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Reactive oxygen species-selective regulation of aortic inflammatory gene expression in Type 2 diabetes

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Vascular diseases are a major complication of diabetes mellitus (DM), although their etiology is poorly understood. NADPH oxidase-dependent reactive oxygen species (ROS) production and inflammation are potential mediators of DM-associated vascular diseases. Using db/db mice as a Type 2 diabetes model, we examined the relationship between NADPH oxidase-derived ROS and vascular inflammation. When compared with control m/+/+ mice, aortas from 4- and 12-wk-old db/db mice had higher NADPH oxidase activity and increased superoxide levels, leading to NADPH oxidase-dependent impaired vasodilation at 12 wk. Diabetes progression from 4 to 12 wk led to increased Nox1, Nox4, and p22^phox subunit mRNAs and induced the expression of a group of matrix remodeling-related cytokines: connective tissue growth factor (CTGF), bone morphogenetic protein 4 (BMP-4), and osteopontin (OPN). After 8 wk of treatment with the superoxide scavenger Tempol, 12-wk-old db/db mice had lower superoxide production, reduced plasma glucose and lipids, and lower BMP-4 and OPN protein expression when compared with nontreated mice. No changes were observed with Tempol in CTGF or m/+/+ mice. The ability of Tempol to reverse ROS production as well as OPN and BMP-4, but not CTGF, induction suggests that DM-induced vascular inflammation involves both ROS-sensitive and -insensitive pathways.

vascular inflammation; NADPH oxidases; cytokines; superoxide; metabolic syndrome

1 Atherosclerosis is an inflammatory disease in which vascular cells and blood-derived inflammatory cells participate in a local chronic response through regulation of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukins (32).

Since the discovery that TNF-α expression also occurs in adipose tissue (21), the contribution of inflammation to diabetes and insulin resistance has been noted. Inflammation is also associated with diabetic vascular disease, the main cause of death in diabetes mellitus patients (39). Vascular disease is characterized by impaired endothelial function, vessel remodeling, and, in severe cases, plaque development and occlusion.1

Inflammatory responses have been mechanistically linked to the production of reactive oxygen species (ROS) (22). In the vasculature, NADPH oxidases are major sources of ROS (18), and they are activated in vessels from Type 1 diabetic rats (20). Both hyperglycemia and insulin activate NADPH oxidases, suggesting that these enzymes may be important in Type 2 diabetes as well. However, the link between ROS and increased inflammation in diabetic vascular disease is not fully understood.

Many factors contribute to inflammation of the vasculature, including matrix remodeling. In addition to matrix metalloproteinas, a number of novel cytokines participate in this response. Osteopontin (OPN), a widely distributed acidic phosphoprotein found in atherosclerotic plaques, contributes to vascular inflammation by regulating macrophage function, vascular smooth muscle cell (VSMC) proliferation, and matrix degradation (13, 15, 16). OPN is upregulated in diabetic animal models (31) and humans with Type 2 diabetes (52) and by high glucose (45). Another important matrix cytokine is bone morphogenetic protein 4 (BMP-4), a member of the transforming growth factor-β superfamily, which is also upregulated in aortic plaques (8, 43), stimulates adhesion molecule expression (43), and causes NADPH oxidase-dependent endothelial dysfunction (33). BMP family members are upregulated in diabetes (34) and may play a role in glucose homeostasis (7).

Finally, connective tissue growth factor (CTGF) is a potent chemotactic and extracellular matrix-inducing growth factor found at high levels in atherosclerotic, but not in normal, vessels (35). CTGF has been implicated in diabetic nephropathy and retinopathy (28, 37). Despite their obvious importance in diabetes, the role of these molecules in diabetic vascular disease is unknown.

