NAD(P)H oxidase and uncoupled nitric oxide synthase are major sources of glomerular superoxide in rats with experimental diabetic nephropathy

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IN EXPERIMENTAL ANIMALS, REACTIVE oxygen species (ROS) are involved in the development of diabetes mellitus induced by streptozotocin (STZ) (15, 26). Oxygen radicals are not only involved in the pathogenesis of diabetes, but the diabetic status itself is associated with increased production of ROS, which in turn has been suggested as one of the pathogenic mechanisms of diabetic complications (3, 4, 7, 25). Experimental and clinical evidence indicates that excessive oxidative stress may also contribute to the initiation and development of diabetic nephropathy (6, 14, 29). In the diabetic state, the presence of high levels of markers for lipid peroxidation, such as malondialdehyde (MDA), protein oxidation, such as pentosidine, and oxidative DNA damage, such as 8-hydroxydeoxyguanosine (8-OHdG), has been reported (14, 40). Previous studies also provided evidence for in vivo oxidative stress in glomeruli of rats with early diabetes (18). The efficacy of dietary antioxidant supplementation or drugs for the blockade of hyperglycemia-induced ROS production in the prevention of diabetic complications has been also examined (24, 34, 39). Thus assessment of the ROS production pathway and blockade of ROS production in diabetes may be important for the prediction and prevention of diabetic complications.

Several pathways are involved in the production of superoxide (O2•−) or ROS in a variety of cells and tissues, such as xanthine oxidase (XO), mitochondrial respiratory chain enzymes, arachidonic acid-metabolizing enzymes, NAD(P)H oxidase, and nitric oxide (NO) synthase (NOS). In cardiovascular diseases, previous studies identified NAD(P)H oxidase as a source for O2•− (10, 33). Another mechanism for ROS production in vascular disease is the uncoupling of NOS. Several recent studies on oxidative stress and endothelial dysfunction in atherosclerosis (27) and diabetes (8, 13) reported an upregulated, but dysfunctional, uncoupled NOS. Based on studies on both neural NOS (nNOS) and endothelial NOS (eNOS), it is clear that in the absence of the substrate l-arginine or cofactor tetrahydrobiopterin (BH4), heme reduction in the enzyme results in the uncoupling of NOS and consequently leads to the production of O2•− rather than NO (41, 44), which may contribute to oxidative stress and endothelial dysfunction. However, it remains to be established whether a similar phenomenon can be demonstrated in the glomeruli of diabetic animals.

The present study was undertaken to investigate ROS production in different pathways in the glomeruli of rats with STZ-induced diabetes. By using fluorescence spectrometric analysis (21, 45), ROS, especially O2•− production, were detected. The results showed that both NAD(P)H oxidase and eNOS uncoupling in diabetic glomeruli potentially contribute to ROS production that accompanies loss of NO production and that these phenomena are improved, at least in part, by BH4 administration.
METHODS

Animal preparation and glomerular isolation. The experimental protocol was approved in advance by the Ethics Review Committee for Animal Experimentation of the Kawasaki Medical School, Kurashiki, Japan. Male Sprague-Dawley rats weighing 180 to 240 g were purchased from Charles River Japan (Kanagawa, Japan). The animals were housed in a temperature- and humidity-controlled room with a 12:12-h light-dark cycle, and they were fed standard laboratory animal chow and had free access to tap water. Diabetes was induced by a single tail-vein injection of STZ (65 mg/kg body wt; Sigma-Aldrich Japan, Tokyo, Japan) diluted in citrate buffer, pH 4.5 (n = 24). Age-matched nondiabetic control rats (n = 12) were each injected with an equal volume of citrate buffer. After 3 days, the induction of diabetes was confirmed by measurement of blood glucose concentrations. Rats were excluded if their blood glucose levels failed to rise above 15 mmol/l. Six weeks after induction of diabetes mellitus, the rats were anesthetized with pentobarbital sodium (50 mg/kg body wt). The NOS cofactor BH4 (Sigma-Aldrich Japan) was administered by gavage to a subgroup of diabetic rats for 2 days before anesthesia with pentobarbital sodium (n = 12). BH4 was compressed into rodent chow pellets without the addition of water or heat to prevent oxidation of the compound. The concentration of BH4 in the pellets (1 mg/g) was calculated to provide a dose of 20–25 mg/day based on an average diabetic rat intake of 20–25 g/kg body wt of diet daily (22). Pellets were stored at −20°C. The abdominal aorta was cannulated, and the kidneys were perfused retrogradely with ice-cold PBS (pH 7.4). The right kidney from each rat was fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemistry. The renal cortex of the left kidney from each rat was cut into small pieces, and glomeruli were isolated by the mechanical graded sieving technique. After isolation, the purity of the final suspension was determined by light microscopic examination. On average, tubular contamination was <5%. The glomerular suspension was then used for O2− production assay, protein isolation, and RNA isolation.

