The bone morphogenic protein inhibitor, noggin, reduces glycemia and vascular inflammation in db/db mice

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PREMATURE ATHEROSCLEROSIS is a hallmark of diabetes mellitus (DM). Although this link is clinically and epidemiologically well documented, the exact biological mechanism by which diabetes causes vascular disease remains inadequately understood. The presence of macrophages and lymphocytes in atherosclerotic lesions is consistent with the current view of atherosclerosis as an inflammatory disease of the vessel wall (28, 44). Molecules associated with the recruitment of leukocytes into the atherosclerotic plaque, such as chemoattractant cytokines and cellular adhesion molecules, are abundant in vascular lesions and seem to mediate ongoing inflammation (14, 37, 39). Chronic vascular inflammation is associated with oxidative stress and has been implicated in the development of diabetic vascular complications (12, 25). ANG II acts as a central mediator of the oxidative and inflammatory responses in vascular disease and has been shown to induce expression of adhesion molecules and cytokines in the vasculature (7, 52).

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BMPs in the vascular complications of diabetes. It is likely due to a combination of increased vascular ROS production and inflammatory gene expression, noggin reduces glycemia, whereas valsartan reduces blood pressure in diabetic mice. We therefore suggest that the vascular complications of DM are because cardiovascular disease is highly prevalent in diabetics, and atherogenic step (51), and that renin-angiotensin system antagonists reduce oxidative stress in the brain (16) and improve diabetic vascular complications such as coronary heart disease, stroke, and nephropathy (22, 45, 50). Taken together, these data suggest that ANG II and BMPs may play causal roles in the increased oxidative stress and vascular inflammation found in db/db mice.

We therefore sought to determine the effectiveness of noggin and valsartan on vascular oxidative stress and inflammation in an animal model of DM. We posited that since both ANG II and BMP-4 stimulate ROS release from the Nox1-dependent NADPH oxidase leading to inflammation (11, 47, 51), either or both might play a causal role in the vascular inflammation found in db/db mice. We found that although both inhibitors block superoxide production and have similar effects on inflammatory gene expression, noggin reduces glycemia, whereas valsartan reduces blood pressure in diabetic mice. We therefore suggest that the vascular complications of DM are likely due to a combination of increased vascular ROS production and a second hit, perhaps elevated glucose and elevated blood pressure. Our data support an important role for BMPs in the vascular complications of diabetes.

Table 1. Measurements of body weight, serum glucose, and blood pressure after noggin and valsartan treatment of db/db mice

<table>
<thead>
<tr>
<th>Age</th>
<th>Treatment*</th>
<th>Genotype, n</th>
<th>Baseline</th>
<th>Vehicle</th>
<th>Valsartan</th>
<th>Noggin</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td></td>
<td></td>
<td>WT</td>
<td>db/db</td>
<td></td>
<td>WT</td>
<td>db/db</td>
</tr>
<tr>
<td>8 wk</td>
<td>Body weight, g</td>
<td>21.1 ± 0.2</td>
<td>53</td>
<td>37.3 ± 0.4</td>
<td>51</td>
<td>24.8 ± 0.3</td>
</tr>
<tr>
<td>12 wk</td>
<td>Weight gain, g</td>
<td>3.4 ± 0.3</td>
<td>25</td>
<td>8.9 ± 0.4</td>
<td>22</td>
<td>3.9 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Serum glucose, mg/dL</td>
<td>93.3 ± 5.7</td>
<td>22</td>
<td>132.2 ± 15.9</td>
<td>14</td>
<td>109.6 ± 5.5</td>
</tr>
<tr>
<td></td>
<td>Systolic blood pressure, mmHg</td>
<td>108.2 ± 1.1</td>
<td>20</td>
<td>101.1 ± 1.4</td>
<td>20</td>
<td>110.1 ± 2.6</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Mice were treated with noggin or valsartan for 4 wk before analysis at age 12 wk. **P < 0.01 compared with wild-type (WT) vehicle; †P < 0.05 compared with db/db vehicle; ††P < 0.01 compared with db/db vehicle; §P < 0.05 compared with WT valsartan; ‡P < 0.05 compared with db/db valsartan.

