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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Satoh, Minoru, Sohachi Fujimoto, Yoshisuke Haruna, Sayaka Arakawa, Hideyuki Horike, Norio Komai, Tamaki Sasaki, Katsuhiko Tsujioka, Hirofumi Makino, and Naoki Kashihara. NAD(P)H oxidase and uncoupled nitric oxide synthase are major sources of glomerular superoxide in rats with experimental diabetic nephropathy. Am J Physiol Renal Physiol 288: F1144–F1152, 2005. First published February 1, 2005; doi:10.1152/ajprenal.00221.2004.—Increased production of reactive oxygen species (ROS) in diabetes may be a common pathway linking diverse pathogenic mechanisms of diabetic vascular complications, including nephropathy. Assessment of the oxidative stress production pathway is therefore important for the prediction and prevention of diabetic complications. However, ROS production mechanisms remain unclear in diabetic glomeruli. To identify the source and determine the mechanisms of ROS production in the diabetic kidney, diabetes was induced with streptozotocin in rats. After 6 wk, glomerular ROS production had increased in the streptozotocin rat kidney, as assessed by dihydrothreotide-derived chemiluminescence. ROS production was increased by the addition of NADH or l-arginine and was partially reduced by the addition of diphenylene iodonium or N3-nitro-l-arginine methyl ester, identifying NAD(P)H oxidase and nitric oxide (NO) synthase (NOS) as ROS sources. The mRNA and protein expression of endothelial NOS (eNOS), as measured by real-time RT-PCR and Western blotting, increased significantly (mRNA level, 1.3-fold; protein level, 1.8-fold). However, the dimeric form of eNOS was decreased in diabetic glomeruli, as measured by low-temperature SDS-PAGE. Production of renal ROS and NO by uncoupled NOS was imaged by confocal laser microscopy after renal perfusion of 2,7’-dichlorofluorescin diacetate (a ROS marker) and diaminohydroamine-4M AM (a NO marker) with l-arginine. Accelerated ROS production and diminished bioavailable NO caused by NOS uncoupling were noted in the diabetic kidney. Administration of tetrahydrobiopterin (BH4), a cofactor for eNOS, reversed the decreased dimeric form of eNOS and glomerular NO production. Our results indicate that NAD(P)H oxidase and uncoupling of eNOS contribute to glomerular ROS production, mediated by the loss of BH4 availability. These mechanisms are potential key targets for therapeutic interventions.

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 increased 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 concentration. 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 BH$_4$ (Sigma-Aldrich Japan) was administered by gavage to a subgroup of diabetic rats for 2 days before anesthesia with pentobarbital sodium ($n=12$). BH$_4$ was compressed into rodent chow pellets without the addition of water or heat to prevent oxidation of the compound. The concentration of BH$_4$ 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 try. 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 $O_2^-$ production assay, protein isolation, and RNA isolation.

Determination of $O_2^-$ 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 coinubcation with 10 mmol/l tiron (Sigma-Aldrich Japan). The fluorescence at an excitation wavelength of 480 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 $O_2^-$ 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 for 5 min at 4°C, the supernatant containing the membrane and cytosolic components, termed the homogenate, was separated. Fluorescence spectrometry of tissue $O_2^-$ production was performed by using a modification of methods described by Zou et al. (45). Briefly, the fluorescent oxidation of DHE to Eth, which requires oxygen, was measured (20 mg/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 610 nm by using a fluorescence microplate reader (FLUOSTAR OPTIMA, Meritex Bio Science, Tokyo, Japan). Xanthine (0.1 mmol/l) was used as a substrate for XO. Succinate (5 mmol/l) was used as a substrate for intramitochondrial $O_2^-$ 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 $O_2^-$ was examined by the addition of NADH (0.1 mmol/l) as a substrate in the reaction mixture. L-Arginine (L-Arg; 1 mmol/l) was used as a substrate for NOS. $N^G$-nitro-L-arginine methyl ester (L-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 $O_2^-$ in glomeruli, we examined the effects of the NAD(P)H oxidase inhibitor diphenylene iodonium chloride (DPI; 0.1 mmol/l) or BH$_4$ (0.01 mmol/l) on Eth-DNA fluorescence intensity. The enzyme activities of different pathways are expressed relative to the control.

