Bone Morphogenic Protein 4 Mediates NOX1-Dependent eNOS Uncoupling, Endothelial Dysfunction, and COX2 Induction in Type 2 Diabetes Mellitus

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Abstract

We have recently shown that angiotensin II-mediated uncoupling of endothelial nitric oxide synthase (eNOS) contributes to endothelial dysfunction in streptozotocin-induced type 1 diabetes mellitus. However, it has remained unclear whether and how eNOS uncoupling occurs in type 2 diabetes mellitus (T2DM) and the consequences of such in regulating vascular function. Here we investigated a role of bone morphogenic protein (BMP)-4 in mediating eNOS uncoupling, endothelial dysfunction, and inflammation in db/db mice. Circulating levels of BMP4 were markedly elevated in db/db mice but not in mice with type 1 diabetes mellitus, in which angiotensin II levels were significantly increased. Infusion of BMP4 antagonist noggin into db/db mice (15 μg/kg/day, 4 weeks) abolished eNOS uncoupling activity while restoring tetrahydrobiopterin (H_4B) bioavailability. The impaired endothelium-dependent vasorelaxation in db/db aortas was significantly improved by noggin infusion. Exposure of aortic endothelial cells to BMP4 (50 ng/mL, 24 hours) resulted in eNOS uncoupling, which was attenuated by H_4B precursor sepiapterin or small interfering RNA silencing nicotinamide adenine dinucleotide phosphate oxidase isoform 1 (NOX1). Interestingly, BMP4-dependent NOX1 up-regulation was abrogated by sepiapterin, implicating a NOX1-uncoupled eNOS-NOX1 feed-forward loop. BMP4 induction of cyclooxygenase 2 (COX2) expression and vascular cell adhesion protein 1 was found in db/db mice. Consistently, COX2 was up-regulated by BMP4 in endothelial cells, which was attenuated by sepiapterin, implicating an upstream role of eNOS uncoupling in COX2-mediated inflammatory activation. Taken together, our data for the first time reveal a novel role of BMP4 in inducing NOX1-dependent eNOS uncoupling in T2DM, which may promote development of novel therapeutics restoring endothelial function in T2DM.

Accumulating evidence has demonstrated a predictive role of endothelial dysfunction in vascular complications in type 1 diabetes mellitus (T1DM) (1) and type 2 diabetes mellitus (T2DM) (2, 3). The uncoupling transformation of endothelial nitric oxide synthase (eNOS), which turns the enzyme into a superoxide generator, contributes to diabetic endothelial dysfunction in T1DM (4, 5). We have recently shown that nicotinamide adenine dinucleotide phosphate oxidase (NOX) isoform 1 (NOX1) activation precedes eNOS uncoupling in streptozotocin (STZ)-induced T1DM mice (6), which is dependent on angiotensin II (Ang II) signaling (7, 8). However, it has remained unclear whether and how eNOS uncoupling occurs in T2DM. Even though Ang II and angiotensin-converting enzyme activity have been known to be elevated in the aortic wall of db/db mice as they develop their obese diabetic phenotype (9), the clinical intervention with Ang II signaling attenuators show only modest effect in the prevention of cardiovascular complications in T2DM (10, 11). Therefore, we hypothesized that pathological stimulus other than Ang II might be dominantly associated with endothelial dysfunction and inflammation in T2DM, which also uncouples eNOS.

Bone morphogenic protein 4 (BMP4) is known to mediate inflammation, endothelial dysfunction, and atherogenesis (12,–14). BMP4 was found involved in impaired endothelium-dependent vasorelaxation in a NOX-dependent manner (12). BMP4 treatment of endothelial cells increased NOX1 protein content, and mediated endothelial dysfunction and inflammation induced by disturbed flow (15, 16). These responses, including NOX activation and induction of endothelial dysfunction and inflammation, are similar to those shown in T1DM in which Ang II-dependent eNOS uncoupling is evident (6). In addition, elevated levels of BMP4 in both mRNA and protein forms have been reported in aortic tissues of db/db mice (17). Given that vascular complications in T2DM are associated with accelerated atherosclerosis, these findings seem to suggest a possible role of BMP4 in mediating endothelial dysfunction and subsequent vascular inflammation in T2DM, which likely involves an eNOS uncoupling phenotype as well.