Determination of O2− production in isolated glomeruli. Superoxide production in isolated glomeruli was detected by dihydroethidium (DHE) conversion to ethidium (Eth), as described previously (21). Isolated glomeruli from each group were incubated in culture vessels kept away from direct light and containing endotoxin-free RPMI 1640 culture medium with 20 mmol/l DHE (Sigma-Aldrich Japan) for 1 h at 37°C. After being rinsed with PBS, the incubated samples were placed on a glass slide and coverslipped. Superoxide signal specificity was confirmed by coincubation with 10 mmol/l tiron (Sigma-Aldrich Japan). The fluorescence at an excitation wavelength of 570 nm was detected at an emission wavelength of 610 nm using confocal laser-scanning microscopy (TCS-NT, Leica-Microsystems, Tokyo, Japan).

Fluorescence spectrometric assay of O2− production in isolated glomeruli. The isolated glomeruli were homogenized with a glass homogenizer in ice-cold HEPES buffer containing 25 mmol/l HEPES, 1 mmol/l (EDTA), and 0.1 mmol/l phenylmethylsulfonyl fluoride. After centrifugation of the homogenate at 6,000 × g, the supernatant containing the membrane and cytosolic components, termed the homogenate, was separated. Fluorescence spectrometry of tissue O2− production was performed by using a modification of methods described by Zou et al. (45). Briefly, the fluorogenic oxidant of DHE to Eth, a fluorescent probe, was used. Briefly, 1 µg tissue equivalents (20 µg/ml) were incubated with DHE (0.02 mmol/l), salmon testes DNA (0.5 mg/ml), and the corresponding substrate for XO, mitochondrial respiratory enzymes, NADH/NADPH oxidase, or NOS in a microtiter plate placed away from direct light at 37°C for 30 min. Eth-DNA fluorescence was measured at an excitation of 480 nm and an emission of 510 nm by using a fluorescence microplate reader (FLUOstar OPTIMA, Meritex Bio Science, Tokyo, Japan). Xanthine (0.1 mmol/l) was used as a substrate of XO. Succinate (5 mmol/l) was used as a substrate for intramitochondrial O2− production, and anti-mycin (0.05 mmol/l) was used to block the normal reaction in the respiratory chain. NADH/NADPH oxidase activity to produce O2− was examined by the addition of NADH (0.1 mmol/l) as a substrate in the reaction mixture. t-Arginine (t-Arg; 1 mmol/l) was used as a substrate for NOS, Nε-nitro-t-arginine methyl ester (t-NAME; 1 mmol/l) was used to block NOS activity. A blank without the homogenate was used to measure background fluorescence, and its level was subtracted from each sample. To further confirm that the activity of NAD(P)H oxidase or uncoupled NOS contributes to the production of O2− in glomeruli, we examined the effects of the NAD(P)H oxidase inhibitor diphenylene iodonium chloride (DPI; 0.1 mmol/l) or BH4 (0.01 mmol/l) on Eth-DNA fluorescence intensity. The enzyme activities of different pathways are expressed relative to the control.

Ferritrochrome c reduction assay for measurement of O2− production in isolated glomeruli. The assay for O2− was carried out by measuring superoxide dismutase-inhibitable (SOD-inhibitable) reduction of ferritrochrome c as described previously (23). Nonhomogenized, isolated glomeruli (10 mg) were suspended in a Krebs bicarbonate buffer (in mmol/l: 118 NaCl, 4.7 KCl, 1.5 CaCl2, 1.1 MgSO4, 1.2 KH2PO4, 5.6 glucose, and 25 NaHCO3) containing the corresponding substrates for XO (0.1 mmol/l xanthine), NOS (1 mmol/l l-Arg), or NOS inhibitor (1 mmol/l l-NAME) in a 24-well microtiter plate, under a 21% O2-5% CO2 gas mixture at 37°C for 4 h. After incubation, the supernatant was collected and ferritrochrome c was added to the supernatant to a final concentration of 70 µmol/l in the absence or presence of SOD (100 U/ml). Reduction of ferritrochrome c in the supernatant was monitored for 30 min at an absorbance of 550 nm using a spectrophotometer (FLUOstar OPTIMA, Meritex Bio Science). The rate of O2− production was calculated as described previously (28), and the results were expressed relative to the control.

