Treatment of diabetic rats with a peroxynitrite decomposition catalyst prevents induction of renal COX-2

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Chen YJ, Santos M, Quilley J. Treatment of diabetic rats with a peroxynitrite decomposition catalyst prevents induction of renal COX-2. Am J Physiol Heart Circ Physiol 300: H1125–H1132, 2011. First published January 14, 2011; doi:10.1152/ajpheart.00768.2010.—Cyclooxygenase (COX)-2 expression is increased in the kidney of rats made diabetic with streptozotocin and associated with enhanced release of prostaglandins stimulated by arachidonic acid (AA). Treatment of diabetic rats with nitro-L-arginine methyl ester (L-NAME) to inhibit nitric oxide synthase or with tempol to reduce superoxide prevented these changes, suggesting the possibility that peroxynitrite (ONOO) may be the stimulus for the induction of renal COX-2 in diabetes. Consequently, we tested the effects of an ONOO decomposition catalyst, 5,10,15,20-tetrakis(N-methyl-4-pyridyl)porphyrinato iron(III) (FeTMPyP), which was administered for 3–4 wk after the induction of diabetes. FeTMPyP treatment normalized the twofold increase in the expression of nitrotyrosine, a marker for ONOO formation, in the diabetic rat and prevented the increase in renal COX-2 expression without modifying the two- to threefold increases in renal release of prostaglandins PGE₂ and 6-ketoPGF₁α in response to AA. FeTMPyP treatment of diabetic rats reduced the elevated creatinine clearance and urinary excretion of TNF-α and transforming growth factor (TGF)-β, suggesting a renoprotective effect. Double immunostaining of renal sections and immunoprecipitation of COX-2 and nitrotyrosine suggested nitrization of COX-2 in diabetic rats. In cultured human umbilical vein endothelial cells (HUVECs) exposed to elevated glucose (450 mg/dl) or ONOO derived from 3-morpholinosydnonimine (SIN-1), expression of COX-2 was increased and was prevented when endothelial cells were treated with FeTMPyP. These results indicate that elevated glucose increases the formation of ONOO, which contributes to the induction of renal COX-2 in the diabetic rat.

There have been numerous reports of increased renal cyclooxygenase (COX) activity and prostaglandin production in diabetes (15, 34), which has been linked to hyperfiltration (13, 25) that precedes renal structural changes and the development of nephropathy (17, 26). Following the discovery of multiple COX isoforms, it was shown that experimental diabetes results in the upregulation of renal COX-2 (22, 30), which has been linked to elevated glomerular filtration rate (GFR) using inhibitors of COX-2 (22). Moreover, inhibition of COX-2 has been shown to attenuate the development of glomerulosclerosis in uninephrectomized diabetic rats (21) and to reduce the appearance of markers of renal damage in a hypertensive diabetic rat model (8).

We have confirmed (30) the increase in renal COX-2 in the diabetic rat and shown an association with enhanced prostaglandin release from the perfused kidney challenged with arachidonic acid (AA), which serves as an index of COX activity but does not distinguish the COX isoforms. Nonetheless, our studies have shown (5, 7, 23) that interventions that prevent the upregulation of renal COX-2 in the diabetic rat are associated with a reduction of the exaggerated renal release of prostaglandins in response to AA, suggesting that increased COX-2 expression/activity contributes to the enhanced prostaglandin production in the diabetic rat.

