The Thromboxane Receptor Antagonist S18886 Attenuates Renal Oxidant Stress and Proteinuria in Diabetic Apolipoprotein E–Deficient Mice

Shanqin Xu, Bingbing Jiang, Karlene A. Maitland, Hossein Bayat, Jiali Gu, Jerry L. Nadler, Stefano Corda, Gilbert Lavielle, Tony J. Verbeuren, Adriana Zuccollo, and Richard A. Cohen

Arachidonic acid metabolites, some of which may activate thromboxane A2 receptors (TPr) and contribute to the development of diabetes complications, including nephropathy, are elevated in diabetes. This study determined the effect of blocking TPr with S1886 or inhibiting cyclooxygenase with aspirin on oxidative stress and the early stages of nephropathy in streptozotocin-induced diabetic apolipoprotein E–/– mice. Diabetic mice were treated with S1886 (5 mg·kg⁻¹·day⁻¹) or aspirin (30 mg·kg⁻¹·day⁻¹) for 6 weeks. Neither S18886 nor aspirin affected hyperglycemia or hypercholesterolemia. There was intense immunohistochemical staining for nitrotyrosine in diabetic mouse kidney. In addition, a decrease in manganese superoxide dismutase (MnSOD) activity was associated with an increase in MnSOD tyrosine-34 nitration. Tyrosine nitration was significantly reduced by S1886 but not by aspirin. Staining for the NADPH oxidase subunit p47<sup>phox</sup>, inducible nitric oxide synthase, and 12-lipoxygenase was increased in diabetic mouse kidney, as were urine levels of 12-hydroxyeicosatetraenoic acid and 8-iso-prostaglandin F<sub>2α</sub>. S18886 attenuated all of these markers of oxidant stress and inflammation. Furthermore, S18886 significantly attenuated microalbuminuria in diabetic mice and ameliorated histological evidence of diabetic nephropathy, including transforming growth factor-β and extracellular matrix expression. Thus, in contrast to inhibiting cyclooxygenase, blockade of TPr may have therapeutic potential in diabetic nephropathy, in part by attenuating oxidative stress. *Diabetes* 55:110–119, 2006

Oxidative stress is widely recognized as a key component in the development and progression of diabetes complications, including diabetic nephropathy (1–3). Enhanced oxidative stress has been documented in renal cells and implicated in mesangial expression of extracellular matrix and early and late events of diabetic nephropathy, including increased glomerular filtration rate, proteinuria, glomerulosclerosis, and tubulointerstitial fibrosis (3–5). One of the many consequences of oxidative stress that has been linked to diabetes is increased production of peroxynitrite, which is generated from the reaction of superoxide and nitric oxide (NO) and is a potent oxidant that can cause protein nitration, lipid peroxidation, and DNA damage. 3-o-nitrotyrosine formed by peroxynitrite is a specific marker of oxidative and nitrosative stress and is increased in the kidney of diabetic patients and diabetic animal models (1,3,6). In one study, the lessening of renal tyrosine nitration observed in db/db mice that overexpress superoxide dismutase (SOD) was associated with decreased nephropathy, suggesting a link between oxidant stress and tyrosine nitration by reactive nitrogen species to diabetic nephropathy (5).

Arachidonic acid derivatives have long been known to be elevated in diabetes. Some of these derivatives, including nonenzymatic lipid peroxidation products, isoprostanes, as well as prostanoids (including thromboxane [TX] A<sub>2</sub> derived from cyclooxygenase [COX] and hydroxyeicosatetraenoic acids [HETEs] derived from lipoxygenase) have been implicated in the development of diabetic nephropathy (7–10). The expression of 12-lipoxygenase is increased in the kidney of diabetic rats, and its major end product, 12-HETE, induces cellular hypertrophy and the expression of the extracellular matrix protein, fibronectin; this implicates renal 12-lipoxygenase in the pathogenesis of diabetic nephropathy (7–9). The increased production and oxidation of arachidonic acid is also evidenced by two- to threefold increases in plasma or urinary 8-isoprostanes in type 1 and type 2 diabetic patients and animal models (11,12). Isoprostanes, TXA<sub>2</sub>, and 12-HETE all can act as agonists of TXA<sub>2</sub> receptors (TPr) (13). Earlier studies reported amelioration of proteinuria by the TPr antagonists Bay U3405 (10) and S-1452 (14) in diabetic models (11,12). Isoprostanes, TXA<sub>2</sub>, and 12-HETE all can act as agonists of TXA<sub>2</sub> receptors (TPr) (13). Earlier studies reported amelioration of proteinuria by the TPr antagonists Bay U3405 (10) and S-1452 (14) in diabetic rats, suggesting that endogenous TPr agonists stimulate the development of diabetic nephropathy. In addition, some studies indicate a benefit of inhibiting TXA<sub>2</sub> synthase...
tion, to consider the role of COX-derived TXA₂ in diabetic hyperlipidemia accelerates nephropathy compared with in...S18886 was previously shown to be efficacious in decreasing atherosclerotic effects of S18886 on oxidative stress and the early stages of...RESEARCH DESIGN AND METHODS

The TPr antagonist, S18886, was obtained from the Institut de Recherches Servier (Suresnes, France). Aspirin, a water-soluble mixture of...S18886 was obtained from Sigma (St. Louis, MO) and prepared fresh on a weekly...DIABETES, VOL. 55, JANUARY 2006 111

Data are means ± SE (n). *P < 0.05 vs. Apo E−/−; †P < 0.05 vs. untreated, diabetic Apo E−/− by ANOVA followed by Fisher’s protected least significant differences test.