In situ detection of NO and ROS. Five rats in each group were killed by intraperitoneal injection of pentobarbital sodium. An 18-gauge needle connected to an infusion pump was inserted into the left ventricle. After the right atrium was cut, the whole body was perfused with 37°C PBS (flow rate, 5 ml/min). Once blood had been removed, the whole body was perfused with PBS containing 0.01 mmol/l diaminobenzidine-4M AM (DAR-4M AM; Daichii Pure Chemicals, Tokyo, Japan); 0.05 mmol/l dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR); 0.1 mmol/l t-Arg; and 2 mmol/l CaCl2 for an additional 10 min at a flow rate of 5 ml/min. All unreacted DAR-4M AM and DCFH-DA were removed by postperfusion with PBS for 10 min. After fixation with 4% paraformaldehyde perfusion, the tissues were cut into 1-mm-thick sections and enveloped on the slide glass. Fluorescent images of NO and ROS were obtained using confocal laser-scanning microscopy (TCS-NT, Leica-Microsystems). The wavelength was as follows: DAR-4M AM, excitation at 560 nm and emission at 575 nm, and DCFH-DA, excitation at 490 nm and emission at 530 nm.

RNA isolation and real-time quantitative PCR. Total RNA was isolated from isolated glomeruli with TRIzol (Invitrogen Japan, Tokyo, Japan). Reverse transcriptase reactions were performed using a Ready-To-Go T-Primed First-Strand Kit (Amersham Biosciences, Piscataway, NJ) for first-strand cDNA synthesis. Real-time quantitative PCR was performed using the ABI Prism 7700 sequence-detection system (Applied Biosystems, Foster City, CA). Primers and probes for TaqMan analysis were designed using Primer Express 1.5 (Applied Biosystems) using information from the supplier based on the sequence information from GenBank or EST databases. The primers and probes used for p22phox, p47phox, p67phox (where phox indicates phagocyte oxidase), nNOS, inducible NOS (iNOS), and eNOS are shown in Table 1. For each gene, 10 ng of the cDNA were analyzed on an ABI PRISM 7700 using TaqMan Universal PCR Master Mix (Applied Biosystems). All TaqMan analysis was performed using three-primer PCR (forward and reverse primers with fluorescent probe) to ensure the specificity of the signal.
Table 1. Sequences of primers and probes used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>p22phox Forward</td>
<td>5'-CTTGGTGTCCCTACTACTTATTGGCA-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GAAGCCCCTTTTTGCTCTTC-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>5'-FAM-TGACTGTTCACTCTGGGATCATGACATTGCA-3'</td>
</tr>
<tr>
<td>p47phox Forward</td>
<td>5'-GTGAGGCTGAGGGATTCCCT-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CCGGCCGCTTCATTAATGTT-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>5'-FAM-CATGACGGCCTACTGCTGGATCATGACATTGCA-3'</td>
</tr>
<tr>
<td>p67phox Forward</td>
<td>5'-CAGACCAGAAACCACGAGAA-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CCGGACAGGGCAAGCAACAT-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>5'-FAM-CTCTGAGGAGAGGACACCCGG-TAMRA-3'</td>
</tr>
<tr>
<td>nNOS Forward</td>
<td>5'-TGCGCTGAGGCTGATGAACTG-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-TTCGGCAGGACAGAATGCA-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>5'-FAM-CAGGAGCTGGCTGCAAGGTATAGTGAAGCTCCA-3'</td>
</tr>
<tr>
<td>iNOS Forward</td>
<td>5'-CCCTCAAGATGGGGATTTGTTG-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-AGGCCCTGGAGGCAAGACCTT-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>5'-FAM-GTCCTTTGCTCATGACATCGACCAG-TAMRA-3'</td>
</tr>
<tr>
<td>eNOS Forward</td>
<td>5'-GAGCCCAGTTTCTCTGAGTAA-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GGAAGCCAACTGAGGCTCTC-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>5'-FAM-CAAGTACAGCCTAGCATACGGCCTGAGCA-3'</td>
</tr>
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nNOS, neuronal nitric oxide synthase; iNOS, inducible NOS; eNOS, endothelial NOS; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-tetramethylrhodamine.

Analysis was based on standardizing RNA levels by correcting for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels in the sample.

**Immunohistochemistry.** Immunohistochemical staining for MDA, 8-OHdG, and nitrotyrosine was performed using a Histofine Simple Stain Max-PO kit (Nichirei, Tokyo, Japan). Microwave heat-induced antigen retrieval in citrate buffer, pH 6.0, was required for optimal staining with all antibodies. After deparaffinization, 5-μm-thick tissue sections were incubated in methanol with 3% hydrogen peroxide to block endogenous peroxidase activity. The sections were incubated overnight with primary antibodies at 4°C. The working concentration of each antibody was 1 μg/ml for the mouse anti-8-OHdG monoclonal antibody (Japan Institute for the Control of Aging, Fukuroi, Shizuoka, Japan), 4 μg/ml for the rabbit anti-nitrotyrosine polyclonal antibody (Upstate Biotechnology, Lake Placid, NY). For negative controls, a monoclonal mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) or normal rabbit serum was used at equivalent concentrations. The primary antibody was detected using a Histofine Simple Stain MAX-PO (MULTI) kit and 3,3′-diaminobenzidine (Sigma-Aldrich Japan).