The stimulus for the induction of renal COX-2 remains to be identified. We investigated the role of oxidative stress based on several observations: first, elevated glucose was shown to increase superoxide generation in a variety of cell types (3, 29); second, reactive oxygen species such as superoxide have been shown to induce COX-2 (20); and third, diabetes is considered a condition of oxidative stress (1). Our study in which we treated diabetic rats with tempol to prevent the upregulation of COX-2 (23) was the first that linked hyperglycemia, oxidative stress, and the induction of COX-2 in vivo. These results do not mean that superoxide is the stimulus for the induction of COX-2 in diabetes, only that it plays a central role. Thus renal nitric oxide (NO) formation is also increased in diabetes (9), and both NO and its product upon interaction with superoxide, peroxynitrite (ONOO), have been reported to lead to induction of COX-2 (12). We reported that treatment of diabetic rats with nitro-L-arginine methyl ester (L-NAME) to inhibit nitric oxide synthase also reduced the expression of COX-2 and the enhanced release of AA-stimulated prostaglandins from the perfused kidney (5). The results with L-NAME, coupled with those of tempol, suggested a potential role of ONOO in the induction of COX-2, particularly as ONOO formation is increased in diabetes, with nitrotyrosine levels as an index (4, 36). Furthermore, we have shown that, in addition to increased renal cortical nitrotyrosine expression (7), renal microvessels from diabetic rats exhibit elevated levels of nitrotyrosine (6).

The experiments reported here were conducted to address the potential role of ONOO in the induction of COX-2 by using a decomposition catalyst, 5,10,15,20-tetrakis(N-methyl-4’-pyridyl)porphyrinato iron(III) (FeTMPyP), that catalyzes the formation of nitrate from ONOO (11, 14, 33). Diabetic rats were treated with FeTMPyP to reduce levels of ONOO, and the effects on renal COX-2 expression and the release of prostaglandins from the isolated, perfused kidney challenged with AA were determined. As diabetes is associated with increases in TNF-α and transforming growth factor (TGF)-β, which have been linked to oxidative stress and implicated in the renal complications of this condition (10, 18, 37), we measured the levels of these cytokines in the urine. Second, we used cultured human umbilical vein endothelial cells (HUVECs) to address the effects of exposure to elevated glucose or ONOO on the induction of COX-2 and the influence of FeTMPyP. The use of endothelial cells was justified by showing that renal microves-

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sels from diabetic rats express higher levels of nitrotyrosine than microvessels from age-matched control rats (6).

METHODS

The studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

Male Wistar rats, 150–175 g, were used in these studies. Rats were made diabetic with streptozotocin (STZ; 70 mg/kg iv), whereas control rats were given an equivalent volume of the vehicle, citrate buffer, pH 4.5. Forty-eight hours later, half of the diabetic rats were treated daily with FeTMPyP (20 mg·kg⁻¹·day⁻¹ ip). This dose of FeTMPyP was based on other studies; for example, Nagle et al. (28) treated mice with 25 mg·kg⁻¹·day⁻¹ for 2 wk. Three weeks later, a 24-h urine collection was obtained by placing rats individually in metabolic cages. Urines were analyzed for the cytokines TNF-α and TGF-β, which are increased in diabetes and may be markers for renal changes that precede the development of nephropathy.

Isolated, perfused kidney. After 3–4 wk of treatment, rats were anesthetized with pentobarbital (65 mg/kg ip) and the kidney prepared for perfusion. Briefly, after a midline laparotomy, the right renal artery was cannulated via the mesenteric artery to avoid interruption of blood flow and the kidney was removed from the rat and perfused with oxygenated Krebs-Henseleit buffer at 37°C at constant flow, which was adjusted to obtain a perfusion pressure of 60–90 mmHg. Glucose levels in tail vein blood were determined with a glucometer (Ames). The left kidney was removed for determination of renal cortical nitrotyrosine and COX-2 expression. After at least 10-min perfusion and once a stable perfusion pressure was obtained, vasoconstrictor responses to 1 and 3 g dose of AA to determine prostaglandine were determined. Perfusate samples were collected for 1 min before and 1 min after the 1-μg dose of AA to determine prostaglandin release.

Prostaglandin measurements. One-minute perfusate collections were measured immediately before and after the administration of 1 μg AA for the measurement of PGE₂ and 6-ketoPGF₁α as an index of cyclooxygenase activity. Levels of the two prostanooids in the renal perfusates were determined by enzyme immunoassay with kits obtained from Cayman Chemical. 6-ketoPGF₁α was chosen as an index of conversion of AA by the endothelium, the presumed site of generation of endoperoxides, whereas PGE₂ levels were determined as an index of total renal prostaglandin formation.