METHODS

Animals. Wild-type (WT) mice and db/db mice on C57BLKS/J background, a model of type 2 diabetes in which leptin receptors are deficient, were purchased from Jackson Laboratory (Bar Harbor, ME) and bred in-house under standard conditions. Mice had free access to water and regular rodent chow from Purina Lab Diets (LabDiet 5001 Rodent Diet). The Emory University Institutional Animal Care and Use Committee approved all animal protocols.

Treatment groups. Eight-week-old male mice were anesthetized with an intraperitoneal injection of ketamin (80 mg/kg), xylazine (10 mg/kg), and acepromazine (3 mg/kg), and micro-osmotic pumps were implanted subcutaneously in the midscapular region for delivery of vehicle (0.1% BSA), noggin (0.048 mg·kg⁻¹·day⁻¹) (5), or valsartan (3 mg·kg⁻¹·day⁻¹) for 4 wk. At 12 wk of age, thoracoabdominal
H2O2 production was measured by lucigenin assay. Data are from aortic H2O2 production in WT and db/db mice, as measured using Amplex Red. ***P < 0.001 compared with WT without noggin; ###P < 0.001 compared with db/db without noggin. B and C: aortic O2•− production in WT and db/db mice, as measured using dihydroethidium (DHE) HPLC (n = 3). **P < 0.01 compared with WT without noggin; ##P < 0.01 compared with with WT without valsartan; #P < 0.05 compared with db/db without valsartan.

**Fig. 2.** Noggin and valsartan reduce reactive oxygen species (ROS) production in db/db mice. Mice were treated with vehicle (white bars), noggin (black bars in A and B), or valsartan (black bars in C) as indicated for 4 wk before analysis at 12 wk. Data are expressed as means ± SE. A: aortic H2O2 production in WT (n = 6) and db/db mice (n = 3), as measured using Amplex Red. ***P < 0.001 compared with WT without noggin; ###P < 0.001 compared with db/db without noggin. B and C: aortic O2•− production in WT and db/db mice, as measured using dihydroethidium (DHE) HPLC (n = 3). **P < 0.01 compared with WT without noggin; ##P < 0.01 compared with db/db without noggin; **P < 0.01 compared with WT without valsartan; #P < 0.05 compared with db/db without valsartan.

Vascular smooth muscle cells (VSMCs) were isolated from the thoracic aorta of male Sprague-Dawley rats by an enzymatic digestion protocol. Cells between the third and the sixth passage were cultured in low-glucose DMEM (Sigma, St. Louis, MO) supplemented with 10% calf serum. Quiescence was achieved by serum-starvation in 0.1% serum medium for 48 h before treatments.

**Fig. 3.** Agonist-induced superoxide production in vascular smooth muscle cells (VSMCs). A: quiescent VSMCs were treated with vehicle, ANG II, noggin, and ANG II plus noggin for 30 min. B: quiescent VSMCs were treated with vehicle, bone morphogenetic protein (BMP)-4, valsartan, and BMP-4 plus valsartan for 30 min. O2•− production was measured by lucigenin assay. Data are expressed as means ± SE. **P < 0.01 compared with vehicle.
Hydrogen peroxide and superoxide measurement. Hydrogen peroxide ($H_2O_2$) production in aortas was measured with an Amplex Red $H_2O_2$/Peroxidase Assay Kit (Molecular Probes) as previously described (56). $H_2O_2$ concentration was calculated using a standard curve and normalized to cellular protein content. To evaluate intracellular production of superoxide ($O_2^-\cdot$) in tissue, we measured the formation of oxyethidium (oxyEth) from dihydroethidium using HPLC analysis as reported previously (10). OxyEth was expressed per milligram protein.

Superoxide production in VSMC membrane fractions was assessed by measuring the reduction of 5 µmol/l Lucigenin by $O_2^-\cdot$ in the presence of NADPH (100 µmol/l, reduced form) as described previously (48).

Data analysis. Results are presented as mean ± SE. Statistical significance was assessed by 1-way or 2-way ANOVA on untransformed data, followed by comparison of group averages by contrast analysis. A $P < 0.05$ value was considered to be statistically significant.

