biosystems (Foster City, CA) as part of Taqman® Gene Expression Assays.

Immunoblotting

Immunoblotting was performed as previously described,\(^13,14\) Equal amounts of cellular
protein or culture medium were used. For optimal detection of VEGF in culture media, VEGF was first immunoprecipitated with anti-VEGF antibodies (Santa Cruz Biotechnology), as previously described\textsuperscript{12} and then analyzed by immunoblotting using specific antibodies to VEGF (200 ng/mL; R&D Systems). For other proteins, blots were incubated with specific antibodies to pSMAD1/5/8, pSMAD2/3 (both diluted to 400 ng/ml; Cell Signaling Technology, Danvers, MA), total SMAD, BMP-4, ALK1, ALK2 (all diluted to 400 ng/ml; Santa Cruz Biotechnology, Santa Cruz, CA), BMP-2, ALK3, ALK6, osteopontin, osterix (all diluted to 200 ng/ml; Santa Cruz Biotechnology), Noggin (diluted to 400 ng/ml; R&D Systems), BMPRII (diluted to 200 ng/ml; R&D Systems), and core binding factor alpha 1 (Cbfα1, diluted to 100 ng/ml; Calbiochem). ß-Actin (1:5000 dilution; Sigma-Aldrich) was used as loading control. The specificity of all antibodies was verified prior to use for immunoblotting and immunostaining as previously described\textsuperscript{13}.

**Immunohistochemistry and Immunofluorescence**

Tissue sections were processed and stained as previously described in detail\textsuperscript{13,15}. Tissue sections were fixed in 4% paraformaldehyde and processed as previously described (6). For immunohistochemistry or immunofluorescence, sections were permeabilized with 0.5% Triton X-100 for 10 minutes, followed by 3 washes with wash buffer (WB, phosphate-buffered saline (PBS) containing 0.1% Tween-20). Non-specific antibody binding sites were blocked by incubating the sections for 30 minutes in blocking buffer (1% BSA, 2% goat serum and 0.5% Triton X-100 in PBS). Primary antibodies were diluted in antibody buffer (PBS containing 1% BSA, 0.5% Triton X-100), and sections were incubated for 60 minutes at room temperature, followed by several washes in WB. Alexa Fluor 488-conjugated (green fluorescence) or Alexa Fluor 594-conjugated (red fluorescence) secondary chick anti-goat or anti-rabbit antibodies (Molecular Probes, Eugene, OR) were applied to the sections and incubated for 30 minutes at room temperature. After several washes in WB and a brief equilibration of the sections with PBS, the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). A DAPI stock solution was diluted to 300 nM in PBS, and 300 µl of the diluted solution was added to the sections, making certain that they were completely covered. The sample was incubated for 1-5 minutes and rinsed several times in PBS. Staining without primary antibodies served as controls. Images were acquired with an inverted Zeiss Axiovert 200 microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY, USA). To eliminate the possibility of false colocalization caused by emission filter bleed through, only images showing signals that were clearly visible by eye through the microscope when using the appropriate filters for the respective antibodies were considered significant and included in the results.

We used specific antibodies to MGP (provided by Dr. Reidar Wallin, Wake Forest University, Winston-Salem, NC), pSMAD1/5/8, pSMAD2/3 (both from Cell Signaling Technology), total SMAD, BMP-2, BMP-4, ALK1, ALK2, ALK3, ALK6 (all from Santa Cruz Biotechnology), Noggin, BMPRII (both from R&D Systems).

**Alkaline Phosphatase (ALP) Assay and Quantification of Calcium Deposition**

Bovine CVC were cultured for up to 8 days in conditioned media from endothelial cells, which were changed every 2 days. Noggin (300 ng/ml, R&D Systems) was added where indicated. ALP activity and calcium accumulation were quantified as previously described\textsuperscript{10,14} after 2 and 8 days, respectively.

**Proliferation Assay**

BAEC transfected with siRNA were seeded in 24-well plates at a density of 100,000 cells per well, and allowed to attach for 4–6 hours. \(^3\)H-Thymidine was added at 1 µCi/ml for 4 days, and \(^3\)H-thymidine incorporation was determined as previously described\textsuperscript{12}. 
Tube Formation Assay
Matrigel™ Matrix (BD Biosciences, Bedford, MA) was diluted 1:3 in medium from HAEC treated with high glucose, and 300 µl was added to each well of a 12-well plate and incubated at 37°C for 30 min to allow polymerization. BAEC were suspended in the same medium at a density of 5 x 10⁴ cells/well, and 400 µl of the cell suspension was added to each well. Photos were obtained after 6 hours.

Enzyme-Linked Immunosorbent Assay (ELISA)
Serum levels of BMP-2 and -4 were determined by Quantikine® ELISA (R&D Systems) as per manufacturer’s instructions, using 100 µl of serum per assay.

Media Thickness and Histochemical Staining of Aortic Sections
The media thickness was measured in hematoxylin-stained sections by light microscopy with a calibrated eyepiece micrometer. Five thickness measurements equally spaced along the ventral surface of the aorta were made on each tissue section, and 15 measurements were used to calculate an average thickness for each of five aortic tissue segments as previously described. Von Kossa staining (30 minutes, 5% silver nitrate) and Alizarin Red S staining were performed to visualize calcification in 10 µm-sections. Alcian Blue staining (0.05% Alcian Blue GX in 0.025 M acetate buffer solution, containing 0.025M MgCl₂, final pH 5.8, for 18 hrs) was performed to visualize cartilage proteoglycans.