**Western immunoblotting.** Portions of isolated glomerular samples were homogenized in lysis buffer containing 0.25 mol/l sucrose, 50 mmol/l dithiothreitol, 3 mmol/l HEPES (pH 7.9), 0.5 mmol/l EGTA, 1 mmol/l 4-(2-aminophenyl)-benzenesulfonyl fluoride, 0.8 μmol/l aprotonin, 21 μmol/l leupeptin, 36 μmol/l bestatin, 15 μmol/l pepstatin A, 14 μmol/l (4-guanidino) butane, and 1% Triton X-100. After centrifugation (8,000 g, 10 min, 4°C), the supernatants were used for Western immunoblotting. Appropriate volumes of the supernatant (100 μg/lane) were mixed with an equal volume of sample buffer (100 mmol/l Tris·HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.02% bromophenol blue) and subjected to SDS-PAGE using 12.5% acrylamide gels. For immunoblot analysis of nitrotyrosine or the monomer of eNOS, samples were heated at 95°C for 5 min before electrophoresis. For immunoblot analysis of the dimeric form of eNOS, samples were not heated and the temperature of the gel was maintained below 15°C during electrophoresis (low-temperature SDS-PAGE) (16, 42). The proteins were transferred by semidy electrophoretic transfer to a polyvinylidene difluoride membrane for 120 min. The blots were then blocked and incubated with rabbit anti-eNOS polyclonal antibody (0.1 μg/ml; Santa Cruz Biotechnology) or rabbit anti-nitrotyrosine polyclonal antibody (1 μg/ml; Upstate Biotechnology) for 120 min at room temperature. Next, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (0.08 μg/ml; Santa Cruz Biotechnology). The antibody was visualized using an enhanced chemiluminescence method (ECL; Amersham Biosciences). The integrated density (density 3 area) of the bands was quantified using National Institutes of Health Image analysis software v1.61.

**Determination of plasma BH4 concentrations.** BH4 concentrations were determined by HPLC as described previously (20, 37). The amount of BH4 was determined from the difference between total (BH4 + BH2 + bioprotein) and alkaline-stable oxidized (BH2 + bioprotein) bioprotein. The A10μm column (250-mm-long, 4.6-mm-inner diameter, 5-μm particle size) was used with 15 mmol/l K2HPO4 buffer, pH 6.0, at a flow rate of 0.8 ml/min. BH4 was detected by emission fluorescence at 350 (excitation) and 440 nm (emission).

**Statistical analysis.** Values are expressed as means ± SE. All parameters were evaluated with the two-tailed unpaired Student’s t-test or compared by one-way ANOVA when a multiple mean comparison was required. Correlation coefficients were determined using linear regression analysis. A P value <0.05 denoted the presence of a statistically significant difference.

**RESULTS**

**ROS production and accumulation of oxidative stress in diabetic glomeruli.** ROS production in isolated glomeruli was evaluated by DHE conversion to Eth and imaged by confocal laser-scanning microscopy. DHE fluorescence was strong, indicating O2− production, in isolated glomeruli from diabetic rats compared with that in glomeruli from control rats (Fig. 1). DHE fluorescence was blocked when the glomeruli were incubated in the presence of the superoxide scavenger tiron, confirming that the signal originated from O2− (data not shown). Immunostaining was employed to evaluate the distribution and expression of MDA and 8-OHdG (Fig. 1). Cells positive for MDA, a marker of lipid peroxidation, were detected in the capillary walls of the glomeruli from diabetic rats but not in those of the control. Cells positive for 8-OHdG, a marker of ROS-induced DNA damage, were almost absent in the glomeruli of control rats but were abundant in the glomeruli of diabetic rats.

**Different pathways responsible for O2− production in diabetic glomeruli.** Incubation of homogenates of diabetic glomeruli with DHE produced strong Eth fluorescence compared with incubation of homogenates of control glomeruli (1.32 ± 0.18-fold, P < 0.05, Fig. 2A).

Incubation of homogenates of isolated diabetic glomeruli with succinate or xanthine, which are substrates for mitochondrial respiratory chain enzymes and XO, respectively, did not increase the formation of O2− compared with incubation without the substrate. Superoxide production in the isolated glomeruli from diabetic rats, as measured by the ferricytochrome c reduction assay, was also higher than in the control (1.69 ± 0.32-fold, P < 0.05, Fig. 2A), but no additional increase was observed by coincubation with xanthine or succinate and antimycin (1.55 ± 0.22- and 1.64 ± 0.23-fold, respectively.) Incubation of homogenates of diabetic glomeruli with NADH resulted in a significant increase in O2− production compared with incubation without a substrate (1.71 ± 0.17-fold, P <
The NAD(P)H-dependent oxidase and NOS, sources of superoxide production in the glomeruli of diabetic but not XO or mitochondrial oxidase, are the predominant results suggest that NAD(P)H-dependent oxidase and NOS, sources of superoxide production in the glomeruli of diabetic but not XO or mitochondrial oxidase, are the predominant sources of superoxide production in the glomeruli of diabetic rats.