Cyclooxygenase 2 (COX2) is undetectable in healthy vascular tissues but is inducible by proinflammatory stimuli such as Ang II (18), cytokines (19), or toxins (20); hence, its activation has been linked to pathological conditions such as atherosclerosis (13, 14, 21) and hypertension (22). Overexpression of COX2 in coronary arteriole was observed in patients with T2DM (23). High glucose is known to induce prostanoids by COX2 activation (24). Moreover, COX2 up-regulation in db/db mice was found associated with hypercontraction of vascular smooth muscle (25). Thus, COX2 induction by BMP4 might be implicated in diabetic vascular dysfunction and inflammation to accelerate diabetic vascular complications in T2DM.

In the present study, we aimed to investigate whether and how eNOS is uncoupled in T2DM, particularly the potential upstream roles of BMP4, Ang II, NOX, and the subsequent activation of inflammatory pathways such as the activation of COX2. We found that BMP4 levels were elevated in db/db T2DM mice, whereas Ang II levels were much higher in STZ induced T1DM mice in comparison. BMP4 induced NOX1-dependent eNOS uncoupling, tetrahydrobiopterin (H_4B) deficiency, impaired endothelium-dependent vasorelaxation, and COX2 activation, all of which were attenuated by the infusion of BMP4 antagonist noggin into db/db mice. Conversely, supplementation of H_4B precursor sepiapterin recoupled eNOS and prevented BMP4 induction of NOX1 and COX2 up-regulation in cultured endothelial cells, indicating eNOS uncoupling dependent COX2 activation and feed-forward activation of NOX1-eNOS uncoupling-NOX1. These data clearly establish a central role of BMP4 in mediating eNOS uncoupling, endothelial dysfunction, and inflammation in T2DM, and that strategies targeting BMP4/NOX1/eNOS uncoupling/COX2 pathway may serve as novel therapeutic options for the prevention and treatment of vascular complications in T2DM.

Materials and Methods

Experimental model

Male BKS.Cgm+/+ *Lepr^{db}*/J (*db*/*db*) diabetic mice and their age-matched nondiabetic littermates (heterozygote *db*/+, homozygote+/+) were used for the experiments. The animals were purchased from the Jackson Laboratory and maintained at the University of California, Los Angeles (Los Angeles, California) under the approval of the Animal Research Committee. Male C57BL/6 mice (3–4-month-old) were purchased from Charles River Laboratories and were used for the STZ-induced T1DM model as well as a high-fat diet-induced obesity model. Mice were injected with STZ (100 mg/kg) iv for 3 consecutive days to induce T1DM as previously published (6, 8). In parallel experiments, mice were fed with a high-fat diet (42% fat; Harlan) for 6 weeks (26).

Detection of circulating BMP4 and Ang II

According to the manufacturer's instructions, the plasma levels of BMP4 and Ang II were determined using an ELISA kit and an enzyme immunoassay kit respectively from Ray Biotech Inc.

Analysis of fasting glucose

Blood glucose was determined using the One Touch Ultra blood glucose meter (Lifescan).

Detection of total cholesterol and triglycerides

Plasma cholesterol levels were determined using a cholesterol reagent colorimetric assay kit (Roche Diagnostics). Triglyceride levels were measured colorimetrically at 540 nm using the triglyceride (glycerol phosphate oxidase) reagent set (Pointe Scientific) as previously described (26).

Noggin infusion

Alzet osmotic pumps releasing noggin (15 μg/kg/day) or vehicle (0.15 mol/L NaCl containing 0.01N acetic acid) were implanted into mice subcutaneously under isoflurane anesthesia. The animals were harvested 4 weeks later.

Cell culture

Bovine aortic endothelial cells (Cell Systems) of passages 4–6 were cultured in Media 199 (Mediatech) containing 10% fetal bovine serum to confluence and starved in 5% media overnight before experiments.