(10) or COX with aspirin, suggesting that COX-derived TXA₂ may be involved to some extent (15,16). Although oxidative stress is recognized as a key factor in the pathogenesis of diabetes complications, the link between activation of TPr and oxidative stress remains elusive. S18886, an antagonist of TPr, inhibits atherogenesis in normoglycemic apolipoprotein E-deficient (ApoE−/−) mice (17) as well as in diabetic ApoE−/− mice (18). The purpose of this study was to investigate the effects of S18886 on oxidative stress and the early stages of nephropathy in streptozotocin (STZ)-induced diabetic ApoE−/− mice. This model of combined diabetes and hyperlipidemia accelerates nephropathy compared with mice with diabetes or hyperlipidemia alone (19). In addition, to consider the role of COX-derived TXA₂ in diabetic nephropathy, the effects of S18886 were compared with those of aspirin. The results indicated that the blockade of TPr with S18886, but not with aspirin, dramatically attenuates renal oxidative stress and improves renal structure and function in diabetic ApoE−/− mice.

Table 1

Metabolic and biochemical parameters

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6</th>
<th>Nondiabetic Apo E−/−</th>
<th>Untreated</th>
<th>Diabetic Apo E−/−</th>
<th>S18886</th>
<th>Aspirin</th>
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<tr>
<td>Body weight (g)</td>
<td>29.5 ± 0.6 (12)</td>
<td>19.5 ± 0.3 (24)</td>
<td>17.3 ± 0.5 (18)*</td>
<td>17.6 ± 0.5 (21)*</td>
<td>18.6 ± 0.4 (18)</td>
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<tr>
<td>Kidney weight (mg)</td>
<td>388 ± 12 (12)</td>
<td>218 ± 7 (24)</td>
<td>343 ± 22 (18)*</td>
<td>321 ± 29 (21)*</td>
<td>341 ± 27 (18)*</td>
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<td>Kidney weight–to–body weight ratio (mg/g)</td>
<td>13.2 ± 0.3 (12)</td>
<td>11.1 ± 0.3 (24)</td>
<td>20.5 ± 1.6 (18)*</td>
<td>18.9 ± 2.0 (21)*</td>
<td>18.6 ± 1.7 (18)*</td>
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</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td>104 ± 4 (10)</td>
<td>107 ± 3 (24)</td>
<td>114 ± 7 (13)</td>
<td>110 ± 5 (18)</td>
<td>109 ± 5 (16)</td>
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<tr>
<td>Blood glucose (mg/dl)</td>
<td>115 ± 10 (7)</td>
<td>140 ± 4 (28)</td>
<td>459 ± 20 (22)*</td>
<td>426 ± 21 (22)*</td>
<td>470 ± 33 (15)*</td>
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<td>Serum cholesterol (mg/dl)</td>
<td>116 ± 7 (8)</td>
<td>381 ± 20 (25)</td>
<td>899 ± 96 (23)*</td>
<td>818 ± 76 (25)*</td>
<td>897 ± 114 (17)*</td>
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<td>Serum thromboxane B₂ (ng/ml)</td>
<td>38 ± 8 (12)</td>
<td>51 ± 6 (14)</td>
<td>77 ± 6 (13)*</td>
<td>62 ± 8 (13)</td>
<td>23 ± 4 (12)*</td>
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<tr>
<td>Urinary 12-HETE–to–creatinine ratio (ng/mmol)</td>
<td>5.3 ± 1.2 (8)</td>
<td>2.1 ± 0.4 (13)</td>
<td>11.5 ± 2.4 (15)*</td>
<td>5.3 ± 0.9 (18)*</td>
<td>10.3 ± 3.5 (12)*</td>
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RESEARCH DESIGN AND METHODS

The TPr antagonist, S18886, was obtained from the Institut de Recherches Servier (Suresnes, France). Aspirin, a water-soluble mixture of acetylsalicylic acid (30%) and the lysine salt of acetylsalicylate (64%) in powder form (Aspegic), was obtained from Laboratoires Synthelabo (Paris, France). STZ was obtained from Sigma (St. Louis, MO) and prepared fresh on a weekly basis from frozen, desiccated powder.