In this study, we hypothesized that NADPH oxidase activity is increased in a mouse model of Type 2 diabetes and metabolic syndrome and that the resulting increase in ROS contributes to impaired vasoreactivity and inflammation. We found that aortic ROS production is higher in diabetic mice, and, as diabetes progresses, NADPH oxidase activity and expression

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increase. At the same time, inflammatory gene expression increases, by ROS-sensitive and ROS-insensitive mechanisms. These findings suggest that ROS selectively regulate specific aspects of the vascular responses to Type 2 diabetes and provide insight into the mechanisms underlying diabetic vasculopathy.

METHODS

Animals. Db/db mice (related genotype, ala + Lepr+/+ - Leprab), a model of Type 2 diabetes and the metabolic syndrome harboring a mutation in the leptin receptor, and misty mice (related genotype, ala m +/m +), expressing wild-type leptin receptors, were purchased from Jackson Laboratory (Bar Harbor, ME) and bred in house. Mice were fed with regular choit diet (LabDiet, rodent diet no. 5001) ad libitum and used after CO2-euthanization at weeks 4 and 12, following overnight fasting. To evaluate the participation of ROS, 4-wk-old db/db and misty mice were treated with Tempol in drinking water (2 mmol/l) freshly made every other day for 8 wk. All protocols involving these mice were approved by the Emory University Institutional Animal Care and Use Committee.

Collection of serum and measurement of metabolic parameters. Blood was collected from the left ventricle using a 21-gauge needle and kept in a 1.5-ml Eppendorf tube for 30 min before centrifugation at 3,000 rpm for 20 min at room temperature. The supernatant/serum was collected and stored at −80°C until processing.

Blood glucose levels were monitored from tail bleeds using a glucometer (Accu-Check, Roche Diagnostics). Insulin levels were analyzed at Yerkes National Primate Research Center (Atlanta, GA) using an ELISA method (ALPCO Diagnostics, Salem, NH). Plasma lipids were analyzed at the Cardiovascular Specialty Laboratories (Atlanta, GA). Triglyceride and total cholesterol levels were determined using colorimetric methods on the CX7 chemistry analyzer with reagents from Beckman Diagnostics (Fullerton, CA), which discriminate glycerol or sterol ring obtained after enzymatic cleavage with reagents from Beckman Diagnostics (Fullerton, CA), which discriminate glycerol or sterol ring obtained after enzymatic cleavage of the fatty acids from the backbone. Free fatty acids were determined using a kit from Wako Chemicals (Richmond, VA). Vascular reactivity study. Thoracic aortas were rapidly removed, cleaned of adventitia, and cut into 3-mm ring segments and studied as previously described (30). Following contraction by PGF2α, relaxations to cumulative concentrations of acetylcholine and nitroglycerin were examined. The degree of preconstriction to PGF2α was chosen to approximate 80% of the maximal response to KCl (80 mmol/l). To examine the role of ROS produced by the NADPH oxidase in inhibiting relaxation, preconstricted isolated vessels were incubated in the organ chamber with apocynin (0.5 mmol/l) for 30 min before dose-response curves were performed.

Measurement of NADPH-dependent superoxide production. Aortas were cleaned of periadventitial fat and placed in 400 μl ice-cold phosphate buffer (PBS), 50 mmol/l phosphate, pH 7.4 (treated for 2 h with 5 g/100 ml Chelex-100 and filtered), containing 0.1 mmol/l diethylenetriaminepentaacetic acid (DTPA), the protease inhibitors aprotinin (10 μg/ml), leupeptin (10 μg/ml), and PMSF (0.5 mmol/l), and homogenized for 2 min. Following centrifugation at 500 g (3–5 min), the supernatant was recentrifuged at 12,000 g for 15 min to sediment mitochondria. The resulting supernatant was transferred into another tube and centrifuged 20 min at 28,000 g. The membrane pellet was resuspended in 100 μl PBS with protease inhibitors. All procedures were performed at 0–4°C. Protein concentration was measured using the Bradford (Bio-Rad) microplate method.