In the experiments to measure creatinine clearance, we also determined the effects of FeTMPyP treatment of diabetic rats on the urinary excretion of 6-ketoPGF₁α. Thus 6-ketoPGF₁α excretion is increased in the diabetic rat, and we found that selective inhibition of COX-2 reduced 6-ketoPGF₁α excretion to levels found in control rats, suggesting a link between expression of renal COX-2 and urinary 6-ketoPGF₁α in the diabetic rat.

Urinary TNF-α. TNF-α levels in urine were measured with an ELISA kit according to the manufacturer’s instructions (BD Biosciences, San Diego, CA).

Urinary TGF-β. TGF-β in 24-h urine samples was measured with an ELISA (Promega, Madison, WI) according to the manufacturer’s instructions.

Creatinine assay. Creatinine in urine and plasma samples was measured with the QuantiChrom Creatinine Assay Kit (BioAssay Systems).

Human umbilical vein endothelial cells. In complementary studies, we used HUVECs to determine the effect of elevated glucose to mimic the diabetic condition and ONOO on the expression of COX-2. We felt justified in using endothelial cells as preliminary studies have shown that renal microvessels from diabetic rats exhibit markedly increased expression of COX-2 and there are several reports of increased vascular COX-2 in human and experimental diabetes (16). Endothelial cells (Cambrex) were cultured in six-well plates coated with rat tail type I collagen in endothelial cell growth medium-2 (EGM-2) from the same company until 95% confluent. Cells from passages 2–6 were used. Six hours before pharmacological treatment, EGM-2 was replaced with serum-free DMEM containing 1 g/l glucose. After 6-h starvation, HUVECs were incubated with DMEM-1 g/l glucose and DMEM-4.5 g/l glucose of identical osmolarity in the presence and absence of 50 μM FeTMPyP for 8 h. In other experiments, cells were exposed to vehicle or 3-morpholinosydnonimine (SIN-1, 0.5 mM) in the absence or presence of 50 μM FeTMPyP for 6 h. SIN-1 was used to generate ONOO as it produces NO and superoxide simultaneously. Preliminary experiments showed that addition of authentic ONOO to endothelial cells resulted in increased expression of COX-2.

Western blot. The cortex was homogenized in RIPA buffer, and, after centrifugation at 17,000 g, protein in the supernate was determined with a Bio-Rad assay kit. For COX-2 and nitrotyrosine, 50 μg and 25 μg protein, respectively, were mixed with 5× SDS-PAGE sample buffer (500 mmol/l DTT, 0.2% bromophenol blue, and 50% glycerol) and boiled for 4 min. Proteins were separated on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, and immunoblotted with a rabbit anti-mouse COX-2 polyclonal antibody (1:1,000 dilution; Cayman Chemical) or a rabbit anti-nitrotyrosine antibody (1:5,000 dilution; Cayman Chemical). Membranes were washed with Tris-buffered saline containing Tween 20 and incubated with horseradish peroxidase-conjugated antisera. COX-2 and nitrotyrosine expression was then detected by enhanced chemiluminescence, and values were normalized against the expression of β-actin.

Preparation of cortex for confocal microscopy. Kidneys were perfused via the mesenteric artery with 10 ml of PBS containing heparin (40 μl/ml) followed by 100 ml of 4% paraformaldehyde. After perfusion, the kidneys were removed and postfixed in 4% paraformaldehyde overnight. The kidneys were dehydrated and cut into 8-μm slices with a Leica1900 cryostat (Leica).

The sections were washed with PBS for 15 min and permeabilized and blocked with 0.3% Triton X-100 dissolved in PBS buffer containing 5% BSA and 0.2 mM glycine for 1 h at room temperature. Kidney sections then were incubated with COX-2 antibody (10 μg/ml) and nitrotyrosine antiserum (10 μg/ml; Alpha Diagnostic International) overnight at 4°C. Sections were thoroughly washed with PBS containing 1% BSA and 2.3% sodium chloride, followed by the separate addition of secondary antibodies (anti-mouse and anti-rabbit, 1:500) in 1% BSA dissolved in PBS for 1 h at room temperature.