Female homozygous Apo E−/− mice (age 7 weeks) were obtained from The Jackson Laboratory (Bar Harbor, ME). Female mice were used because they were used in previous studies of the effect of S18886 on atherosclerosis (18), which is enhanced in female compared with male ApoE−/− mice (20). The mice were maintained on normal mouse chow and given free access to food and water in a temperature- and light-controlled room throughout the study. After they had 1 week to acclimatize to the conditions, they were rendered diabetic by intraperitoneal injection of STZ (dissolved in citrate buffer [pH 4.5]; 70 mg·kg⁻¹·day⁻¹) for 5 days. Control nondiabetic Apo E−/− mice received the same volume of citrate buffer. Blood samples for glucose measurements were taken from the tail vein 1 week after the STZ injection. The presence of diabetes was confirmed by a nonfasting blood glucose level >200 mg/dl, as measured by a Glucometer Elite (TheraSense, Alameda, CA).

The diabetic Apo E−/− mice were then randomly divided into three groups: untreated diabetic, S18886-treated diabetic, and aspirin-treated diabetic. Both S18886 (5 mg·kg⁻¹·day⁻¹) and aspirin (30 mg·kg⁻¹·day⁻¹) were administered for 6 weeks in the drinking water, the volume of which was adjusted for the increased consumption of the diabetic animals. This dosage of S18886 was previously shown to be efficacious in decreasing atherosclerosis in ApoE−/− mice (17,18). In addition, in a subgroup of mice in this study, serum levels of S18886 averaged ~30 nmol/L, ~30-fold higher than the approximate concentration that binds 50% of TP receptors. The dosage of aspirin used was previously shown to be efficacious by significantly decreasing serum TXB₂ levels (17). During the last week of the treatment, a urine sample was collected over 4–10 h from each mouse maintained in a metabolic cage and stored at −80°C until use. The mice were killed under isoflurane anesthesia, and blood samples for serum were collected from the vena cava before the kidneys were removed. The studies were approved by the Boston University Medical Center Institutional Animal Care and Use Committee.

Measurement of systolic blood pressure. Systolic blood pressure was measured in mice during the final 2 weeks of the protocol. Blood pressure was assessed by the tail cuff method in a dark and warm environment only after acclimating the mice during three to five training sessions. Under these conditions, systolic blood pressure agreed well with intra-arterial measurements (21).

Determination of urinary albumin, 8-iso-prostaglandin F₂α, and 12-HETE. Urinary albumin was determined with an enzyme-linked immunosorbent assay (ELISA) kit to measure murine microalbuminuria, and values were normalized to urinary creatinine (Exocell, Philadelphia, PA). Urinary 8-iso-prostaglandin (PG) F₂α was determined with an ELISA kit (Oxford Biomedical Research, Oxford, MI), as was urinary 12-HETE (R&D Systems, Minneapolis, MN).

Determination of serum cholesterol and TXB₂. Serum cholesterol was measured enzymatically using a kit from Sigma. TXB₂ levels were measured using a kit from Cayman Chemical (Ann Arbor, MI), as previously described (17).

Determination of MnSOD activity. SOD activity was measured by inhibiting the reduction of cytochrome c caused by xanthine and xanthine oxidase, as previously described (22,23) and modified (24). Horse heart cytochrome c (type III), xanthine, and xanthine oxidase were purchased from Sigma. After being thawed, mouse kidney samples were washed with 0.9% NaCl solution. The tissue was then homogenized in 2 ml of 50 mmol/l Tris-HCl buffer containing 0.1 mmol/l EDTA at pH 7.0. After being centrifuged at 15,000g for 30 min, the supernatant was removed and the total protein concentration was measured. We used 10 μl of the supernatant in the assay mixture, which had a total volume of 300 μl consisting of 50 mmol/l Tris-HCl, 0.1 mmol/l EDTA, 50 μmol/l cytochrome c, and enough xanthine oxidase (~6 nmol/l) to cause a change in absorbance at 550 nm of 0.025/min at pH 7.8. Under these conditions, 1 unit of SOD activity is defined as the amount that causes 50% inhibition of the initial rate of reduction of cytochrome c. MnSOD activity was measured as the difference between total activity and activity after inhibiting extracellular and cytosolic Cu/Zn SOD activity with sodium cyanide (5 mmol/l).