Electron-spin resonance (ESR) spectroscopy was used for quantitative measurements of superoxide (O2•−) production as described previously (9). In brief, 10 μg of protein were added to the nitrobenzoxadiazole spin trap 1-hydroxy-3-carboxy-pyrrolidine (1 mmol/l), 200 μmol/l NADPH, and 0.1 mmol/l diethylenetriaminepentaacetic acid (DTPA) in a total volume of 100 μl of Chelex-treated PBS. In duplicate samples, NADPH was omitted. Superoxide formation was assayed as NADPH-dependent, SOD-inhibitable formation of 3-carboxy-proxyl (CP•). The ESR spectra were recorded using an EMX ESR spectrometer (Bruker) and a superhigh Q microwave cavity. The ESR instrument settings were as follows: field sweep, 50 G; microwave frequency, 9.78 GHz; microwave power, 20 mW; modulation amplitude, 2 G; conversion time, 656 ms; time constant, 656 ms; 512 points resolution and receiver gain, 1 × 105. Kinetics were recorded using a 1,312-ms conversion time and a 5,248-ms time constant by monitoring the ESR amplitude of the low-field component of the ESR spectrum of CP•. SOD (50 U/ml) added directly to the sample inhibited 95–98% of CP• production.

Detection of intracellular superoxide with high-performance liquid chromatography. To evaluate intracellular production of O2•−, we measured the formation of oxyethidium from dihydroethidium (DHE) using high-performance liquid chromatography (HPLC) analysis as recently reported (9). For each experiment, three 2-mm aortic rings were incubated with 50 μmol/l DHE in fresh Krebs/HEPES buffer and homogenized in 300 μl methanol. Separation of ethidium, oxyethidium, and DHE was performed with the use of an acetonitrile gradient and a C-18 reverse-phase column (Nucleosil 250–4.5 mm) on a Beckman HPLC System. Oxidized ethidium was expressed per milligram protein. In some samples, polyethylene glycol (PEG)-SOD (100 U/ml) was added 1 h before addition of DHE. PEG-SOD inhibited the DHE signal by 60%.

Real-time quantitative reverse transcriptase-PCR. Total RNA was purified from db/db and misty aortas with the use of proteinase K and DNase I digestions and the RNaseasy kit (Qiagen). RNA was reverse transcribed with Superscript II enzyme (Invitrogen) using random primers. Message expression was quantified with the use of the Lightcycler instrument (Roche) with SYBR green dye and specific mouse nox1, nox2, nox4, p22phox, VCAM-1, MCP-1, CTGF, BMP-4, and OPN primers (Table 1) and normalized to 18S ribosomal RNA. Standard curves from genuine cDNA were used to calculate copy numbers.

Table 1. PCR primers used for amplification of cytokine and NADPH oxidase genes

<table>
<thead>
<tr>
<th>Cytokine/oxidase gene</th>
<th>Forward (5′ → 3′)</th>
<th>Reverse (5′ → 3′)</th>
</tr>
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<tbody>
<tr>
<td>OPN</td>
<td>CTTTGCCATTGGATGCTGCCTTTC</td>
<td>CAGCAATCTCTGATTCCTGATGTT</td>
</tr>
<tr>
<td>CTGF</td>
<td>CGGCTGCTAGTGGCCATCAGACTT</td>
<td>GTGGCGCGGGTACACCTTTCTTCTT</td>
</tr>
<tr>
<td>BMP-4</td>
<td>CCTGGGATGAGCAGCTACCTTCT</td>
<td>TCTGCGGGACTTCGAGGCGACACTT</td>
</tr>
<tr>
<td>MCP-1</td>
<td>AGCCCAAGCAACCTCCTCTCATC</td>
<td>TCTGCGGGACTTCGAGGCGACACTT</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>CCTTAAATGCTATGAGGATTG</td>
<td>CATCTGGAGGGGAGTTG</td>
</tr>
<tr>
<td>nox1</td>
<td>CGCTCCGGCAAGAGGCTGATTACCAAGG</td>
<td>CGAGTGGACGCCAAATCCTGGCCACAACCA</td>
</tr>
<tr>
<td>nox2</td>
<td>CTTGGGCGTGAAGTTGCTCTCCTTCT</td>
<td>CCAATACAGGCAGCCCCCTTCAAG</td>
</tr>
<tr>
<td>p22phox</td>
<td>CTGCGGGAGGGGGAAGGATGCTTG</td>
<td>CTCGCCCAACTAAAGGCGAGAAGCTCAG</td>
</tr>
</tbody>
</table>