Ferricytochrome c reduction assay for measurement of $O_2^-$ production in isolated glomeruli. The assay for $O_2^-$ was carried out by measuring superoxide dismutase-inhibitable (SOD-inhibitable) reduction of ferricytochrome 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 CaCl$_2$, 1.1 MgSO$_4$, 1.2 KH$_2$PO$_4$, 5.6 glucose, and 25 NaHCO$_3$) containing the corresponding substrate for XO (0.1 mmol/l), ferricytochrome c (5 mmol/l succinate and 0.05 mmol/l anti-myco- cytin); NADH/NADPH oxidase (0.1 mmol/l NADH; NOS (1 mmol/l L-Arg); or NOS inhibitor (1 mmol/l L-NAME) in a 24-well microtiter plate, under a 21% O$_2$–5% CO$_2$ gas mixture at 37°C for 4 h. After incubation, the supernatant was collected and ferricytochrome c was added to the supernatant to a final concentration of 70 mmol/l in the presence or absence of SOD (100 U/ml). Reduction of ferricytochrome c in the supernatant was monitored for 30 min at an absorbance of 550 nm using a spectrophotometer (FLUOSTar OPTIMA, Moritex Bio Science). The rate of $O_2^-$ 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 in 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 diamonohydramine-4M AM (DAR-4M AM; Daiichi Pure Chemicals, Tokyo, Japan); 0.05 mmol/l dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR); 0.1 mmol/l L-Arg; and 2 mmol/l CaCl$_2$ 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 and the tissues were cut into 1-mm-thick sections and envel- ooped 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 quantita- tive PCR was performed using the ABI Prism 7700 sequence-detect- ing 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 correspond to the control.

NOS and NO IMBALANCE IN DIABETIC NEPHROPATHY F1145

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**Table 1. Sequences of primers and probes used in this study**

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**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 O$_2^-$ 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 O$_2^-$ (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 O$_2^-$ 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 O$_2^-$ compared with incubation without the substrate. Superoxide production in the isolated glomeruli from diabetic rats, as measured by the ferricicytochrome 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 O$_2^-$ production compared with incubation without a substrate (1.71 ± 0.17-fold, P < 0.05, Fig. 2A).
ROS and NO Imbalance in Diabetic Nephropathy

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 lipid oxidation and DNA oxidation, respectively. Bars = 50 μm.

0.05, Fig. 2A). The NADH-induced O$_2^-$ overproduction was significantly inhibited by coinubcation 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 ± 0.08-fold), L-Arg increased O$_2^-$ formation by diabetic glomeruli relative to the control (1.57 ± 0.16-fold, P < 0.05, Fig. 2, A 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 coinubcation increased O$_2^-$ production in diabetic glomeruli (2.62 ± 0.26- or 2.15 ± 0.11-fold, respectively, Fig. 2A), whereas coinubcation 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.

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.

- **Fig. 3** shows ROS and NO imaging in control and diabetic glomeruli.

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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-

Fig. 3. In situ detection of nitrate oxide (NO) and reactive oxygen species (ROS). Representative images of ROS (top) and NO (bottom) in renal cortex of control, DM rats, and DM rats treated with tetrahydrobiopterin (DM + BH4) are shown. Generation of ROS and NO was evaluated by the fluorescent intensity of dichlorofluorescein (DCF) and diaminorhodamine-4M (DAR-4M), respectively; n = 5/group. Bars = 50 μm.

Fig. 4. mRNA expression in isolated glomeruli detected by real-time PCR analysis. A: components of NADPH oxidase subunits, p22phox, p47phox, and p67phox, were checked in glomeruli of control and DM rats. B: 3 NOS isoforms, endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS), were examined in the glomeruli of control and DM rats. No iNOS mRNA expression was detected in either the control and DM glomeruli. Values are means ± SE of 7 rats/group and are expressed relative to the control. *P < 0.05 vs. control.

Fig. 5. Nitrotyrosine formation in glomeruli. A: representative immunohistochemical localization of nitrotyrosine in the renal cortex of control and DM rats. Bars = 50 μm. B: representative Western blot analysis of nitrotyrosine abundance in isolated glomeruli of control and DM rats. Densitometric quantification of the corresponding bands is performed using NIH Image software. Values are means ± SE and are expressed relative to the control. *P < 0.05 vs. control.
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) 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.

**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 ROS 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. 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.