Tissue preparation and histology. The kidneys were fixed in 10% buffered formalin acetate overnight. After the adherent fat was removed, the kidneys were cut transversely, processed, and embedded in paraffin. To assess the renal pathology and extracellular matrix deposition, Masson’s trichrome staining was performed in 5-μm-thick renal tissue sections with an Accustain Trichrome Stain Kit (Sigma) following the manufacturer’s protocol. Briefly stated, the comparable sections of kidneys were extracted of paraffin, hydrated with water, and immersed in Bouin’s solution for 15 min. The sections were then rinsed and stained with Mayer’s hematoxylin for 5 min. After the sections were rinsed again, Biebrich scarlet-acid fuchsin was added for 5 min and the sections were rinsed once more; then phosphotungstic/phosphomolybdic acid solution was added for 5 min, followed by aniline blue solution for 5 min. After the sections were rinsed, 1% acetic acid was added for 2 min, and the slides then were dehydrated and mounted. Degenerated tubules were identified by the absence of cytoplasm staining (red color), Matrix deposition (blue color) within the glomeruli and tubular interstitium on Masson’s trichrome stained sections was analyzed using computer-assisted image analysis. Briefly, staining images were captured and digitalized using an Olympus microscope attached to an Olympus HC5000 digital camera. Blue
color was extracted from the images with color range selection function of Photoshop (version 7.0). Glomerular matrix deposition was evaluated semi-quantitatively by two investigators who were unaware of the identity of the samples. The scoring was based on the proportion of glomeruli with obvious matrix deposition at the glomerular basement membrane versus total glomeruli counted from four nonoverlapping regions in each renal section viewed at 100× magnification. The scoring was estimated on a scale of grade 0–4: grade 0, the proportion of glomeruli with obvious matrix deposition <10%; grade 1, 10–20%; grade 2, 20–30%; grade 3, 30–40%; and grade 4, >40%.

To determine diabetic glomerular hypertrophy, five random nonoverlapping fields of each section from seven mice per group were analyzed. A line was manually drawn around the boundary of each of the glomerular tufts that were transected at random levels in each section. The glomerular tuft area (area inside the line) of at least 100 glomeruli of each section was measured, and the mean values calculated from individual sections were compared statistically among groups.

### Immunohistochemistry

After paraffin was removed and the tissue was rehydrated, 5-μm thick renal tissue sections were treated with 10 mmol/l citric acid buffer (pH 6.0) and then heated by microwave (2 min, three times at 700 W) to recover antigenicity. Nonspecific binding was blocked with 10% normal goat serum in PBS (pH 7.4) for 30 min. The tissue sections were then incubated with the respective primary antibodies in PBS with 1% BSA overnight at 4°C. The primary antibodies and the respective concentration or dilution used were: polyclonal anti-nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY), 1 μg/ml; polyclonal anti-inducible NO synthase (iNOS) antibody (BioMol Research, Plymouth Meeting, PA), 1:2,000; polyclonal anti-p47phox antibody, 5 μg/ml; polyclonal anti-12-lipoxygenase antibody (25), 3.3 μg/ml; and polyclonal anti-transforming growth factor-β (TGF-β) 1 antibody, 1 μg/ml (Santa Cruz Biotechnology, Santa Cruz, CA). A sequence-specific antibody toward nitrosylation on the active site tyrosine 34 (Tyr34) of MnSOD was obtained from Bethyl Laboratories (Montgomery, TX). The polyclonal antibody was generated in rabbits immunized with a peptide chemically synthesized with the constituent amino acids, including 3-o-nitrotyrosine within the human MnSOD sequence (25LHSHKH-HAA[nY]VNVLNV40). To remove antibodies against the non-nitrated sequence, antisera were processed over immunosorbents consisting of non-nitrated peptides immobilized on agarose. Subsequently, specific antibodies against nitrated peptide were column-purified from the processed antisera using immunosorbents consisting of the nitrated peptide immobilized on agarose. The yields of antibody were 5–20 μg/ml. Site-specific staining with the antibody was demonstrated by showing that staining was blocked by the tyrosine-nitrated peptide, but not the non-nitrated peptide or free nitrotyrosine (data not shown), and was appropriately prevented by dithionite, which reduces nitrotyrosine to aminoxyxine. The antibody was used at 4 μg/ml on kidney sections. The tissue sections were then incubated for 30 min at room temperature with a biotinylated anti-rabbit IgG secondary antibody (1:200–800) using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). VectorRed alkaline phosphatase substrate was used to visualize positive immunoreactivity. The specificity of the nitrotyrosine antibody was confirmed by preincubation of antibody with free 3-nitrotyrosine (10 mmol/l). In addition, on every slide, specificity was also ensured by using a nonimmune rabbit IgG (Vector Laboratories) isotype control. The alkaline phosphatase reaction was stopped before any nonspecific staining occurred. Semiquantitative analysis of tissue immunoreactivity was done in a blinded fashion by trained medical research laboratory personnel using the arbitrary 0–4 grading system previously described (26) to estimate the degree of overall staining on the kidney cross section.

### Statistical analysis

All quantitative or semiquantitative data are presented as means ± SE. Data were analyzed by one-way ANOVA followed by Fisher’s protected least significant differences test. P < 0.05 was considered statistically significant.