NOX1 small interfering RNA (siRNA) transfection

Proliferating endothelial cells of 85%–90% confluence were transfected with control siRNA or NOX1 siRNA (25 nmol/L; Dharmacon Inc; NOX1 sequence: 5′-ACACCUGUUUAACCUUGAA-3′) using Oligofectamine (Invitrogen) for 4 hours in Opti-MEM serum-free media (Invitrogen) before the addition of growth media containing 10% fetal bovine serum. Forty-eight hours later, cells were subjected to measure eNOS uncoupling activity using electron spin resonance (ESR).

ESR detection of superoxide production

Freshly isolated aortas were carefully and thoroughly cleaned of adhering fat in ice-cold modified Krebs/HEPES buffer and cut into 3-mm rings. The superoxide ($O_2^{\bullet-}$) specific spin trap methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH; 1 mmol/L; Alexis) solution was prepared freshly in nitrogen gas bubbled Krebs/HEPEs buffer containing diethyldithiocarbamic acid (5 μmol/L; Sigma) and deferoxamine (25 μmol/L; Sigma). Aortic rings were mixed with spin trap solution and loaded into glass capillary for analysis of $O_2^{\bullet-}$ signal using an e-Scan ESR spectrophotometer (Bruker Biospin). Some of the aortic rings were preincubated with 100 μmol/L of N-ω-nitro-L-arginine methyl ester (L-NAME) on ice for 1 hour before the addition of spin trap solution. The ESR settings used were as follows: center field, 3475; sweep width, 9 G; static field, 3484.98; microwave frequency, 9.75 GHz; microwave power, 21.02 mW; modulation frequency, 86 KHz; modulation amplitude, 2.47 G; resolution in X, 512; and number of x-scans, 10.

HPLC for determination of H_4B bioavailability

Fifty microliters of plasma were mixed with an equal volume of H_4B lysis buffer (0.1 M phosphoric acid; 1 mM EDTA, pH 8.0; 10 mM dithiothreitol). An isolated clean aorta was cut into 2-mm pieces, ground in liquid nitrogen, and lysed with 100 μL of H_4B lysis buffer. After centrifugation at 12 000 × *g* for 3 minutes, the supernatant either from lysed serum or lysed aortas was subjected to HPLC analysis for the determination of circulating levels or aortic levels of H_4B as we previously published (6, 27,–29).

Western blotting

Bovine aortic endothelial cells (BAECs) were lysed in ice-cold Tris buffer (50 mmol/L Tris, pH 7.4; 2 mmol/L EDTA; 1 mmol/L EGTA) containing 1% Triton X-100, 0.1% sodium dodecyl sulfate, 50 mmol/L NaF, 10 mmol/L $Na_4P_2O_7$, 1 mmol/L Na_3VO_4, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethylfonylsulfonyl fluoride, and 10 μl/ml of the protease inhibitor cocktail (Sigma). Twenty micrograms of proteins were subjected to SDS-PAGE (12% gel) and transferred to nitrocellulose membrane (Amersham Inc). Blots were subjected to immunostaining with antibodies of COX2 (160106; Cayman), vascular cell adhesion protein 1 (VCAM1; ab134047; Abcam), BMP4 (ab39973; Abcam), NOX4 (58849; Novus Biologicals), NOX2 (SC5827; Santa Cruz Biotechnology), GTPCH1 (250680; Abbiotech), dihydrofolate reductase (DHFR; ab49881; Abcam), sepiapterin reductase (SPR; customized; YenZym), NOX1 (SC5821; Santa Cruz Biotechnology), or actin (Sigma) at 4°C overnight, followed by incubation with peroxidase-conjugated secondary antibody at room temperature for 1 hour, and then protein targets were visualized using the enhanced chemiluminescence technique.

Assessment of endothelium-dependent vasorelaxation

An aortic ring was prepared in Krebs solution (119 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1 mmol/L KH_2PO_4, 2.5 mmol/L $CaCl_2$, 25 mmol/L $NaHCO_3$, and 5.6 mmol/L glucose) gassed with 5% CO_2 in O_2. A triangle vessel hook was placed through the lumen of a 3-mm vessel segment under a microscope, which was then mounted into organ bath (Myobath II; World Precision Instruments [WPI]). The aortic rings were stretched with 800 mg of tension and then equilibrated for 90 minutes. After equilibration, the rings were precontracted with 1 μmol/L of phenylephrine and then exposed to incremental concentrations of acetylcholine (10^{-9} to 10^{-6} mol/L). Data acquisition was performed using hardware data-trax and software lab-trax (WPI).