OPN, osteopontin; CTGF, connective tissue growth factor; BMP-4, bone morphogenetic protein 4; MCP-1, monocyte chemoattractant protein-1; VCAM-1, vascular cell adhesion molecule 1.
Western blotting. Previously frozen aortas were extracted with a 1% Triton X-100 and 1% SDS lysis buffer, mixed with scissors and sonicated for 10 s on ice, and analyzed by Western blotting as described previously (9). Primary antibodies were against BMP-4 (Santa Cruz, monoclonal catalog no. sc-12721) and CTGF (polyclonal, catalog no. sc-14939, Santa Cruz). After incubation with horseradish peroxidase (HRP)-conjugated secondary antibody, proteins were detected by ECL chemiluminescence. The films were analyzed by densitometry with ImageJ software (National Institutes of Health).

Histochromy in aorta. After euthanization, the heart and aorta were pressure perfused at 100 mmHg with 0.9% sodium chloride solution, followed by pressure fixation with a 10% formalin solution. Aortas were embedded in paraffin, and 5-μm cross sections were cut. After 10 mmol/l citrate buffer antigen retrieval, BMP-4 was immunolocalized using a monoclonal antibody (monoclonal antibody, catalog no. sc-12721, Santa Cruz) followed by a MOM biotinylated anti-mouse (catalog no. MK-2202, Vector Laboratories, Burlingame, CA) and visualized with HRP/diaminobenzidine (Jackson ImmunoResearch). For OPN, a polyclonal antibody was used (catalog no. 18621, IBL-Japan) and visualized using the avidin-biotin complex system/alkaline phosphatase (ABC kit, Vector).

Statistical analysis. Data are shown as means ± SE. Statistical significance was assessed by two-way ANOVA on untransformed data, followed by comparison of group averages by contrast analysis. In the indicated cases, one-way ANOVA or nonparametric test was used. SuperANOVA statistical program (Abacus Concepts, Berkeley, CA), and SPSS 14 for Windows were used for the analyses. A P value <0.05 was considered to be statistically significant.

RESULTS

Metabolic parameters. Previous work showed that db/db mice become fully diabetic at 12 wk (14). Our measurements confirmed this time course (Table 2). When compared with age-matched controls, 12-wk db/db mice were obese, hyperglycemic, hyperlipidemic, and hyperinsulinemic. Glucose and insulin were not significantly elevated at 4 wk in these animals; however, their weight was greater and their lipid levels tended to be higher compared with 4-wk-old misty mice. Systolic blood pressure was 100 ± 2 mmHg in db/db mice at 8 wk, 117 ± 4 mmHg in db/db mice (n = 17) at 12 wk, and 95 ± 3 mmHg in misty mice (n = 13) at 12 wk (P < 0.001). The combination of hypertension and the above metabolic abnormalities make these mice an excellent model of the metabolic syndrome.

Aortic superoxide production and NADPH oxidase activity and expression. Both vascular disease and diabetes are associated with increases in ROS, and NADPH oxidases are among the primary ROS-generating enzymes in vessels (18). To determine the effect of diabetes in these enzymes, we first examined changes in aortic NADPH-dependent oxidase activity. We found a small, but significant, increase in aortic oxidase activity from 4-wk db/db mice compared with misty mice (Fig. 1A). This difference was greater in older animals (160 ± 0.4%, P < 0.00001).