In situ detection of ROS and NO in diabetic glomeruli. Figure 3 shows ROS and NO imaging in control and diabetic glomeruli. Generation of ROS and NO in the glomeruli was evaluated by determining the fluorescent intensity of DCF and DAR-4M, respectively. Oxidation of DCFH to the fluorescent compound DCF was applied as a qualitative marker of cellular oxidant stress, because of the multiple pathways that can lead to DCF fluorescence, such as H$_2$O$_2$, ONOO$^-$, and HOCl (29). DCF fluorescence was measured with excitation at 490 nm and emission at 530 nm, and thus it could be distinguished from DAR-4M fluorescence (excitation at 560 nm and emission at 575 nm). To activate NOS, L-Arg and CaCl$_2$ were added to the perfusion solution. In control kidneys, ROS production was rarely observed, but abundant NO production was noticed in the capillary wall cells in the glomeruli. In contrast, in diabetic kidneys, ROS levels were increased and NO levels were decreased in the glomeruli.

![Image](http://ajpregnal.physiology.org/)

Fig. 1. Superoxide production and accumulation of oxidative stress in diabetic glomeruli. Representative images of dihydroethidium (DHE; top) staining, malondialdehyde (MDA; middle), and 8-hydroxydeoxy guanosine (8-OHdG; bottom) immunohistochemical staining of control (Control) and diabetic rats (DM) are shown. The fluorescent intensity of DHE indicates superoxide production. Images were collected with a confocal laser-scanning microscopy. MDA and 8-OHdG are markers of lipidoxidation and DNA oxidation, respectively. Bars = 50 μm.

0.05, Fig. 2A). The NADH-induced O$_2^-$ overproduction was significantly inhibited by coincubation with NAD(P)H oxidase inhibitor DPI (1.19 ± 0.14-fold, P < 0.05, Fig. 2B). Incubation of control glomeruli with BH$_4$ or L-Arg reduced O$_2^-$ formation compared with incubation without the substrate (0.70 ± 0.12- and 0.77 ± 0.06-fold, respectively, P < 0.05, Fig. 2C). In contrast, although BH$_4$ reduced O$_2^-$ formation (1.18 ± 0.88-fold), L-Arg increased O$_2^-$ formation by diabetic glomeruli relative to the control (1.57 ± 0.16-fold, P < 0.05, Fig. 2A and C). Moreover, L-NAME, a NOS-inhibitor, decreased O$_2^-$ production by diabetic glomeruli (1.17 ± 0.09-fold, P < 0.05, Fig. 2A). The amount of O$_2^-$ production assessed by the ferricytochrome c reduction assay was similar to that determined by the DHE assay in both the control and diabetic rats. NADH or L-Arg coincubation increased O$_2^-$ production in diabetic glomeruli (2.62 ± 0.26- or 2.15 ± 0.11-fold, respectively, Fig. 2A), whereas coincubation with L-NAME reduced production (1.30 ± 0.11-fold, Fig. 2A). Taken together, these results suggest that NAD(P)H-dependent oxidase and NOS, but not XO or mitochondrial oxidase, are the predominant sources of superoxide production in the glomeruli of diabetic rats.

![Image](http://ajpregnal.physiology.org/)

Fig. 2. Production pathway of superoxide in diabetic glomeruli. A: different pathways are responsible for superoxide production in diabetic glomeruli. Glomeruli of control and DM rats were separately incubated with substrates of different enzymes and superoxide productions were measured by DHE and ferricytochrome c reduction assays. Mito, mitochondria; Xan, xanthine; L-Arg, L-arginine; L-NAME, N$^\omega$-nitro-L-arginine methyl ester. Values are means ± SE of 7 rats/group and are shown relative to the control. *P < 0.05 vs. Control. #P < 0.05 vs. DM(–). B: effects of NADH oxidase inhibitor on superoxide production in glomeruli assessed by DHE assay. Data depict the effects of diphenylene iodonium (DPI) on NADH-induced superoxide production in control (open bars) and DM (filled bars). Data are expressed relative to control with NADH(–) plus DPI(–). Values are means ± SE of 7 rats/group. *P < 0.05 vs. NADH(–) plus DPI(–). #P < 0.05 vs. NADH(+) plus DPI(–). C: effects of nitric oxide synthase (NOS) cofactor on superoxide production in diabetic glomeruli assessed by DHE assay. Data show effects of tetrahydrobiopterin (BH$_4$) on L-Arg-induced superoxide production in control (open bars) and DM (filled bars). Data are expressed relative to the control with L-Arg(–) plus BH$_4$(–). Values are means ± SE of 7 rats/group. *P < 0.05 vs. L-Arg(–) plus BH$_4$(–). #P < 0.05 vs. L-Arg(+).
Expression of NAD(P)H oxidase component in diabetic glomeruli. The mRNA expression levels of NADPH oxidase components were determined by real-time PCR (Fig. 4A). We investigated the membrane-associated p22phox and cytosolic components of p47phox and p67phox. mRNA expression levels of all examined components were higher in the diabetic glomeruli compared with control glomeruli (1.55 ± 0.37-, 1.25 ± 0.07-, and 1.39 ± 0.41-fold, respectively, P < 0.05).