Statistical analysis

An ANOVA was used to compare means of multiple experimental groups, and Tukey's multiple test was used as a posthoc. The vasorelaxation data were analyzed using a two-way ANOVA, followed by a Bonferroni posthoc test. A *t* test was used to compare means between two groups. A value of $P < .05$ was considered significant. All grouped data shown in the figures are presented as mean ± SEM.

Results

Elevated circulating BMP4 levels in db/db T2DM mice vs elevated circulating Ang II levels in STZ-induced T1DM mice

We have previously shown that eNOS uncoupling occurs in T1DM to result in endothelial dysfunction and that the response was mediated by Ang II. In the present study, we tested an alternative BMP4-dependent pathway in potentially inducing eNOS uncoupling in T2DM. We started with measuring levels of circulating BMP4 and Ang II in representative mouse models, namely STZ-induced T1DM diabetic mice or db/db T2DM mice. As shown in Figure 1A, circulating BMP4 levels in plasma were significantly elevated in db/db mice compared with +/+ and db/+ littermates (572.3 ± 11.83 and 403.9 ± 33.63 vs 969 ± 68.51 pg/mL, for +/+ and db/+ vs db/db, respectively, $P < .001$). Even though the levels of circulating BMP4 were modestly increased in STZ-induced diabetic mice compared with wild-type (WT) mice, the amplitude and degree of increase in BMP4 was substantially lower than that of db/db mice. Of note, BMP4 levels were significantly increased in high-fat diet-fed mice compared with WT control. It should be noted that genetic backgrounds were different between db/db mice and STZ-induced diabetic mice, which are C57BLKS/J and C57BL6, respectively. These backgrounds seem to affect basal levels of circulating BMP4, which needs to further be investigated to identify the underlying mechanism. Nonetheless, the differences are much more prominent if compared with their diabetic group counterparts. In contrast to BMP4, Ang II levels were significantly elevated in STZ-induced diabetic mice and high-fat diet-fed mice but not in db/db mice (318.5 ± 35.04 and 156.9 ± 34.19 vs 82.11 ± 7.956 pg/mL for STZ, high-fat-fed mice vs db/db mice, respectively) (Figure 1B). Of note, blood glucose levels were significantly increased to 463.6 ± 35.75 mg/dL in db/db mice (119.7 ± 5.028 and 157.6 ± 7.541 mg/dL in +/+ and db/+ littermate controls, Figure 1C), which is similar to what was observed in STZ-induced diabetic mice. Body weights were increased in db/db mice compared with littermates, and in high-fat-fed mice compared with chow-fed control mice (Figure 1D). Cholesterols and triglycerides were measured to see the correlation between dyslipidemia and circulating BMP4 levels. Levels of both cholesterol and triglyceride were significantly increased in db/db mice (Figure 1, E and F). In high-fat-fed mice, cholesterols were increased. As shown in Figure 1, G and H, levels of cholesterol and triglyceride were significantly correlated with circulating levels of BMP4 (values are $P = .0433$ and $P = .0051$, respectively). These positive correlations were not found in STZ-induced T1DM mice. Taken together, these data suggest that BMP4 elevation in db/db mice might be associated with dyslipidemia, at least in part, and that BMP4 and Ang II are differentially elevated in T2DM and T1DM.

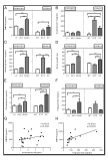

Figure 1.

BMP4 is elevated in db/db mice, whereas Ang II is elevated in STZ-induced diabetic mice. A, Levels of BMP4 measured by BMP4 ELISA in db/db mice, STZ-induced diabetic mice, and high-fat-fed mice. B, Circulating Ang II determined by enzyme immunoassay in ...