Immunoprecipitation. To preclar the tissue extract of proteins that can bind nonspecifically to the beads, 25 μl of protein G magnetic beads (New England Biolabs) was added to 200 μg of renal cortical homogenates, which were adjusted to equal volume (200 μl). Subsequently, the supernatant was incubated with 2.5 μg of COX-2 antibody (Santa Cruz Biotechnology) overnight at 4°C. Immune complexes were precipitated by 1-h incubation at 4°C with 25 μl of beads. The beads were washed three times with RIPA buffer and then

![Image of Western blot](http://ajpheart.physiology.org/DownloadedFrom/10.1161.335.on October 14, 2017)
resuspended in 2× SDS-PAGE sample buffer and boiled for 5 min. The supernate was separated on 10% SDS-PAGE gel and probed with a biotinylated nitrotyrosine monoclonal antibody.

Analysis of data. All data are expressed as means ± SE and were compared with an unpaired t-test or ANOVA in which individual points were compared with a modified t-statistic (Newman-Keuls). A value of $P < 0.05$ was considered statistically significant.

RESULTS

Body weights for the untreated control and diabetic rat groups were 372 ± 8 and 247 ± 16 g, respectively, at the time of euthanization. The mean body weight in the treated diabetic group was 228 ± 17 g, which was not different from the untreated group ($n = 7–10$). Blood glucose levels were 499 ± 20 mg/dl in the untreated diabetic group compared with 119 ± 4 mg/dl in the untreated control group. Treatment of diabetic rats with FeTMPyP resulted in lower blood glucose levels of 432 ± 9 mg/dl ($P < 0.01$).

Fig. 2. Increases ($\Delta$) in perfusion pressure (PP) in response to arachidonic acid (AA) and phenylephrine (PE) in isolated, perfused kidneys from control (CON) and diabetic (STZ) rats with and without treatment with FeTMPyP (FeT, 20 mg·kg$^{-1}$·day$^{-1}$) for 3–4 wk. *$P < 0.05$ vs. CON; $n = 7–10$.

Fig. 4. Western blot analysis for nitrotyrosine (3-NT) expression, standardized against $\beta$-actin, in samples of renal cortex obtained from control (citrate) and untreated and FeTMPyP-treated diabetic (STZ) rats ($n = 4$ for each group). FeTMPyP was given for 3–4 wk at a dose of 20 mg·kg$^{-1}$·day$^{-1}$ (STZ+FeT). The space between lanes shows where images that were obtained from the same blot were spliced together. *$P < 0.05$ vs. citrate group.

Fig. 3. Increase in release of 6-ketoPGF$_{1\alpha}$ (A) and PGE$_2$ (B) in response to 1 $\mu$g AA from isolated, perfused kidneys obtained from untreated control (CON) and diabetic (STZ) rats and those treated with the peroxynitrite decomposition catalyst FeTMPyP for 3–4 wk (STZ+F). *$P < 0.05$ vs. respective control; $n = 7–10$.