Nitrotyrosine formation in diabetic glomeruli. Diabetes-induced alterations in glomerular protein tyrosine nitration are summarized in Fig. 5. Diabetic rats showed positive staining for nitrotyrosine in glomerular cells and tubules (Fig. 5A), whereas there was little or no staining in the control glomeruli or tubules. Western blot analysis revealed ~60-kDa nitroty-
BH₄ is an essential cofactor of NOS, and its deficiency decreases NO bioactivity. The plasma levels of BH₄, assessed by HPLC, in diabetic rats (4.9 ± 1.3 μg/ml) to levels similar to the control rats. To investigate whether BH₄ administration improves the eNOS dysfunction, we examined O₂⁻ production in isolated diabetic glomeruli in rats treated with BH₄. The addition of BH₄ reversed L-Arg-induced ROS increase in diabetic glomeruli (Fig. 2C). Moreover, diabetic rats treated with BH₄ showed normalized ROS and NO production in the glomeruli (Fig. 3). In this method, to activate NOS, L-Arg and CaCl₂ were added to the perfusion solution. Hence, the NO and ROS detected by this method reflect the statement of in situ NOS coupling. These data indicate that BH₄ normalized eNOS function to produce NO instead of ROS in diabetic glomeruli. The immunoreactivity for total eNOS protein in diabetic glomeruli was not significantly affected by BH₄ (DM, 1.83 ± 0.10-fold; DM + BH₄, 1.71 ± 0.15-fold, Fig. 6A). However, BH₄ treatment restored the low eNOS dimer/monomer ratio in the diabetic glomeruli (DM, 0.46 ± 0.11; DM + BH₄, 1.77 ± 0.18, P < 0.05, Fig. 6B). These results suggest that BH₄ administration improves eNOS dysfunction in diabetic glomeruli.

**DISCUSSION**

The major findings of the present study were the following. 1) There was excessive oxidative stress in glomeruli of diabetic rats. 2) The sources of ROS are NAD(P)H oxidase and uncoupled NOS, resulting in a reduction of glomerular expression of NO despite increased expression of eNOS; the uncoupled NOS was further confirmed by the decrease in the eNOS dimer form and by in vivo NO imaging. 3) In diabetic rats, the plasma level of BH₄ was reduced compared with that of normal rats, and administration of BH₄ normalized NO bioavailability in diabetic glomeruli in parallel with an increase in the eNOS dimer form.

The involvement of oxidative stress in diabetic nephropathy was demonstrated by the presence of lipid peroxidation products and 8-OHdG in the glomeruli of STZ-induced diabetic rats. These data are consistent with those of previous studies (9, 14, 40). Moreover, we also provided evidence for the presence of excessive oxidative stress in glomeruli of diabetic rats by using DHE, a fluorescent dye that has been used to estimate the formation of O₂⁻ (38). These results are in agreement with the previous report showing strong DCF immunofluorescent staining for O₂⁻ in glomeruli of diabetic rats but not in those of diabetic glomeruli. Control glomeruli showed a faint band of nitrotyrosine-positive protein, whereas the band intensity of diabetic glomeruli was 2.10 ± 0.18-fold stronger (P < 0.05). Blots run in the absence of anti-nitrotyrosine antibody confirmed that no immunoreactive products were detected by the secondary antibody alone (data not shown).

**Expression of NOS in diabetic glomeruli.** NOS mRNA expression in glomeruli was examined by real-time PCR (Fig. 4B). There was no difference in nNOS mRNA expression between control and diabetic glomeruli. No iNOS mRNA expression was detected in either control and diabetic glomeruli. On the other hand, expression of eNOS mRNA was higher in diabetic glomeruli than in control glomeruli (1.30 ± 0.09-fold, P < 0.05). We also evaluated the expression of eNOS protein in isolated glomeruli. In conventional Western blot analysis (Fig. 6A), the immunoreactivity of total eNOS protein was significantly higher in diabetic glomeruli (1.83 ± 0.10-fold) than the control (P < 0.05). On the other hand, immunoblot analysis after low-temperature SDS-PAGE (Fig. 6B) showed a lower immunoreactivity for the eNOS dimer and higher immunoreactivity for the eNOS monomer in diabetic glomeruli. The expression ratio of the dimer to monomer was significantly lower in glomeruli of diabetic rats than those of control rats (2.21 ± 0.11 vs. 0.46 ± 0.11, P < 0.05).