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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 \( \text{O}_2^- \) production in glomeruli in the presence of different substrates for \( \text{O}_2^- \)-producing enzymes by the use of fluorescence spectrometry, which is based on the fluorogenic oxidation of DHE to Eth as a measure of \( \text{O}_2^- \) (38, 45), and the ferricytochrome \( \text{c} \) 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 \( \text{O}_2^- \) is increased by the addition of the NOS substrate \( \text{l-Arg} \) and is decreased by NOS-inhibition with \( \text{l-NAME} \) in glomeruli of diabetic rats. Under normal conditions, NO is synthesized from \( \text{l-Arg} \) through a five-electron oxidation step in the presence of sufficient cofactor (BH4) (30).

BH4-deficient isolated NOS, when activated, cannot catalyze the 5-electron oxidation of \( \text{l-arginine} \) into NO. However, the same BH4-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, \( \text{O}_2^- \), resulting in a one-electron reduction to form superoxide anion (31, 35). Under this condition, addition of the NOS substrate \( \text{l-Arg} \) may generate \( \text{O}_2^- \) instead of NO. In contrast, NOS inhibition by \( \text{l-NAME} \) may decrease \( \text{O}_2^- \) production. Mitochondrial respiratory chain enzymes and XO in the diabetic glomeruli expressed relatively similar activity to produce \( \text{O}_2^- \) as that in the control glomeruli. These results suggest that under diabetic conditions, \( \text{O}_2^- \) 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 \( \text{O}_2^- \) 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 \( \text{O}_2^- \) 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 glomerulus. 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 \( \text{O}_2^- \) rather than NO when its cofactor BH4 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 BH4 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 \( \text{O}_2^- \) is quenched by its interaction with NO in the diabetic glomeruli. BH4 serves as a critical cofactor for eNOS. A deficiency of BH4 results in eNOS uncoupling, which is associated with increased \( \text{O}_2^- \) and decreased NO production. We found that the plasma level of BH4 in the diabetic rats was lower than that in the control rats. The underlying reason for the decreased BH4 bioavailability in diabetic nephropathy has not been fully elucidated but may be related to impaired synthesis or increased catabolism for oxidation to BH2. Peroxynitrite was shown to uncouple eNOS by oxidation of BH4 (19). Oxidation of NOS cofactor BH4 leads to eNOS uncoupling (monomerization) and results in increased ROS and reduced NO production by the enzyme. Therefore, the initial oxidative loss of BH4 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 \( \text{O}_2^- \) generation (20).

Our results showed the beneficial effects of BH4 administration in diabetic nephropathy. Short-term BH4 treatment reversed the plasma level of BH4 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 BH4 synthesis, the restoration of NO-mediated endothelial function under hyperglycemic conditions. Importantly, several clinical studies have demonstrated the beneficial effects of BH4 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 BH4 (5). Recently, it was demonstrated that folate is also capable of BH4-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 BH4 synthesis, thereby improving eNOS function (11). Thus the mechanisms that modulate BH4 status in human vascular disease, including diabetic nephropathy, represent promising targets for therapeutic interventions. We showed the effect of short-term BH4 treatment in correcting NOS uncoupling. However, with this short-term BH4 treatment, staining for MDA and 8-OHdG, which indicate accumulation of oxidative stress, was not different in glomeruli of diabetic rats and those of BH4-treated diabetic rats (data not shown). Therefore, it would be useful to investigate whether reversal of glomerular oxidative stress by long-term BH4 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 \( \text{O}_2^- \) and decreased bioavailability of NO in diabetic...
glomeruli. First, high glucose levels increase the formation of O$_2^-$ through NAD(P)H oxidase activation. NO produced by NOS and O$_2^-$ combine to form peroxynitrite anion. Oxidation of BH$_4$ by ROS such as peroxynitrite results in the formation of BH$_2$, which inactivates eNOS cofactor function, suggesting that reduction in availability of BH$_4$ uncouples NOS, leading to decreases in bioavailable NO and further increases in formation of O$_2^-$ in diabetic glomeruli. The initial oxidative loss of BH$_4$ by ROS production via NAD(P)H oxidases seems to result in amplification of oxidative stress (20). This paradigm illustrates how small changes in ROS production may be amplified and modulated through interactions between different oxidase systems.

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 BH$_4$ availability. These mechanisms are potential key targets for therapeutic interventions in diabetic nephropathy. Further studies are needed to clarify the effectiveness of BH$_4$ treatment on the progression of diabetic nephropathy.

REFERENCES