To determine whether this increase in oxidase activity is due to increased catalytic subunit expression, we measured nox1, nox2, and nox4, as well as p22phox mRNA levels. Fig. 1B shows the pattern of expression at 4 and 12 wk in db/db and control misty mice. p22phox was significantly upregulated in db/db mice compared with control mice in both 4-wk-old (211 ± 42.9%, P < 0.05) and 12-wk-old animals (233 ± 53.5%, P < 0.001). This increase was much greater by 12 wk. Similarly, nox1 and nox4 were increased at 12 wk, and their expression tended to be higher in db/db mice at both times, reaching statistical significance in nox4 at 12 wk. In the case of nox1, this increase was mostly a consequence of age, rather than diabetes, because it was also apparent in misty mice. We found no difference in nox2 expression over time or between animals.

We then studied whether NADPH oxidase activation leads to an overall increase in vascular O2•− production. As shown in Fig. 2, O2•− levels increased in diabetic animals at both 4 and 12 wk. Thus aortic O2•− production and NADPH oxidase expression and activity are increased in db/db mice during diabetes mellitus progression.

Impaired endothelium-dependent relaxation is reversed by NADPH oxidase inhibition. To determine whether this increase in NADPH oxidase activity influenced endothelial function, we measured endothelium-dependent and -independent relaxation in aortas in the presence and absence of the NADPH oxidase inhibitor apocynin. As shown Fig. 3A, acetylcholine-induced relaxation was reduced in aortas from 12-wk-old db/db mice compared with misty mice. Acute treatment with apocynin did not affect relaxation in misty mice but improved the acetylcholine response in db/db mice, suggesting that NADPH oxidases contribute to impaired endothelial function. In contrast, nitroglycerin-induced relaxation was unaffected in db/db mice, and apocynin had no effect (Fig. 3B).

Aortic inflammation is increased in diabetic mice. Both diabetes and ROS are known to cause inflammation (22). Therefore, we hypothesized that vascular inflammatory genes are upregulated in db/db mice. We examined a series of proinflammatory genes, including VCAM-1, MCP-1, CTGF, OPN, and BMP-4. We found no change in VCAM-1 and MCP-1 (not shown), but we observed significant increases in the latter three genes. As demonstrated in Fig. 4, in 4-wk-old db/db mice, aortic CTGF gene expression was increased compared with that from misty mice (446.4 ± 166.6%, P < 0.05). No changes in BMP-4 or OPN expression were observed at 4 wk. In aortas from 12-wk-old db/db mice, the increase in

Table 2. Metabolic parameters

<table>
<thead>
<tr>
<th></th>
<th>m+/+ 4 wk</th>
<th>db/db 4 wk</th>
<th>m+/+ 12 wk</th>
<th>db/db 12 wk</th>
<th>m+/+ 12 wk Tempol</th>
<th>db/db 12 wk Tempol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, g</td>
<td>12.8 ± 0.5</td>
<td>18.8 ± 4.3*</td>
<td>24.0 ± 0.6†</td>
<td>43.4 ± 1.0*†</td>
<td>21.2 ± 0.3</td>
<td>45.7 ± 0.5*</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>77.2 ± 5.7</td>
<td>101.8 ± 5.5</td>
<td>100.2 ± 5.7</td>
<td>327.4 ± 26.3*‡</td>
<td>90.3 ± 5.2</td>
<td>236.1 ± 15.7*‡</td>
</tr>
<tr>
<td>FFA, meq/l</td>
<td>0.51 ± 0.02</td>
<td>0.83 ± 0.05*</td>
<td>0.42 ± 0.03</td>
<td>0.82 ± 0.05*</td>
<td>0.51 ± 0.04</td>
<td>0.81 ± 0.13*</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>55.0 ± 10.9</td>
<td>96.2 ± 6.7</td>
<td>98.2 ± 16.0</td>
<td>143.3 ± 16.8*</td>
<td>119.7 ± 21.5</td>
<td>104.4 ± 12.0§</td>
</tr>
<tr>
<td>Triglyceride, mg/dl</td>
<td>13.1 ± 4.6</td>
<td>68.8 ± 10.8*</td>
<td>44.4 ± 9.2</td>
<td>191.1 ± 33.2*‡</td>
<td>50.3 ± 11.8</td>
<td>95.4 ± 19.4§</td>
</tr>
<tr>
<td>Insulin, μg/l</td>
<td>1.8 ± 0.4</td>
<td>3.9 ± 0.9</td>
<td>0.9 ± 0.3</td>
<td>10.0 ± 2.1*†</td>
<td>0.8 ± 0.3</td>
<td>14.0 ± 2.6*</td>
</tr>
</tbody>
</table>