**Effects of BH₄ on eNOS dysfunction in diabetic glomeruli.** BH₄ is an essential cofactor of NOS, and its deficiency decreases NO bioactivity. The plasma levels of BH₄, assessed by HPLC, in diabetic rats (1.8 ± 0.3 μg/ml) were significantly lower than in control rats (4.3 ± 0.2 μg/ml, P < 0.05, Fig. 7). BH₄ supplementation significantly increased plasma levels of BH₄ in diabetic rats (4.9 ± 1.3 μg/ml) to levels similar to the control rats. To investigate whether BH₄ administration improves the eNOS dysfunction, we examined O₂⁻ production in isolated diabetic glomeruli in rats treated with BH₄. The addition of BH₄ reversed L-Arg-induced ROS increase in diabetic glomeruli (Fig. 2C). Moreover, diabetic rats treated with BH₄ showed normalized ROS and NO production in the glomeruli (Fig. 3). In this method, to activate NOS, L-Arg and CaCl₂ were added to the perfusion solution. Hence, the NO and ROS detected by this method reflect the statement of in situ NOS coupling. These data indicate that BH₄ normalized eNOS function to produce NO instead of ROS in diabetic glomeruli. The immunoreactivity for total eNOS protein in diabetic glomeruli was not significantly affected by BH₄ (DM, 1.83 ± 0.10-fold; DM + BH₄, 1.71 ± 0.15-fold, Fig. 6A). However, BH₄ treatment restored the low eNOS dimer/monomer ratio in the diabetic glomeruli (DM, 0.46 ± 0.11; DM + BH₄, 1.77 ± 0.18, P < 0.05, Fig. 6B). These results suggest that BH₄ administration improves eNOS dysfunction in diabetic glomeruli.
nondiabetic control rats (18). An excessive amount of ROS production occurs before structural changes such as mesangial matrix deposition. Therefore, ROS production in the glomeruli is considered an upstream event in the pathophysiology of diabetic nephropathy.

The present study detected $O_2^-$ production in glomeruli in the presence of different substrates for $O_2^-$-producing enzymes by the use of fluorescence spectrometry, which is based on the fluorogenic oxidation of DHE to Eth as a measure of $O_2^-$ (38, 45), and the ferricytochrome $c$ reduction assay. We found that the activity of NAD(P)H oxidase in the diabetic glomeruli was much higher than in the control glomeruli. We also showed that formation of excess $O_2^-$ is increased by the addition of the NOS substrate L-Arg and is decreased by NOS-inhibition with L-NAME in glomeruli of diabetic rats. Under normal conditions, NO is synthesized from L-Arg through a five-electron oxidation step in the presence of sufficient cofactor (BH$_4$) (30). BH$_4$-deficient isolated NOS, when activated, cannot catalyze the 5-electron oxidation of L-arginine into NO. However, the same BH$_4$-deficient NOS can still receive electrons from NADPH and store them in its bound flavins and then can donate them one at a time to its other substrate, O$_2$, resulting in a one-electron reduction to form superoxide anion (31, 35). Under this condition, addition of the NOS substrate L-Arg may generate O$_2^-$ instead of NO. In contrast, NOS inhibition by L-NAME may decrease O$_2^-$ production. Mitochondrial respiratory chain enzymes and XO in the diabetic glomeruli expressed relatively similar activity to produce $O_2^-$ as that in the control glomeruli. These results suggest that under diabetic conditions, $O_2^-$ in the glomeruli may be produced primarily by NAD(P)H oxidase and the NOS system. In peripheral circulation and tissue cells, NAD(P)H oxidase and eNOS dysfunction are suspected as the major enzymes responsible for $O_2^-$ production, which may contribute to endothelial dysfunction and remodeling of the vascular wall in hypertension (20), atherosclerosis (27), and diabetes (8, 13). Our results confirmed the existence of the same mechanisms of NAD(P)H oxidase and uncoupled NOS in the generation of $O_2^-$ in diabetic glomerular injury.