## RESULTS

### Metabolic and biochemical parameters

Table 1 summarizes the metabolic and biochemical effects of diabetes and treatment with S18886 or aspirin. Reference values obtained in C57BL/6 mice at age 15 weeks are included for comparison. As expected, there were significant increases in blood glucose and serum cholesterol levels in diabetic ApoE−/− mice compared with nondiabetic ApoE−/− mice. Treatment with S18886 or aspirin did not affect hyperglycemia or hypercholesterolemia, suggesting that the treatments had no effect on the abnormalities of glucose or cholesterol metabolism in diabetic ApoE−/− mice. In ad-
dation, diabetes was accompanied by significant decreases in body weight and increases in kidney weight and the kidney weight-to-body weight ratio; this was not affected by either drug treatment, which also indicated that the metabolic parameters were similar. Furthermore, no significant changes among the groups were found in systolic blood pressure that could have affected kidney function.

To confirm the inhibition of COX activity by aspirin, the serum level of TXB₂, a stable metabolite of TXA₂, was measured. TXB₂ levels were significantly higher in diabetic than in nondiabetic ApoE−/− mice (Table 1). Treatment with aspirin significantly reduced serum TXB₂ levels (by >70%), indicating that aspirin effectively inhibited the production of TXA₂ by COX. S18886 did not significantly affect serum TXB₂ levels in diabetic ApoE−/− mice.

Diabetic ApoE−/− mice had significantly increased urinary levels of 12-HETE, the product of 12-lipoxygenase, compared with nondiabetic ApoE−/− mice (Table 1). The levels of 12-HETE in diabetic ApoE−/− mice treated with S18886 were significantly lower than those in untreated diabetic ApoE−/− mice. Urinary 12-HETE levels in diabetic ApoE−/− mice were not significantly affected by treatment with aspirin.

Compared with nondiabetic ApoE−/− mice, diabetic mice had significantly increased microalbuminuria (Fig. 1A), indicating the presence of early-stage diabetic nephropathy. Treatment with S18886 significantly reduced urinary albumin excretion by ~70%. In contrast, treatment with aspirin had no significant effect.

Renal histological changes. Glomerular tuft size was significantly increased in diabetic ApoE−/− mice, consistent with early diabetes-induced renal changes. This increase in glomerular tuft size was ameliorated by treatment with S18886 but not by treatment with aspirin (Fig. 1B).

Semiquantitative scoring showed a significantly increased matrix deposition in glomeruli in diabetic ApoE−/− mice, which was significantly decreased by treatment with S18886 but not with aspirin (Fig. 1C). Representative kidney sections stained with Masson’s trichrome are shown in Fig. 2 (left and middle columns). Appreciable increases in matrix in the glomeruli and renal interstitium and degenerative changes in tubules were seen in the diabetic as compared with in the nondiabetic ApoE−/− mice. These changes were attenuated by treatment with S18886.

Almost no TGF-β staining was observed in normal C57BL/6 mouse kidney, but a significant increase in staining was seen in the tubules of nondiabetic ApoE−/− mice. In diabetic ApoE−/− mice, there was a further significant increase in staining seen extensively in both proximal and distal tubules as well as in glomeruli (Fig. 1D and Fig. 2, right column). Treatment with S18886 dramatically decreased TGF-β staining throughout the diabetic ApoE−/− mouse kidney to levels that were not significantly different from those of C57BL/6 mice. However, in the aspirin-treated group, the staining pattern was nearly as intense as that of untreated diabetic ApoE−/− mice.

Renal nitrotyrosine and urinary 8-iso-PGF₂α. Immunohistochemical staining for nitrotyrosine was increased in renal glomerular mesangial cells and capillary vessels as well as in tubular epithelial cells in the outer medulla (Fig. 3, left column) and papilla (not shown) in both nondiabetic and diabetic ApoE−/− mice. In diabetic ApoE−/− mice, the staining was significantly more intense, particularly in medullary and papillary tubular epithelial cells.
Treatment with S18886 essentially eliminated nitrotyrosine staining in diabetic ApoE⁻/⁻ mouse kidneys, whereas aspirin showed no significant effect. A similar increase in nitrotyrosine staining was observed in renal medullary rays in diabetic ApoE⁻/⁻ mice. In this renal structure, both S18886 and aspirin decreased the intensity of the staining, although the effect of S18886 was much more pronounced (Fig. 3, middle column). Figure 4A summarizes the scoring results from seven mice in each group. The score, which reflects overall staining on the kidney cross section, was significantly increased in nondiabetic ApoE⁻/⁻ mice compared with in C57BL/6 mice and was further increased by ~50% in diabetic ApoE⁻/⁻ mice. S18886, but not aspirin, significantly decreased the nitrotyrosine staining in diabetic ApoE⁻/⁻ mice, preventing any significant increase caused by diabetes.