Fig. 5. Results of Western blot analysis for cyclooxygenase (COX)-2 expression, standardized against $\beta$-actin, in renal cortical samples from control (citrate) and untreated and FeTMPyP-treated diabetic (STZ) rats ($n = 6$ for each group). FeTMPyP was given for 3–4 wk at a dose of 20 mg·kg$^{-1}$·day$^{-1}$ (STZ+FeT). The space between lanes shows where images that were obtained from the same blot were spliced together. *$P < 0.05$ vs. citrate group.
In a subsequent experiment, we measured the effect of FeTMPyP treatment of diabetic rats for 4 wk on creatinine clearance as an index of GFR ($n = 4$–$6$/group). Body weight for the control group was $447 \pm 15$ g compared with $301 \pm 13$ g for the diabetic group and $321 \pm 21$ g for the treated diabetic group. Blood glucose levels were $110 \pm 5$, $423 \pm 18$, and $478 \pm 24$ mg/dl for the control, untreated diabetic, and treated diabetic groups, respectively. Creatinine clearance was increased in the diabetic group compared with age-matched control rats, and the increase was prevented by treatment with FeTMPyP (Fig. 1). As FeTMPyP treatment reduced creatinine clearance in diabetic rats to levels not different from those of nondiabetic rats, it was necessary to examine whether FeTMPyP treatment reduced creatinine clearance in nondiabetic rats. Consequently, in another experiment, we determined the effect of FeTMPyP treatment for 3 wk on creatinine clearance. FeTMPyP treatment was without effect on creatinine clearance in nondiabetic rats, $1.56 \pm 0.09$ ml/min compared with $1.69 \pm 0.13$ ml/min for the untreated group ($n = 5$/group).

Isolated, perfused kidney. Mean perfusion pressures were $69 \pm 1$, $70 \pm 2$, and $71 \pm 4$ mmHg for the control, diabetic, and FeTMPyP-treated diabetic groups, respectively, with perfusate flow rates of $11.3 \pm 0.9$, $12.5 \pm 0.7$, and $12.7 \pm 0.9$ ml/min, respectively ($n = 7$–$10$/group).

In response to AA, vasoconstriction that is mediated by endoperoxides (32) and reflected as increases in perfusion pressure was greatly enhanced in kidneys from diabetic rats (Fig. 2), as we have previously reported (31). For example, $1 \mu$g of AA elevated perfusion pressure by $89 \pm 20$ mmHg in the diabetic rats compared with $13 \pm 7$ mmHg in the nondiabetic group. Treatment of diabetic rats with FeTMPyP was without effect on vasoconstrictor responses to AA. In contrast to the
responses to AA, renal vasoconstrictor responses to 100 ng of phenylephrine were not different between diabetic (22 ± 4 mmHg) and nondiabetic (21 ± 4 mmHg) rats. However, treatment of diabetic rats with FeTMPyP increased responses to phenylephrine from 22 ± 4 to 56 ± 6 mmHg (P < 0.001) (Fig. 2).

As we have observed before, increased prostaglandin release was associated with enhanced vasoconstrictor responses to AA in diabetic rat kidneys. After administration of 1 μg of AA to the isolated, perfused kidney, the increase in PGE2 release was more than threefold that of the control rat kidney, whereas the release of 6-ketoPGF1α was almost twofold that of the control and neither was affected by treatment with FeTMPyP (Fig. 3, where n = 7–10/group).

Nitrotyrosine and COX-2 expression. Figure 4 shows part of a Western blot of the renal expression of nitrotyrosine and the effects of FeTMPyP treatment (n = 4/group). There were multiple bands that reacted to the nitrotyrosine antibody, and all were increased in diabetic compared with control renal cortex. When the primary antibody was omitted, no bands were observed. The two bands that are shown are those closest to the molecular mass of COX-2 (72 kDa), the focus of this study; we were justified, as confocal microscopy revealed colocalization of COX-2 and nitrotyrosine and immunoprecipitation indicated that COX-2 was nitrosylated, an effect reduced by FeTMPyP treatment. Expression of nitrotyrosine was almost twofold greater in renal cortex from diabetic rats compared with control rats, and the increase was prevented by treatment with the OONO decomposition catalyst, confirming that OONO formation was reduced by FeTMPyP. Similarly, renal cortical COX-2 expression was increased in the diabetic rats compared with the nondiabetic rats, and this increase was prevented in diabetic rats treated with FeTMPyP (Fig. 5, where n = 6/group).