RESULTS

Metabolic parameters. Consistent with previous data, db/db mice are fully diabetic at 12 wk (15, 46). As expected, body weight and serum glucose levels were much higher in db/db mice compared with WT mice (Table 1). There was no significant difference in body weight between treatment groups in WT and db/db mice at 8 and 12 wk of age. Even though nogenin treated-mice had a significantly lower weight gain over time than valsartan treated-mice regardless of genotype, this was primarily due to body weight variability at baseline rather than a treatment effect. Nogenin treatment did not change serum glucose levels in WT mice compared with vehicle-treatment. In contrast, in db/db mice, serum glucose levels were decreased by nogenin compared with vehicle (Table 1). There was no significant difference in blood pressure between animal strains. Nogenin infusion did not significantly alter blood pressure in either genotype. As expected, valsartan significantly decreased blood pressure in WT and db/db mice, but had no effect on serum glucose (Table 1). Of note, there was no difference in insulin levels of mice among treatment groups (Fig. 1).

Aortic superoxide and hydrogen peroxide production. Hyperglycemia stimulates NADPH oxidases in VSMCs to generate ROS (23). Furthermore, blood vessels isolated from diabetic patients exhibit increased $O_2^-\cdot$ production and expression of several NADPH oxidase subunits, suggesting that NADPH oxidases are more active in diabetes (18). Importantly, ROS production and NADPH oxidase activity are also markedly increased in atherosclerotic lesions (29, 49). To investigate the effect of nogenin and valsartan on ROS production, we measured $O_2^-\cdot$ and $H_2O_2$ production in mouse aortas. As we reported previously (46), $O_2^-\cdot$ and $H_2O_2$ generation was increased in db/db mice compared with WT mice (Fig. 2). In WT mice, nogenin had no significant effect on $H_2O_2$ or $O_2^-\cdot$ production; however, in db/db mice, nogenin treatment decreased $O_2^-\cdot$ and $H_2O_2$ generation compared with vehicle (Fig. 2, A and B). Similarly, $O_2^-\cdot$ production was reduced in db/db mice treated with valsartan (Fig. 2C). The addition of ANG II to VSMCs in vitro led to a significant increase in $O_2^-\cdot$ production (Fig. 3). In the presence of nogenin, however, ANG II failed to increase $O_2^-\cdot$ production. This suggests that BMP activation downstream of ANG II is necessary for ROS production in VSMCs. BMP-4, on the other hand, did not cause VSMCs to generate more $O_2^-\cdot$.

![Fig. 4. Noggin reduces inflammatory gene expression in db/db mice. Aortas from WT (n = 6) and db/db (n = 8) mice treated with vehicle (white bars) or nogenin (black bars) were harvested at 12 wk and processed for real-time quantitative PCR analysis. A: VCAM-1. B: ICAM-1. C: BMP-2. D: BMP-4. Data are expressed as means ± SE. ***P < 0.001, *P < 0.05 compared with WT without nogenin; ##P < 0.01, #P < 0.05 compared with db/db without nogenin.](http://ajpheart.physiology.org/)

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Aortic inflammatory markers are increased in diabetes and differentially reduced by noggin and valsartan. Vascular oxidative stress is closely linked to vascular inflammation. Therefore, we hypothesized that both noggin and valsartan would also reduce vascular inflammatory gene expression in db/db mice. We examined a series of proinflammatory genes, including VCAM-1, ICAM-1, CTGF, BMP-2, and BMP-4. Aortic VCAM-1, ICAM-1, BMP-2, and BMP-4 mRNAs were uniformly increased in db/db mice compared with WT controls (Figs. 4 and 5). In db/db mice, VCAM-1, ICAM-1, and CTGF were also significantly increased at the protein level (Figs. 6 and 7). These results confirm a state of vascular inflammation in our model.

In aortas from WT mice, we found no change in VCAM-1, ICAM-1, or CTGF after noggin infusion, but we observed that noggin treatment considerably reduced expression of these proteins in aortas from db/db mouse (Fig. 6). Consistent with this observation, noggin treatment also significantly reduced VCAM-1, ICAM-1, and BMP-4 mRNAs in diabetic mice (Fig. 4). BMP-2 mRNA expression also tended to be lower after noggin treatment, but this was not statistically significant.

Valsartan treatment significantly reduced VCAM-1, ICAM-1, and BMP-4 mRNAs in db/db mice; however, unlike noggin, it did not significantly reduce BMP-2 mRNA levels (Fig. 5). At the protein level, valsartan treatment reduced BMP-4, VCAM-1, and CTGF expression in db/db mice, but did not significantly reduce ICAM-1 expression despite its effect on mRNA levels (Fig. 7).