A similar pattern of staining was observed in kidneys from diabetic ApoE⁻/⁻ mice with a site-specific antibody directed toward nitrotyrosine on Tyr³⁴ in the active site of MnSOD (Fig. 3, right column). Treatment with S18886, but not aspirin, attenuated staining of MnSOD tyrosine nitration in diabetic ApoE⁻/⁻ mice. Because tyrosine nitration at this site is associated with complete inhibition of MnSOD activity, MnSOD activity was measured in kidney homogenates and found to be significantly decreased in diabetic ApoE⁻/⁻ mice compared with in C57BL/6 mice (Fig. 4B). S18886 but not aspirin significantly increased MnSOD activity to levels not significantly different from those of C57BL/6 mice. These changes in MnSOD activity were not accompanied by changes in MnSOD protein expression in renal homogenates evaluated by Western blot (data not shown).

There was a significant increase in urinary 8-iso-PGF₂αα excretion in diabetic ApoE⁻/⁻ mice compared with in nondiabetic ApoE⁻/⁻ mice (Fig. 4C). S18886 markedly decreased urinary excretion of 8-iso-PGF₂αα (by >70%), whereas aspirin had no significant effect.

**Immunohistochemistry for p47phox, 12-lipoxygenase, and iNOS.** Immunohistochemical staining with a polyclonal anti-p47phox antibody was significantly enhanced in diabetic ApoE⁻/⁻ mouse kidneys compared with in normal or nondiabetic ApoE⁻/⁻ mouse kidneys. Staining was most marked in distal tubule and collecting duct cells, where nitrotyrosine staining also was most intense, whereas glomeruli were less involved. S18886, but not aspirin, significantly decreased staining for p47phox (Fig. 5, left column, and Fig. 6A).

Immunohistochemical staining for 12-lipoxygenase in the renal cortex of normal, nondiabetic ApoE⁻/⁻, diabetic ApoE⁻/⁻ mice, and those treated with S18886 or aspirin is shown in Fig. 5 (middle column) and Fig. 6B. 12-lipoxygenase staining was significantly increased in nondiabetic ApoE⁻/⁻ mice and significantly further increased in diabetic ApoE⁻/⁻ mice. S18886 decreased the staining to levels that were not significantly different from those seen in C57BL/6 mouse kidney, but aspirin had no effect. The changes in 12-lipoxygenase were consistent with the changes in urinary 12-HETE levels shown in Table 1.

Minimal immunohistochemical staining for iNOS was present in the kidneys of C57BL/6 and nondiabetic...
ApoE<sup>−/−</sup> mice. In contrast, iNOS was expressed in glomeruli, cortical tubules, medullary collecting ducts, and papillary tubular cells of the diabetic ApoE<sup>−/−</sup> mice (Fig. 5, right column, and Fig. 6C). Staining for iNOS was dramatically attenuated in the cortex and medulla of diabetic mice treated with S18886 but not in those treated with aspirin. The changes in iNOS staining in these areas were similar to those that occurred in nitrotyrosine and p47<sub>phox</sub> staining.

**DISCUSSION**

Although it is widely recognized that oxidative stress is a key component in the pathogenesis of diabetic nephropathy, the most important new finding in the present study is that renal oxidative stress is dependent on activation of TPr in diabetic ApoE<sup>−/−</sup> mice. We used the ApoE<sup>−/−</sup> mouse model in our study, which has previously been shown to develop accelerated atherosclerosis (27) and nephropathy (19) when made diabetic with STZ. Although the nondiabetic ApoE<sup>−/−</sup> mouse develops little evidence of albuminuria, it does display moderate pathological evidence of nephropathy (19). The dramatic acceleration of renal disease in the diabetic ApoE<sup>−/−</sup> mouse is due to the combined effects of hyperglycemia and hyperlipidemia, because the degree of pathology and albuminuria exceeds that in the nondiabetic ApoE<sup>−/−</sup> mouse and the diabetic C57BL/6 mouse. With the notable exceptions of TGF-β, nitrotyrosine, and 12-lipoxygenase staining, which were significantly increased in nondiabetic ApoE<sup>−/−</sup> mice, the induction of diabetes accounted for the major changes in the parameters measured. Thus the mouse model used in this study combined the effects of two clinically important risk factors for nephropathy (28). Treatment of the diabetic ApoE<sup>−/−</sup> mice with S18886, a competitive TPr antagonist, attenuated the development of early nephropathy induced by these risk factors, as evidenced by the decreased proteinuria and histopathological changes, including extracellular matrix and TGF-β. TGF-β has been implicated as a pathophysiological mediator of the matrix deposition induced in diabetic renal cells by their exposure to high glucose (29). Because there was no effect of the treatment on hyperglycemia, hypercholesterolemia, or body weight, the beneficial effect of S18886 was independent of any appreciable influence on the metabolic severity of diabetes. In addition, no significant changes in blood pressure appeared to explain the effect of diabetes or of S18886 to prevent it. Rather, the present study showed that S18886 dramatically decreases renal oxidative stress, as evidenced by the decreased renal nitrotyrosine deposition and urinary 8-isoprostanexcretion. Although TPr antagonists have previously been shown to reduce diabetic nephropathy in diabetic rats, this is the first study indicating that the effect of inhibiting TPr can be attributed to a novel role of TPr in mediating oxidant stress in the kidney.