Figure 6A shows double immunostaining for COX-2 (red) and nitrotyrosine (green) in kidney sections from diabetic and control rats. Both COX-2 and nitrotyrosine immunofluorescence were increased in kidneys of diabetic rats. The overlay demonstrates that part of COX-2 was colocalized with nitrotyrosine in the diabetic rat kidney cortex. When primary antibody to nitrotyrosine or COX-2 was omitted, no immunofluorescence was detected; similarly, blocking the nitrotyrosine antibody with excess 3-nitrotyrosine prevented binding to the renal sections. Immunoprecipitation of COX-2 and nitrotyrosine confirmed the association and strongly supported the interpretation that COX-2 is nitrated in the diabetic rat kidney, an effect that was reduced when diabetic rats were treated with FeTMPyP (Fig. 6B).

Urinary 6-ketoPGF1α excretion. As FeTMPyP treatment of diabetic rats prevented the induction of renal COX-2 but failed to reduce the increased release of prostaglandins from the perfused kidney in response to AA, we addressed whether this discrepancy was a manifestation of an isolated, perfused organ system. Consequently, we measured urinary 6-ketoPGF1α as an index of in vivo prostaglandin production that is increased in the diabetic rat. As we have reported, urinary 6-ketoPGF1α excretion was increased approximately twofold in diabetic rats compared with control rats (4.09 ± 0.37 ng/h and 2.12 ± 0.31 ng/h, respectively). FeTMPyP treatment of diabetic rats failed to reduce the increase in 6-ketoPGF1α excretion (4.64 ± 0.40 ng/h).

Urinary TNF-α and TGF-β. Both renal TNF-α and TGF-β are increased in diabetes, and oxidative stress resulting from hyperglycemia increases the production of these cytokines from endothelial cells (24, 27). Accordingly, we found that urinary excretion of TNF-α and TGF-β was markedly increased in rats with diabetes. TNF-α excretion was increased sevenfold over the respective age-matched control rats, and treatment of diabetic rats with FeTMPyP reduced this to threefold that of the control rats (Fig. 7, where n = 5/group). Similarly, excretion of TGF-β in diabetic rats was increased 14-fold over that in control rats, and treatment with the peroxynitrite decomposition catalyst resulted in a 50% decrease (Fig. 7; n = 5/group).

HUVECs. We addressed whether endothelial cells, which are constantly exposed to high glucose in vivo in the diabetic rat, respond to exposure to elevated glucose with increased expression of COX-2. Figure 8A shows a Western blot of COX-2 in endothelial cells incubated in the presence of normal glucose and high glucose for 8 h where the solutions were of equal osmolarity (n = 4/group). High glucose led to a more than twofold increase in COX-2 expression over that observed in cells exposed to normal glucose; moreover, this increase was prevented when cells were treated with FeTMPyP, suggesting a role for ONOO, and we have shown that nitrotyrosine expression is also increased by high glucose. Supporting this idea, we showed that endothelial cells incubated with SIN-1, an NO donor that also generates superoxide and, thereby, ONOO, responded with increased expression of COX-2, an effect that is...
was similarly prevented when FeTMPyP was included in the incubating medium (Fig. 8B; n = 4).

**DISCUSSION**

The findings from these studies support the concept that ONOO is a stimulus for the induction of renal COX-2 in the diabetic rat and are consistent with our previous findings implicating a role for superoxide and NO (5, 23). The present study confirmed the role of oxidative stress in the induction of renal COX-2 in diabetes, which is hardly surprising when considering that exposure to elevated glucose increases oxidative stress (3, 29) and, in turn, has been shown to increase COX-2 expression in a variety of cell types (20). Our study with tempol (23) was the first to link hyperglycemia, oxidative stress, and induction of COX-2 in vivo. We have extended these findings to support the idea that ONOO is the stimulus for the induction of COX-2. Thus ONOO increases COX-2 expression (12), as does SIN-1 in endothelial cells, an effect prevented by a ONOO decomposition catalyst. ONOO formation is increased in human and experimental diabetes as reflected by increased expression of nitrotyrosine, which we have shown for the renal cortex and renal microvessels of diabetic rats (6). Moreover, nitrotyrosine and COX-2 were colocalized in the kidney, and increases in both were evident in renal cortical sections from diabetic rats, although this does not establish cause and effect, indicating only that COX-2 is nitrated, a possibility supported by the results of immunoprecipitation.