BMP4 mediates eNOS uncoupling in db/db mice

The eNOS uncoupling activity, reflected by L-NAME-sensitive superoxide production, was increased markedly in db/db mice (100% ± 21.43% vs 262.2% ± 47.79%, for +/+ vs db/db, $P < .05$, Figure 2A). This was accompanied by deficiencies in both circulating and aortic levels of H_4B, an essential cofactor for eNOS as shown in Figure 2B (2.418 ± 0.386 vs 0.7127 ± 0.2124 pmol/mg protein for +/+ vs db/db, $P < .05$) and Figure 2C (2.3 ± 0.434 vs 1.346 ± 0.086 pmol/mg protein for +/+ vs db/db, $P < .05$), respectively. As shown in Figure 2D, levels of circulating H_4B are correlated positively with that of aortic H_4B (value is $P = .0008$). Similarly, high-fat-induced obesity also induced eNOS uncoupling (172.2% ± 22.95% over control, Figure 2E), which is accompanied by a decrease in H_4B bioavailability (Figure 2F). The effect of BMP4 can be antagonized by binding to noggin or chordin. In subsequent experiments, BMP4 antagonist noggin was infused into db/db mice at a dose of 15 μg/kg/day (equals to ~600 ng/day or 420 μg/kg for mice with 40 g body weight). After 4 weeks of infusion, db/db mice were harvested for analysis of eNOS uncoupling activity. As shown in Figure 3, A and B, noggin infusion was highly effective in attenuating eNOS uncoupling activity (122.4% ± 41.91% vs 323% ± 59.87%). It also abolished H_4B deficiency in db/db mice. Of note, endothelium-dependent vasorelaxation was impaired in db/db mice, which was substantially improved by noggin infusion, implicating an intermediate role of BMP4 in inducing endothelial dysfunction in T2DM (Figure 3C). Neither blood glucose level nor body weight was affected by noggin infusion (Figure 3, D and E). Taken together, eNOS uncoupling and endothelial dysfunction in T2DM obese db/db mice can be reversed by inhibition of BMP4 signaling.

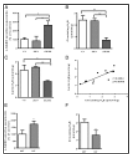

Figure 2.
Uncoupling of eNOS in db/db mice. A, L-NAME-sensitive superoxide production, reflective of eNOS uncoupling activity, was increased in db/db mice. The deficiencies of circulating H_4B (B) and aortic H_4B (C) were observed in db/db mice. D, Correlation between ...

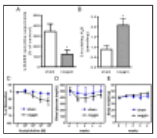

Figure 3.
Noggin attenuates eNOS uncoupling and endothelial dysfunction in db/db mice. A, The eNOS uncoupling activity was attenuated in db/db mice infused with noggin, BMP4 antagonist, for 4 weeks. B, H_4B bioavailability was restored in db/db mice infused with ...

BMP4-induced eNOS uncoupling in cultured endothelial cell: recoupling by sepiapterin

Next, we examined whether BMP4 induces eNOS uncoupling in vitro, and the efficacy of recoupling eNOS with H_4B precursor sepiapterin. Sepiapterin is a known precursor of H_4B that has been used as an eNOS recoupling agent in both cell and animal studies. However, it has been found that sepiapterin is an ineffective agent to recouple eNOS in deoxycorticosterone acetate-salt hypertension due to a sepiapterin reductase (SPR) deficiency (30). Therefore, we first examined whether BMP4 induces a SPR deficiency prior to using sepiapterin to recouple eNOS in db/db mice. Whereas the protein expression of dihydrofolate reductase was diminished by BMP4 treatment in vitro, SPR protein expression was not affected (Supplemental Figure 1). Cultured bovine aortic endothelial cells were treated with 50 ng/mL of BMP4 for 24 hours with or without pretreatment of H_4B precursor sepiapterin (5 μmol/L, 2 hours). As shown in Figure 4A, BMP4-induced superoxide production was completely abolished by sepiapterin pretreatment (to 117.2 ± 73.45 from 477.58% ± 96.99% of control). Indeed, BMP4 was capable of inducing eNOS uncoupling in cultured endothelial cells, which was attenuated by pretreatment of sepiapterin (285.8% ± 25.22% vs 147.52% ± 5.2% of control, BMP4 treated vs sepiapterin pretreated cell, respectively) (Figure 4B). BMP4-mediated eNOS uncoupling was accompanied by a deficiency of H_4B (Figure 4C), which is consistent with observations in vivo. These results support the observation in vivo that BMP4 mediates eNOS uncoupling.