Marked nitrotyrosine staining was present in renal cross sections of the diabetic ApoE<sup>−/−</sup> mice, consistent with previous reports by others who detected increased nitrotyrosine deposition in diabetic rat kidneys (3,30) and renal biopsies of diabetic patients (1). Peroxynitrite, the reaction product of NO and superoxide anion, is one cause of nitrotyrosine deposition in tissue, and the fact that nitrotyrosine is decreased in mice overexpressing SOD has been interpreted as evidence of peroxynitrite formation in the diabetic kidney (5). Not only does nitrotyrosine represent a marker of oxidative stress, but also the modification of protein structure can cause abnormal protein function. As examples, we have previously demonstrated that the nitration of MnSOD, prostacyclin synthase, and sarcoplasmic reticulum calcium ATPase is associated with decreased enzyme activities (24,26,31). Furthermore, the marked improvement of renal function associated with S18886 is likely to be causally related to the dramatic improvement in tyrosine nitration observed. This is suggested by the finding that overexpression of SOD in db/db mice, which prevents renal tyrosine nitration, also greatly ameliorates renal function (5). The alternative interpretation, that a reduction in oxidant stress caused by the TPr is the result of improvement in renal function mediated by other mechanisms, is made less likely by this observation. In addition, by using a site-specific antibody directed toward the nitrated Tyr<sup>34</sup> of MnSOD, we demonstrated nitration of a specific mitochondrial protein in diabetic ApoE<sup>−/−</sup> mouse kidney. Nitration at this site in MnSOD is a marker of oxidative stress, but also the modification of protein structure can cause abnormal protein function.
associated decrease in enzyme activity that we found suggests one mechanism of worsened oxidant stress in diabetic nephropathy. We speculate that MnSOD nitration and dysfunction may be associated with increased oxidants and that generalized mitochondrial dysfunction may be associated with abnormalities in electron transport and ATP generation and related changes in calcium and cell swelling. Treatment with S18886, but not aspirin, attenuated the nitration of MnSOD as well as increased its enzymatic activity. No changes in MnSOD expression, as assessed by Western blot (data not shown), were observed to explain these changes in activity, indicating that the oxidant stress mediated by TPr activation in diabetic ApoE<sup>−/−</sup> mouse kidney is mediated in part by inactivation of MnSOD and that treatment with a TPr antagonist preserves MnSOD activity. MnSOD is expressed heavily in renal tubules, where it is also thought to protect against oxidative changes induced by ischemia (33). This study points to the regulation of MnSOD activity by oxidant-induced tyrosine nitration secondary to TPr activation as an important contributor to diabetic nephropathy.

Increases in superoxide anion that yield peroxynitrite and nitrotyrosine have been previously attributed to NADPH oxidase (34). The multicomponent phagocyte-type NADPH oxidase is a major source of reactive oxygen species production in many nonphagocytic cells, including fibroblasts, vascular smooth muscle cells, endothelial cells, and renal mesangial and tubular cells (2,3,35). Under physiological conditions, nonphagocytic NADPH oxidases have a low level of constitutive activity. However, enzyme activity can be upregulated in response to high glucose, hyperlipidemia, growth factors including angiotensin II, and cytokines (35). The p47<sub>phox</sub> subunit of NADPH oxidase is overexpressed in diabetic nephropathy (2,3). In the present study, p47<sub>phox</sub> was increased in the diabetic ApoE<sup>−/−</sup> mouse kidney and was preferentially located in distal tubule and collecting ducts, where we also observed the most intense nitrotyrosine staining. Treatment with S18886 fully prevented the increased p47<sub>phox</sub> expression, suggesting that the increased formation of reactive oxygen species in the diabetic kidney may be at least partially related to TPr-mediated stimulation of p47<sub>phox</sub> expression and NADPH oxidase activity. Indeed, several TPr agonists increase expression of NADPH oxidase subunits and superoxide production in vascular endothelial and smooth muscle cells (36). Our observation that MnSOD is tyrosine nitrated and inactivated is direct evidence for increased oxidants in renal tubular mitochondria. This suggests a possible relationship between mitochondrial oxidants and other sources of oxidants in renal tubular cells of diabetic ApoE<sup>−/−</sup> mice.

NO may contribute to the pathogenesis of several renal diseases, including immune-mediated glomerulonephritis, postischemic renal failure, diabetic nephropathy, obstructive nephropathy, and acute and chronic renal allograft rejection (37). Low concentrations of NO are generated by eNOS present in the vascular endothelium, whereas high concentrations are synthesized by iNOS at sites of inflammation (38). Superoxide anion may also be generated by iNOS (39). The expression of iNOS significantly increases in the diabetic rat kidney in response to lipopolysaccharide (40) but has not been widely reported in untreated diabetic nephropathy.
diabetic rodents (41). Our data showed that iNOS is increased in glomeruli, distal and proximal tubules, collecting ducts, and the papilla of the diabetic ApoE⁻/⁻ mouse kidney, suggesting that iNOS may contribute to the peroxynitrite generation and nitrotyrosine observed in this model. S18886 treatment suppressed iNOS staining in most parts of the diabetic kidney in association with decreased nitrotyrosine levels. Particularly in sites of the most intense nitrotyrosine staining, the effect of S18886 on iNOS expression may contribute to the decrease in nitrotyrosine and therefore to the amelioration of diabetes-induced renal damage that occurred.