Nitrotyrosine expression has been considered a footprint for ONOO formation, but its use as a marker for ONOO is limited without the additional qualifications outlined by Szabo et al. (35) to show that antioxidants as well as inhibitors of NO synthesis reduce nitrotyrosine expression as we have done with tempol and L-NAME treatment of diabetic rats (unpublished data).

The results with FeTMPyP provide the most convincing evidence for a role of ONOO in the induction of renal COX-2 in the diabetic rat. Thus the ONOO decomposition catalyst reduced renal nitrotyrosine expression and coincidentally prevented the upregulation of COX-2. Although the results with FeTMPyP support a role of ONOO in the induction of COX-2, it should be pointed out that FeTMPyP has been reported to scavenge superoxide and hydroxyl radicals, and removing superoxide would be expected to reduce ONOO formation in much the same way as tempol reduces nitrotyrosine expression. Thus Kang et al. (19) reported that FeTMPyP was a potent scavenger of superoxide at 10 μM. However, Xie et al. (38) reported that FeTMPyP has no SOD catalytic activity, and Salvemini et al. (33) reported differential effects of FeTMPyP and a SOD mimetic in a model of intestinal damage induced by LPS, suggesting selectivity of the decomposition catalysts as affirmed by Esposito and Cuzzocrea (14). Nonetheless, we cannot exclude the possibility that the effect of FeTMPyP to prevent upregulation of renal COX-2 may not only relate to its ability to catalyze the decomposition of ONOO to nitrate, as we did not assess the effects of FeTMPyP treatment on superoxide levels the diabetic rat kidney. The same caveat applies to the experiments in endothelial cells in which a concentration of 50 μM FeTMPyP prevented the induction of COX-2 in response to high glucose. The latter has been shown to increase superoxide generation (3) as well as ONOO formation (40). High glucose increased nitrotyrosine expression in endothelial cells that preceded the upregulation of COX-2, which was prevented by the ONOO decomposition catalyst. FeTMPyP
also prevented the increase in COX-2 expression in response to SIN-1, an effect that could theoretically result from scavenging superoxide, which is formed with NO to generate ONOO. In our previous studies, treatment of diabetic rats with tempol, l-NAME, and fenofibrate all prevented the increase in renal COX-2. Subsequently, these treatments were shown to reduce nitrotyrosine expression, and it is likely that the effects of l-NAME are independent of superoxide. When considered together, the in vivo and in vitro results provide considerable evidence for the role of ONOO in inducing COX-2. Further support is based on the demonstration that exposure of endothelial cells to high glucose produced increased expression of nitrotyrosine, confirming the results of other studies (40). Thus the use of FeTMPyP is not the only intervention to link glucose, increased COX-2 expression, and ONOO.

Of potentially great significance, the results of this study suggest that FeTMPyP provides a renal protective effect in the diabetic rat as it prevented increases in urinary TNF-α and TGF-β, which may result from hyperglycemia and which have been implicated in the renal complications of diabetes (18, 37). Thus oxidative stress of endothelial cells to high glucose, which increases oxidative stress and ONOO, increases the production of TNF-α and TGF-β (24, 27). Our results are consistent with those from the study of Wang et al. (37) in which urate was used as a scavenger of ONOO in diabetic rats and was shown to reduce the glomerular expression of nitrotyrosine and the associated increase in the renal cortical expression of TGF-β.