We confirmed glomerular NOS dysfunction and ROS production by the NO fluorescent indicators DCFH and DAR, respectively. A common feature of endothelial dysfunction is a reduction in the amount of bioavailable NO produced in the glomeruli. However, specification of the site of NO action is difficult using conventional NO detection methods such as electron spin resonance (2). DAR, a fluorescence probe based on rhodamine chromophore that can detect intracellular NO, has higher photostability, longer excitation wavelength, and can be applied over a wide pH range. Hence, this compound is useful for bioimaging NO in samples that have strong autofluorescence in the case of 490-nm excitation, or with intracellular pH $<$6.0 (17). The use of DCFH plus DAR in our technique facilitated simultaneous detection of ROS and NO production. The ROS fluorescence indicator DCFH could be visualized because the wavelength was different from that of DAR, the NO fluorescence indicator. NOS uncoupling refers to a process in which the enzyme generates $O_2^-$ rather than NO when its cofactor BH$_4$ is limited. This phenomenon has been implemented in vitro studies (32), although direct detection of a NOS uncoupling site in the kidney has not been demonstrated previously. Administration of BH$_4$ improved NOS function, as determined by increased glomerular NO production and decreased glomerular ROS production. These results also suggest the existence of uncoupled NOS in the diabetic glomeruli.

Another possible mechanism of reduced bioactivity of glomerular NO is the presence of an excess amount of ROS in the diabetic glomeruli. We confirmed that both the expression and activity of NAD(P)H oxidase were increased in the diabetic rats. Moreover, we demonstrated the accumulation of nitrotyrosine, an indicator of peroxynitrite, indicating that a significant amount of $O_2^-$ is quenched by its interaction with NO in the diabetic glomeruli. BH$_4$ serves as a critical cofactor for eNOS. A deficiency of BH$_4$ results in eNOS uncoupling, which is associated with increased $O_2^-$ and decreased NO production. We found that the plasma level of BH$_4$ in the diabetic rats was lower than that in the control rats. The underlying reason for the decreased BH$_4$ bioavailability in diabetic nephropathy has not been fully elucidated but may be related to impaired synthesis or increased catabolism for oxidation to BH$_2$. Peroxynitrite was shown to uncouple eNOS by oxidation of BH$_4$ (19). Oxidization of NOS cofactor BH$_4$ leads to eNOS uncoupling (monomerization) and results in increased ROS and reduced NO production by the enzyme. Therefore, the initial oxidative loss of BH$_4$ in response to increased ROS production by NAD(P)H oxidases seems to result in amplification of oxidative stress due to the resulting loss of NO production and increased NOS-dependent $O_2^-$ generation (20).

Our results showed the beneficial effects of BH$_4$ administration in diabetic nephropathy. Short-term BH$_4$ treatment reversed the plasma level of BH$_4$ and eNOS dimer formation to a normal state, which was associated with increased NO and decreased ROS production in the glomeruli. In this regard, Alp et al. (1) demonstrated in transgenic mice with endothelial-targeted overexpression of guanosine triphosphate-cyclohydrolase I, the rate-limiting enzyme in BH$_4$ synthesis, the restoration of NO-mediated endothelial function under hyperglycemic conditions. Importantly, several clinical studies have demonstrated the beneficial effects of BH$_4$ administration on endothelial function in patients with cardiovascular risk factors, such as hypercholesterolemia, smoking, hypertension, diabetes, and coronary artery disease (12, 36). Supplementation of vitamin C also has been shown to prevent oxidation of BH$_4$ (5). Recently, it was demonstrated that folate is also capable of BH$_4$-dependent potentiation of eNOS function in vitro and improvement of NO-mediated endothelial function in vivo (43). Interestingly, recent studies showed that statins, in addition to augmenting eNOS expression, may also potentiate GTP cyclohydrolase I gene expression and BH$_4$ synthesis, thereby improving eNOS function (11). Thus the mechanisms that modulate BH$_4$ status in human vascular disease, including diabetic nephropathy, represent promising targets for therapeutic interventions. We showed the effect of short-term BH$_4$ treatment in correcting NOS uncoupling. However, with this short-term BH$_4$ treatment, staining for MDA and 8-OHdG, which indicate accumulation of oxidative stress, was not different in glomeruli of diabetic rats and those of BH$_4$-treated diabetic rats (data not shown). Therefore, it would be useful to investigate whether reversal of glomerular oxidative stress by long-term BH$_4$ treatment prevents or reduces glomerular injury of diabetes.

Based on the present findings, we propose the following working model for the effects of hyperglycemia on the formation of $O_2^-$ and decreased bioavailability of NO in diabetic nephropathy.
ROS and NO Imbalance in Diabetic Nephropathy

In conclusion, we have demonstrated in the present study that NAD(P)H oxidase and uncoupling of eNOS contribute to diabetic glomerular ROS production, mediated by the loss of BH4 availability. These mechanisms are potential key targets for therapeutic interventions in diabetic nephropathy. Further studies are needed to clarify the effectiveness of BH4 treatment on the progression of diabetic nephropathy.

REFERENCES