Values are means ± SE. FFA, free fatty acids. *P < 0.01 vs. wild type; †P < 0.01 vs. 4 wk; ‡P < 0.01 vs. 12 wk.
CTGF mRNA was maintained (295.5 ± 93.8%, P < 0.02), and BMP-4 (230.3 ± 50.4%, P < 0.01) and OPN (188 ± 54.7%, P < 0.01) expression was significantly increased when compared with controls. The latter two genes also increased with age in misty animals, although to a lesser extent.

Effect of Tempol treatment on superoxide and metabolic parameters. To understand the contribution of ROS-mediated pathways to diabetes-induced inflammation, we treated misty and db/db mice with the O$_2^•^{-}$ scavenger Tempol from 4 to 12 wk. This treatment effectively reduced O$_2^•^{-}$ production in 12-wk-old diabetic mice (Fig. 2). There was no change in NADPH-dependent oxidase activity (data not shown).

Body weight was unchanged by Tempol treatment (misty, 24.0 ± 0.6 vs. 21.2 ± 0.3; db/db, 43.4 ± 1 vs. 45.7 ± 0.5 g, n = 6; not significant). However, plasma glucose, total cholesterol, and triglyceride levels were all reduced by Tempol (Fig. 5).
Inflammation in db/db mice is induced by ROS-sensitive and -insensitive pathways. We then examined changes in cytokine protein expression. As is shown in Fig. 6A, BMP-4 was dramatically upregulated exclusively in response to diabetes progression; misty mice had no significant increase in BMP-4 expression with age. Of importance, db/db mice treated with Tempol showed a significant reduction in aortic BMP-4 protein production as evaluated by Western blot (Fig. 6A) and confirmed by immunohistochemistry (Fig. 6B). OPN was also ROS sensitive (Fig. 6C). No OPN expression was detected in misty mice at any time. However, aortas from diabetic animals were strikingly positive for OPN in the media, and this staining was partially reduced after Tempol treatment (Fig. 6C). In contrast, CTGF protein showed a very different pattern of expression from the other inflammatory proteins. CTGF was strongly induced in aortas from both misty and db/db mice at 12 wk of age but was significantly higher in db/db animals. Of importance, CTGF protein and mRNA expression was unaffected by Tempol treatment (Fig. 7), suggesting that it is ROS independent.

DISCUSSION

Previous studies of diabetic vascular complications focused largely on impaired reactivity and microvascular remodeling. However, inflammatory molecules such as TNF-α can induce insulin resistance (46), and diabetes upregulates inflammatory genes in adipose tissue (49). On the basis of the relationship between inflammation and atherosclerosis, we evaluated whether, in addition to impaired vasodilation and remodeling, the vasculature is a direct target of the diabetes-induced proinflammatory response. We found a NADPH oxidase-dependent reduction in the vasodilatory response to acetylcholine. More importantly, expression of a group of inflammatory cytokines, CTGF, OPN, and BMP-4, was increased in aortas from diabetic animals. Our results also revealed that both redox-sensitive and redox-insensitive pathways are responsible for activation of these genes, illustrating the complexity of diabetes pathology.