Statistical Analysis
Data were analyzed for statistical significance by two-way analysis of variance with post hoc Tukey’s analysis. The analyses were performed using the GraphPad Instat® 3.0 software (GraphPad Software, San Diego, CA). P-values less than 0.05 were considered significant. All experiments were repeated a minimum of three times.
REFERENCES
**Supplemental Table I.** Serum levels of glucose and phosphate in wild type and diabetic mice (C57BL6/J background), 20 weeks of age.

<table>
<thead>
<tr>
<th>Mice</th>
<th>Glucose (mg/dl)</th>
<th>Phosphate (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>109.3±25.1</td>
<td>0.836±0.004</td>
</tr>
<tr>
<td>Ins2Akita/+</td>
<td>336.7±53.6***</td>
<td>0.837±0.010</td>
</tr>
<tr>
<td>db/db</td>
<td>327.8±45.4***</td>
<td>0.837±0.011</td>
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</table>

Asterisks indicate statistically significant differences compared to wild type. ***<0.001, Tukey’s test (n=4).
**Supplemental Table II.** Serum levels of glucose and phosphate in wild type and HIP rats, aged 3-18 months.

<table>
<thead>
<tr>
<th>Rats</th>
<th>Glucose (mg/dl)</th>
<th>Glucose (mg/dl)</th>
<th>Glucose (mg/dl)</th>
<th>Glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 months</td>
<td>6 months</td>
<td>12 months</td>
<td>18 months</td>
</tr>
<tr>
<td>Wild type</td>
<td>79.0±11.5</td>
<td>99.0±7.4</td>
<td>90.3±12.8</td>
<td>72.0±8.5</td>
</tr>
<tr>
<td>HIP</td>
<td>104.7±19.9*</td>
<td>139.3±38.5**</td>
<td>292.1±99.8***</td>
<td>263.0±15.6***</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Rats</th>
<th>Phosphate (ng/ml)</th>
<th>Phosphate (ng/ml)</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 months</td>
<td>6 months</td>
<td>12 months</td>
<td>18 months</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.837±0.027</td>
<td>0.834±0.024</td>
<td>0.837±0.031</td>
<td>N.D.</td>
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<tr>
<td>HIP</td>
<td>0.839±0.019</td>
<td>0.833±0.022</td>
<td>0.835±0.014</td>
<td>N.D.</td>
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</table>

Asterisks indicate statistically significant differences between wild type and HIP rats. *<0.05, **<0.01, ***<0.001, Tukey's test (n=6 for glucose, n=4 for phosphate).
**Supplemental Table III.** Serum levels of glucose and phosphate in wild type and diabetic mice (C57BL6/J background) with or without the MGP transgene, 20 weeks of age.

<table>
<thead>
<tr>
<th>Mice</th>
<th>Glucose (mg/dl)</th>
<th>Phosphate (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>120±11.3</td>
<td>0.826±0.005</td>
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<tr>
<td>MGP&lt;sup&gt;tg/&lt;/sup&gt;wt</td>
<td>121±3.6</td>
<td>0.826±0.014</td>
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<tr>
<td>Ins2&lt;sup&gt;Akita/+&lt;/sup&gt;</td>
<td>360±7.9***</td>
<td>0.828±0.018</td>
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<tr>
<td>Ins2&lt;sup&gt;Akita/+&lt;/sup&gt;; MGP&lt;sup&gt;tg/&lt;/sup&gt;wt</td>
<td>371.7±32.6***</td>
<td>0.826±0.014</td>
</tr>
</tbody>
</table>

Asterisks indicate statistically significant differences compared to wild type. ***<0.001, Tukey’s test (n=3).
Supplemental Figure I

A

<table>
<thead>
<tr>
<th></th>
<th>wt</th>
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</table>

Scale bars: 100 μm
Supplemental Figure 1

A. Localization of BMP components in the aortic wall of Ins2<sup>Akita/+</sup>. 
To examine the localization of the expression of the different BMP components in the aortic wall, we selected the Ins2<sup>Akita/+</sup> mouse and compared it to littermate controls at age 20 weeks. We co-localized expression of BMP-2, BMP-4, ALK1, ALK2, ALK3, ALK6, BMPRII, Noggin, MGP and VEGF with that of smooth muscle α-actin, a marker of the medial SMC, using immunofluorescence. 
(Top) BMP-4, MGP, ALK1 and ALK2 (green) were predominantly detected in on the luminal side (up) in proximity to the endothelium, and did not co-localize with smooth muscle alpha-actin (red).  
(Bottom) BMP-2, Noggin, ALK3, ALK6, BMPRII and VEGF (green) were detected throughout the vascular wall, and co-localized with alpha-actin (red).

B. High magnification of MGP immunofluorescent staining in wild type mouse aorta. 
High magnification of MGP staining in wild type mice (aged 20 weeks), with and without DAPI nuclear stain, demonstrates that the MGP staining (green) is associated with the endothelial layer. Autofluorescence of the elastic lamellae was minimal with green fluorescence in most of our stained specimens, but visible with blue fluorescence.
Supplemental Figure II

Increased serum levels of BMP-2 and BMP-4 in diabetic rats.

(A) Levels of BMP-2 (left) and BMP-4 (right) in serum from HIP rats aged 3-12 months, as determined by ELISA (n=6 animals in each group).

(B) Osteogenic differentiation in CVC incubated with serum from HIP rats and wt rats aged 3-12 months. The cells were treated with medium containing 10% serum from the HIP rats or controls, and alkaline phosphatase activity (left) and calcium accumulation (right) were determined in absence and presence of Noggin (300 ng/ml) after 2 and 8 days, respectively.

Asterisks indicate statistically significant differences compared to wt for the respective age. *<0.05, ***<0.001, Tukey’s test.