There is now considerable evidence supporting a role for 12-lipoxygenase in promoting diabetes and diabetic nephropathy (42,43). 12-lipoxygenase expression is increased in diabetic rat glomeruli and tubular epithelial cells (7). In agreement with these previous findings, in our study, marked staining for 12-lipoxygenase was present in the renal cortical and medullary tubules, collecting ducts, and papilla of diabetic ApoE⁻/⁻ mice. Consistent with increased 12-lipoxygenase expression, the urinary excretion of 12-HETE was significantly increased. The blockade of TPr with S18886 decreased both 12-lipoxygenase expression and 12-HETE excretion. 12-lipoxygenase, for which NADPH is also a cofactor, may also directly contribute to oxidative stress by generating superoxide (44). Indeed, because S18886 decreased expression of p47phox, iNOS, and 12-lipoxygenase, it is possible that all three sources contributed to superoxide anion production in the diabetic ApoE⁻/⁻ mouse kidney. Thus the present results point to the fact that diabetes induces a broad array of proteins that contribute to oxidant levels and that TPr are a common key to their induction.

Elevated glucose in diabetes has long been recognized to increase levels of arachidonic acid metabolites, many of which stimulate TPr and therefore could explain the effect of S18886. The high dose of aspirin used in this study, which was equivalent to gram doses in patients and was sufficient to inhibit platelet COX as demonstrated by decreased serum TXB₂, was ineffective in decreasing the nephropathic changes in the diabetic ApoE⁻/⁻ mouse. This result also suggests that although its levels may be elevated, TXA₂ produced in platelets or tissues is not the most important agonist that stimulates TPr in the kidney of diabetic ApoE⁻/⁻ mice. 8-isoprostanates are potent activators of TPr that form in association with lipid peroxidation. Nonenzymatic-derived isoprostane formation is inhibited by SOD or butylated hydroxytoluene but not by COX inhibitors (45) and can be detected in plasma and urine in conditions of oxidative stress in 10- to 100-fold higher concentrations than COX-derived eicosanoids (46).

Thus the elevated levels of 8-iso-PGF₂α detected in the urine of diabetic ApoE⁻/⁻ mice may not only have reflected evidence of oxidant stress but may also have stimulated TPr. Although the effect of aspirin did not reach statistical significance, 8-iso-PGF₂α levels averaged ~50% of those in untreated diabetic ApoE⁻/⁻ mice. This may suggest that a major component of isoprostanates was derived from platelet arachidonic acid release, which may have been inhibited by aspirin. 12-HETE, the product of 12-lipoxygenase, is also a potent activator of TPr, adding to the elevated levels of potential mediators that may explain the effect of blocking TPr with S18886. Because this TPr agonist is not affected by COX inhibitors, its stimulation of TPr helps to explain the oxidant stress and nephropathy that persisted despite aspirin treatment.

The present study indicates that S18886 potently inhibits nephropathic changes in diabetic ApoE⁻/⁻ mice and links the ability of the TPr antagonist to do so to a role of TPr in mediating oxidant stress. S18886 in concentrations up to 100 μmol/l is not a scavenger of reactive oxygen species generated by xanthine and xanthine oxidase (18), indicating that its effects on oxidant stress are consistent with its ability to block TPr rather than any role as an oxidant scavenger. A structurally similar TPr antagonist has been shown to have a similar ability to attenuate nephropathy in the diabetic rat (10). The inability of aspirin to affect diabetic nephropathy in the mouse model studied here indicates not only that TXA₂ is not the major activator of TPr but also that COX is not essential to oxidant stress. S18886 not only decreased evidence of oxidants, as indicated by nitrotyrosine and 8-isoprostanates, but also decreased expression of p47phox, iNOS, and 12-lipoxygenase.
Improvement in MnSOD tyrosine nitration was accompanied by increased MnSOD activity as well. This suggests that blockade of TPR interrupts a positive feedback loop in which activation of TPR stimulates the expression of NADPH oxidase, 12-lipoxygenase, and iNOS that gives rise to oxidants that, in turn, participate in tissue damage by producing activators of TPR, including 8-isoprostanes and 12-HETE. Because of the complex relationship among the levels of these participants in oxidant stress, the most important elements remain uncertain. What is apparent from the present study is the potent therapeutic potential represented by targeting TPR and the important role they play in the oxidant stress and nephropathy that develops in diabetic ApoE⁻/⁻ mouse kidney.

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