Although a relationship between ROS and diabetes is well established, the sources of ROS production remain controversial. Multiple studies in culture showed that hyperglycemia increases ROS production via activation of mitochondrial respiration and NADPH oxidases (6). However, in vivo, the source of ROS depends not only on the type of diabetes being studied but also on the tissue. For example, in animal models of diabetes Type 1, mitochondrial ROS are implicated in cardiomyopathy (41), whereas NADPH oxidase-derived ROS are associated with proteinuria and vascular injury (2, 47). In the Goto-Kakizaki rat model of Type 2 diabetes, liver mitochondrial activity is not increased (23), but that of retina and kidney is elevated (27, 38). In vascular tissue, less is known about the role of mitochondria and NADPH oxidases in Type 2 diabetes. Whereas there is undoubtedly a contribution of mitochondria to vascular ROS production (38), the role of NADPH oxidases is more controversial. In Otsuka Long-Evans Tokushima Fatty rats, aortic NADH oxidase activity and gp91phox/p22phox expression is increased (25), whereas in Goto-Kakizaki rats, no increase in aortic NADPH oxidase...
activity is observed (38). Our study was not designed to discriminate between mitochondria- and NADPH oxidase-derived ROS, but rather to determine whether NADPH oxidases are regulated by the onset of Type 2 diabetes. We found a significant increase in NADPH oxidase activity during the development of diabetes in aortas from \( \text{db/db} \) mice (Fig. 1A).

The reversal of endothelial dysfunction by apocynin clearly demonstrates the physiological importance of this enzymatic activity in at least some of the vascular complications of diabetes.

Consistent with a role for NADPH oxidases in diabetic vascular dysfunction, we found an increase in NADPH oxidase expression in aortas from \( \text{db/db} \) mice (Fig. 1B). Expression of the catalytic Nox1 and Nox4 subunits, as well as the stabilizing subunit p22\( \text{phox} \), increased with age and diabetes similar to previous findings in kidney and aorta from streptozotocin-induced diabetic animals (11, 17, 50). However, NADPH oxidase activity and ROS production were significantly increased only as a consequence of diabetes and did not change with age in either misty or \( \text{db/db} \) mice (Figs. 1A and 2). Furthermore, the greater increase in NADPH oxidase activity compared with \( \text{O}_2^-/\text{H}_2\text{O}_2 \) levels in 12-wk-old diabetic animals suggests that the age-related increase in ROS can be compensated, perhaps by induction of antioxidant enzymes, but that diabetes-associated ROS production overwhelms compensatory mechanisms.

In this study, we observed two vascular manifestations of elevated ROS in \( \text{db/db} \) mice. First, endothelium-dependent vasodilation was impaired and was restored by treatment with apocynin (Fig. 4A), providing clear evidence for involvement of NADPH oxidases that contain p47\( \text{phox} \) as part of their active complex (Nox1 or Nox2 in the vasculature). Previous work in other models of Type 1 or Type 2 diabetes using different...
methods to block ROS provided similar results (19, 24, 40). Second, vascular inflammation in db/db mice appears to be partially related to excess ROS. Surprisingly, induction of only some cytokines was reduced by Tempol treatment, suggesting that, in this model, inflammatory responses are triggered by both ROS-dependent and ROS-independent mechanisms. This is not surprising, because both hyperglycemia and hyperinsulinemia activate multiple signaling pathways that regulate the transcriptional and posttranscriptional control of cytokine expression, some of which are regulated by ROS (e.g., NF-κB).