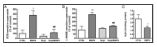

Figure 4.
BMP4 induces eNOS uncoupling in bovine aortic endothelial cells, which was attenuated by sepiapterin (Sepi) pretreatment (5 μM, 2 hours). A, BMP4 treatment (50 ng/mL, 24 hours) increased total superoxide production, which was attenuated by sepiapterin ...

NOX1 mediates BMP4 uncoupling of eNOS

NOX has been suggested to play a crucial role in eNOS uncoupling. In transgenic mice overexpressing NOX1 in smooth muscle cells, Ang II infusion caused an enhanced production of reactive oxygen species derived from uncoupled eNOS (31). We have also recently demonstrated that NOX1 is responsible for Ang II uncoupling of eNOS in an STZ-induced T1DM mice (6). Nonetheless, which specific isoforms(s) of NOX is/are involved in eNOS uncoupling in T2DM animals remain unclear. To address this question, expressions of NOX isoform(s) were examined in aortas of db/db mice. As shown in Figure 5, protein abundance of NOX1 was significantly increased in db/db mice, whereas NOX2 and NOX4 were not changed in expression. Based on this observation, we investigated whether BMP4-induced eNOS uncoupling is mediated by NOX1 activation. RNA interference of NOX1 was effective in reducing NOX1 expression (Figure 6A). As shown in Figure 6B, BMP4 induction of eNOS uncoupling was diminished in NOX1 siRNA-transfected cells. These data indicate that NOX1 is the primary NOX isoform that mediates eNOS uncoupling provoked by BMP4, which represents a newly defined mechanistic pathway of endothelial dysfunction in T2DM.

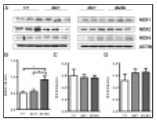

Figure 5.
NOX1 is up-regulated in aortas of db/db mice. A, Representative Western blots. B, Grouped data for NOX1 expression. C, Grouped data for NOX2 expression. D, Grouped data for NOX4 expression. Data are presented as mean ± SEM. *, $P < .05$ ...

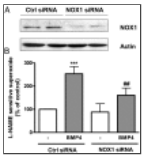

Figure 6.
NOX1 mediates BMP4 induction of eNOS uncoupling. Bovine aortic endothelial cells were transfected with 25 nM of siRNA for NOX1 for 48 hours prior to exposure to BMP4 (50 ng/mL, 24 hours). A, NOX1 was effectively down-regulated by RNA interference. B, ...

BMP4 up-regulates COX2 in cultured endothelial cells: reversal by sepiapterin

Given the known role of BMP4 in atherosclerosis, we investigated whether BMP4 induces COX2 in an eNOS uncoupling dependent fashion to promote inflammation. Aortic expression of COX2 was significantly increased in db/db mice in which BMP4 expression was elevated, whereas their up-regulations were attenuated by noggin infusion (Figure 7, A–C). Increased expression of VCAM-1 was also observed in db/db mice as shown in Figure 7C. These data indicate that BMP4 induced COX2 activation might promote inflammation. Next, we confirmed whether BMP4 activation of COX2 is eNOS

uncoupling dependent in cultured endothelial cells. In vitro data consistently demonstrated that the expression of COX2 was significantly increased by BMP4 treatment (1.37 ± 0.05-fold, $P < .01$ vs control, Figure 8, A and B). Intriguingly, pretreatment of sepiapterin, an eNOS recoupling agent, attenuated BMP4-induced COX2 expression, indicating that COX2 lies downstream of BMP4 induction of eNOS uncoupling.

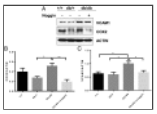

Figure 7.
Expressions of inflammatory proteins are increased in aortas from db/db mice. A, Representative Western blots. B, Grouped data for expression of COX2. C, Grouped data for expression of VCAM-1. Data are presented as mean ± SEM. *, $P < .05$; ...

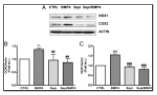

Figure 8.
BMP4 up-regulates NOX1 and COX2 in endothelial cells. A, Representative Western blots for NOX1 and COX2. B, Significant reduction in BMP4 induced NOX1 by sepiapterin (Sepi) pretreatment. C, Significant reduction in BMP4-induced COX2 by sepiapterin pretreatment. ...