DISCUSSION

Previous studies in diabetic animal models have shown an activation of the BMP signaling system in the vasculature (4), and a multitude of experimental and clinical studies indicate the importance of the renin-angiotensin system in atherosclerosis. Vascular inflammation occurs in the vessel wall of spontaneously hypertensive rats, which can be abolished by ACE-Is (8). Furthermore, inhibition of the renin-angiotensin system in diabetic patients reduces cardiovascular end points more than other antihypertensives (1). Moreover, we have previously shown that inflammatory proteins including BMP-4 are increased in aortas from diabetic mice involving both redox-sensitive and redox-insensitive pathways (46). Based on these prerequisites, we sought to compare the effects of valsartan and noggin on vascular superoxide generation and adhesion molecule expression in db/db mice. In this study we find that both valsartan and noggin reduce vascular superoxide production in diabetic animals. As expected, valsartan treatment also significantly reduces blood pressure, whereas noggin, to our surprise, reduces serum glucose levels in db/db mice. Both noggin and valsartan diminish mRNA expression of BMP-4 in diabetic mice, and both appear to have the tendency to reduce BMP-2 mRNA, albeit not significantly. At the protein level, noggin reduces VCAM-1, ICAM-1, and CTGF, whereas valsartan reduces VCAM-1, CTGF, and BMP-4. This suggests that both noggin and valsartan are strong anti-inflammatory agents in this mouse model of diabetic atherogenesis.

Recent evidence implicates BMPs as proinflammatory and proatherogenic mediators in the vessel wall (26). Both BMP-2

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Fig. 5. Valsartan reduces inflammatory gene expression in db/db mice. Aortas from WT and db/db mice treated with vehicle (white bars) or valsartan (black bars) were harvested at 12 wk and processed for real-time quantitative PCR analysis. A: VCAM-1. B: ICAM-1. C: BMP-2. D: BMP-4. Data from 4 animals in each group are expressed as means ± SE. ***P < 0.001, **P < 0.01 compared with WT without valsartan; ###P < 0.001, ##P < 0.01 compared with db/db without valsartan.
and BMP-4 have been found in calcified atherosclerotic plaques and in calcified aortic valves, suggesting a role in atherosclerosis (3, 36). In addition, it has been shown that endothelial cells overlying foam cell lesions express BMP-4, but endothelial cells in nondiseased areas do not (51). Importantly, vascular BMPs are upregulated in DM (40), and our data (Figs. 4 and 5) strongly suggest that BMP-2 and -4 may play a role in vascular inflammation in \textit{db/db} mice.

Noggin treatment not only blocks BMP activity but also reduces gene expression of BMP-4 (Fig. 4). It has been suggested that BMP-2/-4 gene transcription has an autoregulatory feedback mechanism (19, 41). Because noggin acts as an extracellular blocker of BMP activity, one can speculate that it disrupts a potential positive feedback mechanism on gene expression in the diabetic vasculature. However, the effect of noggin on BMP-2 was not significant. Because we did not investigate BMP-2 protein levels, a translational effect on BMP-2 protein expression cannot be excluded. Interestingly, others have reported no effect of noggin on BMP-2 expression. Zhu et al. (57) found that noggin does not affect BMP-2 gene expression, and Liu et al. (32) showed that noggin did not reduce glucose-induced BMP-2 expression in human aortic smooth muscle cells, but it did block vascular calcification. In contrast, bovine VSMCs secrete more BMP-2 when stimulated with high glucose (6). Therefore, another possibility is that BMP-2 transcription in vivo is glucose sensitive. We speculate that noggin does not directly inhibit BMP-2 expression but may potentially influence it by reducing glycemia. Our finding that noggin reduces glucose levels in \textit{db/db} mice is consistent with this possibility. It is thus important to understand the interplay between glucose and noggin on the BMP pathway, although the mechanism by which noggin decreases serum glucose levels needs to be further addressed. The effect of valsartan on BMP-2 and -4 expression is essentially the same as that of noggin; however, valsartan did not reduce serum glucose. Like noggin, however, valsartan reduces ROS production, suggesting an additional potential mechanism of BMP regulation. Alternatively, blood pressure reduction may exert a similar effect as serum glucose reduction. These possibilities need to be further evaluated. BMP-2 plays a distinct role in diabetic atherosclerosis. For instance, Boström and coworkers (4) showed that BMP-2 is a stronger stimulator of osteogenesis than BMP-4 in calcifying vascular cells. In contrast with BMP-2, BMP-4 seems to be regulated by serum cholesterol levels (55). In this context, blunting both BMP-2 and BMP-4 may achieve a more comprehensive blockade of the BMP system in diabetic vascular disease and may therefore be advantageous for the treatment of diabetic vascular disease.