We reported in 1990 (31) an exaggerated release of prostaglandins from the isolated, perfused diabetic rat kidney challenged with AA; however, the association with induction of COX-2 awaited the report of 2003 (30). The increased renal release of prostaglandins from the diabetic rat may not solely be a function of induction of COX-2, although preventing the upregulation of COX-2 with tempol, l-NAME, or fenofibrate also attenuated the exaggerated release of prostaglandins by AA (5, 7, 23). This pattern was not apparent when diabetic rats were treated with FeTMPyP, which prevented the induction of renal COX-2 but did not reduce AA-stimulated prostaglandin release. The lack of effect of FeTMPyP on 6-ketoPGF1α release may be explained by removal of an inhibitory influence on prostacyclin synthase (39) that offsets the decrease in COX-2 expression. Thus nitration of prostacyclin synthase by ONOO has been reported to result in inhibition of the synthase. As prostacyclin synthase should be tonically inhibited under conditions of nitrosative stress, a reduction in basal release of 6-ketoPGF1α might be expected as well as an increase in basal and stimulated release when the negative influence is eliminated. However, the basal release was not different in control and diabetic rats and was not increased after FeTMPyP treatment.

FeTMPyP reduced creatinine clearance in diabetic rats while not affecting that of control rats, excluding a generalized effect to reduce GFR and suggesting that the effect in the diabetic rat relates to decreased nitrosative stress and, possibly, the reduction in COX-2. However, it is difficult to make a case for reduced expression of COX-2 when renal prostaglandin release was not reduced, a finding supported by measurements of urinary 6-ketoPGF1α, which was increased in diabetic rats but not modified by FeTMPyP treatment. Our previous studies (5, 7, 23) showed that interventions that prevented the upregulation of COX-2 also reduced renal prostaglandin release, and, more recently, we have found that chronic inhibition of COX-2 reduced GFR in the diabetic rat in association with reduced renal and urinary prostaglandins (unpublished data). In light of these results, the lack of effect of FeTMPyP on renal prostaglandin release while preventing the induction of COX-2 and moderating the increase in GFR was totally unexpected and suggests that the effect of FeTMPyP treatment on GFR is not related to reduced prostaglandin synthesis. It is possible that the renal efflux or urinary excretion of prostaglandins does not reflect those produced at sites critical to the regulation of GFR, although this idea is difficult to reconcile with earlier studies showing an association of COX-2 expression and renal prostaglandin release (5, 6, 23). An alternative explanation relates to deficient renal 20-hydroxyeicosatetraenoic acid (20-HETE) in the STZ-diabetic rat (6), which could contribute to increased GFR. Thus we have found that treatment of diabetic rats with FeTMPyP prevents the deficit in 20-HETE (unpublished observations).

In our previous studies we have found an association between the enhanced prostaglandin release from the diabetic rat kidney in response to AA and the greatly increased vasoconstrictor response to AA (31), which we have shown to be mediated by endoperoxides (32). However, we have also found that interventions that prevent the upregulation of COX-2 and reduce prostaglandin release do not reduce the vasoconstrictor effect of AA, suggesting that COX-2 may not contribute to the vasoconstrictor effect (23). Similarly, in this study, we found that treatment of diabetic rats with FeTMPyP did not affect the increased vasoconstrictor response to AA. In contrast, treatment of diabetic rats with FeTMPyP enhanced the renal vasoconstrictor effect of phenylephrine, which is consistent with reports that ONOO inhibits responses to α-adrenergic receptor stimulation (2). It is unlikely that differences in blood pressure in response to FeTMPyP contribute to the changes in COX-2 expression, as an earlier study revealed no difference in blood pressure between diabetic and nondiabetic rats (5).

In summary, treatment of diabetic rats with a ONOO decomposition catalyst reduced renal nitrotyrosine expression and prevented the induction of COX-2, which were colocalized. Taken together with previous studies invoking a role for superoxide and NO, these results strongly suggest a role for ONOO in the induction of COX-2. Complementary studies in endothelial cells showed that the ONOO decomposition catalyst prevented the induction of COX-2 in response to elevated glucose and SIN-1, which generates ONOO. Of functional significance, treatment of diabetic rats with FeTMPyP prevented increased in creatinine clearance as well as increases in TNF-α and TGF-β, suggesting that removal of ONOO may exert a renal protective effect.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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