Uncoupled eNOS feed-forwardly up-regulates NOX1 expression

Consistent with previous finding in endothelial cell, BMP4 caused a significant increase of NOX1 protein content (1.55 ± 0.11-fold, $P < .001$ vs control, Figure 8C). Of interest, supplementation of H_4B precursor, sepiapterin, significantly attenuated BMP4 induced NOX1 expression. These data seem to suggest that BMP4 induces NOX1-dependent eNOS uncoupling and eNOS uncoupling-derived reactive oxygen species induce NOX1 expression to aggravate oxidative stress, which might be the underlying mechanism for diabetic endothelial dysfunction and vascular complications in T2DM (Figure 9).

Figure 9.
Schematic illustration of mechanisms of eNOS uncoupling, endothelial dysfunction, and inflammatory activation in T1DM and T2DM. In STZ-induced T1DM mice, Ang II mediates NOX1 activation, which induces a deficiency of H_4B and consequent uncoupling of eNOS. ...

Discussion

In the present study, we have established for the first time that BMP4 mediates eNOS uncoupling in obese T2DM by observing the following: 1) marked elevation of circulating levels of BMP4 in db/db mice but not in STZ induced type 1 diabetic mice in which Ang II is predominantly increased; 2) attenuation of eNOS uncoupling by infusion of BMP4 antagonist noggin into db/db mice; 3) restoration of H_4B bioavailability, a determining factor for eNOS coupling activity, by noggin infusion; and 4) NOX1-dependent eNOS uncoupling in response to BMP4 in cultured endothelial cells and its reversal by H_4B precursor sepiapterin. In addition, our data further demonstrate that BMP4 induction of eNOS uncoupling is associated with subsequent activation of endothelial inflammation and endothelial dysfunction in obese T2DM, as evidenced by the following: 1) aortic up-regulation of inflammatory proteins COX2 and VCAM-1 in db/db mice in which the BMP4 is elevated; 2) BMP4 induction of COX2 in cultured endothelial cells and its reversal by sepiapterin treatment, indicating the upstream role of BMP4 uncoupling of eNOS in COX2 induction; and 3) improvement of the impaired endothelium-dependent relaxation by noggin infusion in db/db mice, indicating a role of BMP4 in endothelial dysfunction in T2DM (Figure 9).

Previously we have shown that NOX activation of eNOS uncoupling was mediated by Ang II in T1DM (8). Consistently, AT-1R blockade with telmisatan prevented eNOS uncoupling by restoration of GTPCH1, a rate-limiting enzyme in de novo synthesis of H_4B, and by attenuation of NOX1, NOX2, p67phox, p47phox, and Rac1 in the STZ-induced diabetic rat (32). However, targeting Ang II has been shown to be beneficial only to microvascular complications in T2DM. Therefore, we hypothesized that instead of Ang II, BMP4, a known proinflammatory and proatherosclerotic protein, might play a major role in endothelial dysfunction in T2DM.

BMP4 is one of the structurally related members of TGF-β superfamily, and it was originally implicated in bone and cartilage formation during embryonic development (33). In a recent report, its circulating concentration was measured to be approximately 17.8 pg/mL in healthy human patients (34), which is in similar magnitude to what we found in WT C57BL6 mice (Figure 1). Even though data on circulating BMP4 levels under pathological settings have not been available, up-regulation of BMP4 has been found associated with inflammation at atherosclerotic loci and endothelial dysfunction in hypertension. BMP4 can be increased by several factors such as hyperglycemia in venous endothelial cells (35), high-fat diet in bone of the diabetic prone BB/OK rat (36), and advanced glycation end product in diabetic mesangial cells (37), all of which are relevant to T2DM.