The differential effect of noggin and valsartan on the adhesion molecules ICAM-1 and VCAM-1 is intriguing, since it may imply that each inhibitor may target a specific aspect of inflammation. Vascular inflammation begins with a well-orchestrated sequence of interaction between leucocytes and the vascular endothelium. Specific endothelial cell adhesion molecules, including VCAM-1, mediate the initial interaction of circulating inflammatory leucocytes with endothelial cells, which results in tethering and rolling of leucocytes along the endothelial surface. Subsequently, members of the immuno-
globulin superfamily of endothelial adhesion molecules, particularly ICAM-1, mediate arrest of the rolling monocytes and firm adhesion. Therefore, reduced ICAM-1 protein expression is likely to have a strong anti-inflammatory effect in the vessel wall. Previous studies have shown that expression of VCAM-1 and ICAM-1 is regulated by distinct stimuli. For example, unstable flow is a strong inducer of ICAM-1, but not VCAM-1, expression (38). Why, in our study, we found that valsartan treatment reduces ICAM-1 mRNA but has no significant effect on protein level remains unclear. It may be due to a contribution of soluble ICAM-1 in the interstitium of the wall segment. It is conceivable that noggin and ARB treatment, therefore, may exert an additive effect, which needs to be further investigated.

Both valsartan and noggin reduced ROS production (Fig. 2), in confirmation of previous studies in which the ARB candesartan was shown to reduce O$_2^-$ and H$_2$O$_2$ in an animal model of atherosclerosis, and noggin reduced NADPH oxidase activity in BMP-4-induced hypertension (11, 35). Our previous work showed that treatment of db/db mice with the superoxide scavenger tempol inhibits the expression of some (e.g., BMP-4 and osteopontin), but not all (e.g., CTGF) inflammatory genes in the aorta (46). Together, these observations strongly suggest that the inflammatory response is dependent upon more than...
just an increase in ROS in the vessel wall. We found two differences between the effects of valsartan and noggin on the end points in this study. First, valsartan was more effective than noggin at reducing blood pressure in these mice (Table 1). Second, noggin reduced serum glucose levels, whereas valsartan did not (Table 1). It is very likely that both a reduction in glucose and a reduction in blood pressure differentially contribute to the anti-inflammatory effects of noggin and valsartan, since elevated glucose is closely correlated with the inflammatory response (17, 24). Our data do not elucidate if the effect of noggin on vascular inflammation is a direct effect or indirect due to reduction of glycemia. The noggin effect on ROS production in VSMCs suggests that a BMP family member acts downstream of ANG II, and yet there are slight differences between the inhibitors regarding their effect on transcriptional and translational control of inflammatory protein expression. We therefore suggest that vascular inflammation in DM is likely a consequence of a combination of increased vascular ROS production and a second hit, perhaps elevated glucose and elevated blood pressure. Hence, inhibition of the BMP-2/-4 pathway by either mechanism appears to be effective in reducing subsequent inflammation.

A limitation of this study is the lack of serial glucose measurements to exclude short-term fluctuations throughout the treatment period. However, the early characterization of the db/db mouse strain showed that these mice exhibit progressive hyperglycemia with levels remaining elevated at 400 mg/dL or higher until death at 5 to 8 mo (9). Therefore, it is very likely that a significantly lower blood glucose level as we have observed it in our noggin-treated mice at 12 wk can have effects on vascular inflammation. Another caveat inherent to all animal studies is the translatability of findings to human pathophysiology. However, one can argue that with the use of valsartan we are using an agent that is approved in human use and does have a proven effect on hypertension in humans, although we are using an agent that is approved in human use and does have a proven effect on hypertension in humans.}

**ACKNOWLEDGMENTS**

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**REFERENCES**


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31. Circulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the vessel wall.