Fig. 6. Effect of Tempol on osteopontin and BMP-4 expression in aortas from 12-wk-old mice. A: aortas were harvested, and proteins were analyzed for BMP-4 by Western blot. Representative Western blot (top) and graph (bottom) show the means ± SE of 4–7 independent experiments. **P < 0.01 vs. 4-wk-old mice of the same genotype; +P < 0.05 vs. nontreated mice of the same genotype. B: the heart and aorta were pressure fixed, and sections were subjected to immunohistochemistry using a BMP-4 monoclonal antibody. Brown staining indicates BMP-4 immunoreactivity. Each image is representative of 8–10 sections from 2–4 animals. C: sections were stained for osteopontin using a polyclonal antibody and were visualized with alkaline phosphatase. Each image is representative of 8–10 sections from 2–4 animals.
and some of which, like those that regulate CTGF (e.g., ERK1/2 in vascular smooth muscle) (44), are not. Previous work on inflammatory gene expression in diabetic animals centered on MCP-1 and VCAM-1 (53). We first examined the expression of these canonical molecules and found no change in their expression. This differs from results reported in db/db mice crossed into the apolipoprotein E-deficient background, in which VCAM-1, tissue factor, and matrix metalloproteinase-9 were induced when plaques were present (51). Without the additional stress of excessive hypercholesterolemia, perhaps these markers of inflammation are undetectable. Therefore, we concentrated on proteins that regulate matrix remodeling, are redox associated, and have been implicated in cellular inflammation. OPN expression is increased in atherosclerotic plaques (15) and regulates macrophage and VSMC migration, matrix degradation, and VSMC proliferation (13, 15, 16). OPN has previously been shown to be induced by oxidative injury (36). Of importance, we found that OPN is upregulated at 12 wk in aortas from diabetic animals, and this increase is partially reversed by Tempol. Another important proinflammatory molecule is BMP-4, which mediates monocyte adhesion (42, 43) and is upregulated in atherosclerosis (8, 33), restenosis (48), and diabetes (34). It has also been implicated in impaired endothelium-dependent vasodilation in hypertension (33). BMP-4 was one of the most highly regulated genes both at the protein and mRNA levels, and its expression was abrogated by Tempol. Not only are both BMP-4 and OPN upregulated by ROS, but also they activate NADPH oxidases (29, 42), suggesting that they may play a causal role in the increased oxidative stress found in db/db mice. Recent work has shown that CTGF can negatively regulate BMP-4 signaling (1), raising the possibility that the increase in CTGF that we observed in diabetic mice compensates for increased BMP-4 expression. CTGF itself is a potent chemotactic and extracellular matrix-inducing growth factor that has been implicated in progression of inflammatory and fibroproliferative disorders (5). The net effect of these changes in gene expression is to create a proinflammatory environment in the vessel wall of diabetic animals that may facilitate the development of more advanced vasculopathies.

Db/db mice have been established as a model of diabetic dyslipidemia (26). An unexplained finding of this study was that Tempol also decreases glucose, cholesterol, and triglycerides in the blood of these mice. Consistent with our findings, Tempol treatment of old rats decreased plasma glucose and triglycerides (3), and apocynin caused similar changes in KKAy obese mice (12). Furthermore, Blenda et al. (4) showed that in Ren-2-overexpressing mice, Tempol improved the insulin resistance index. The mechanism of these effects is unknown, but the observation raises the interesting question of
whether the protective effects of Tempol are due to a direct antioxidant effect on the vessel wall or to its ability to improve the lipid profile and hyperglycemia in these animals. It is possible that ROS may mediate glucose and lipid metabolism, and some inflammatory responses, without affecting weight gain or insulin levels, indicates that inflammation is not related to insulin or obesity in this model.

One caveat to this work is that some of the vascular manifestations of diabetes in these animals may be attributed to the small increase in blood pressure observed in 12-wk-old db/db mice. However, diabetes and the accompanying impaired relaxation and inflammatory responses developed during 4–12 wk. As noted, blood pressure measurements taken at 8 wk show no difference between misty and db/db animals, suggesting that blood pressure is more likely to be a consequence than a cause of ROS-related vascular dysfunction. Regardless of the trigger, patients with the metabolic syndrome are often hypertensive, and our results suggest that a similar combination of high blood pressure and dyslipidemia in mice is accompanied by impaired vascular responses that can be corrected in part by Tempol treatment. While we were unable to measure blood pressure in the Tempol-treated animals, others have shown that this dose of Tempol effectively lowers blood pressure in other hypertensive models (10). Thus, in addition to its salutary effect on the vessel wall or to its ability to improve whether the protective effects of Tempol are due to a direct antioxidant therapy.

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GRANTS

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REFERENCES