In the present study, we found that BMP4 levels were indeed markedly elevated in db/db T2DM mice, whereas Ang II levels were much higher in STZ-induced T1DM mice by comparison. Infusion of BMP4 inhibitor noggin attenuated eNOS uncoupling activity along with the recovery of H_4B bioavailability, indicating a critical and novel role of BMP4 in mediating eNOS uncoupling, which subsequently causes endothelial dysfunction and inflammation in T2DM. Of note, high-fat-fed mice exhibited a significant increase in the BMP4 level, even though its level was lower than T2DM mice in this study. Given that BMP4 levels were increased in db/db mice in which cholesterol and triglyceride levels were higher, dyslipidemia in T2DM might be associated with BMP4 elevation, at least in part. In addition, leptin is known to induce eNOS uncoupling to result in endothelial dysfunction in obesity (38). Given the deficiency of leptin receptor in db/db T2DM mice, leptin might be the one of the factors involved in BMP4 uncoupling of eNOS in T2DM. Taken together, several factors such as hyperglycemia, dyslipidemia, and leptin might contribute to the elevation of BMP4 levels in T2DM. Of note, eNOS uncoupling activity was increased in high-fat diet-fed mice, in which cholesterol and BMP4 levels were elevated, indicating that common factors between high-fat-fed mice and db/db mice contribute to an elevation in BMP4 and subsequent BMP4-dependent eNOS uncoupling to sustain endothelial dysfunction in T2DM. Our data also showed that impaired endothelium-dependent vasorelaxation in db/db aortas is improved by noggin infusion in db/db mice.

Activation of NOX has been implicated in BMP4-mediated endothelial dysfunction. BMP4 activates NOX in arterial endothelial cells, resulting in impaired endothelial function (39). Infusion of BMP4 stimulated NOX activity in mice, which was reversed by noggin and apocynin (12). BMP4 induced NOX1 mRNA expression, and monocyte adhesion was inhibited in $p47^{phox}$-deficient cells (15). Likewise, BMP4-induced eNOS uncoupling was NOX1 dependent in cultured endothelial cell in this study. Therefore, BMP4-induced eNOS uncoupling in db/db mice might be mediated by NOX1 activation as shown in T1DM (6). Even though NOX1 seems to function as a common mediator of eNOS uncoupling in both T1DM and T2DM, its upstream activators differ. These may suggest that targeting BMP4/Ang II or NOX1 at different stage of the disease might be important in achieving an effective inhibition of eNOS dysfunction.

In particular, BMP4 is known to induce COX2 up-regulation, an inflammatory regulator, in both endothelial cells (39) and vascular smooth muscle cells (25). COX2 is activated under pathological conditions such as atherosclerosis (13, 14) and hypertension (22). In T2DM, expression of COX2 is known to be enhanced in db/db mice (25), and its up-regulation was found associated with hypercontraction of VSMCs (25). In the present study, it was newly demonstrated that the BMP4 up-regulation of COX2 is mediated by eNOS uncoupling in T2DM. Given that elevated circulating levels of ICAM-1 and VCAM-1 have been proposed to be biomarkers reflecting endothelial dysfunction in T2DM (2, 40), COX2 activation by BMP4 and the subsequent increase of VCAM-1 in aortas might deteriorate inflammation and endothelial dysfunction via interaction of endothelial cells and vascular smooth muscle. Taken together, targeting BMP4-mediated eNOS uncoupling may prove to be an effective therapeutic strategy to restore eNOS function and in turn prevent vascular complications, in particular in T2DM.

In conclusion, our present study for the first time reveals a critical role of BMP4 in mediating eNOS uncoupling and inflammatory protein expression in T2DM obese db/db mice, which may represent novel mechanisms by which endothelial dysfunction and atherosclerotic risk develop. This pathway is different from the Ang II-dependent mechanisms in T1DM, at least for conduit arteries, although both seem to uncouple eNOS via activation of NOX1 in different types of diabetes. However, these data provide novel insights as to targeting differential pathways at an early stage of the disease may be differentially beneficial for different type of diabetes.

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Footnotes

Abbreviations:

Ang II
: angiotensin II

BMP4
: bone morphogenic protein 4

COX2
: cyclooxygenase 2

eNOS
: endothelial nitric oxide synthase

ESR
: electron spin resonance

H_4B
: tetrahydrobiopterin

L-NAME
: N-ω-nitro-L-arginine methyl ester

NOX
: nicotinamide adenine dinucleotide phosphate oxidase

NOX1
: NOX isoform 1

siRNA
: small interfering RNA

SPR
: sepiapterin reductase

STZ
: streptozotocin

T1DM
: type 1 diabetes mellitus

T2DM
: type 2 diabetes mellitus

VCAM1
: vascular cell adhesion protein 1

WT
: wild-type.

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