Deficient renal 20-HETE release in the diabetic rat is not the result of oxidative stress

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Chen YJ, Li J, Quilley J. Deficient renal 20-HETE release in the diabetic rat is not the result of oxidative stress. Am J Physiol Heart Circ Physiol 294: H2305–H2312, 2008. First published March 7, 2008; doi:10.1152/ajpheart.00868.2007.—We confirmed that release of 20-hydroxyeicosatetraenoic acid (20-HETE) from the isolated perfused kidney of diabetic rats is greatly reduced compared with age-matched control rats. The present studies were undertaken to examine potential mechanisms for the deficit in renal 20-HETE in rats with streptozotocin-induced diabetes of 3–4 wk duration. A role for oxidative stress was excluded, inasmuch as treatment of diabetic rats with tempol, an SOD mimetic, for 4 wk did not affect the renal release of 20-HETE. Similarly, chronic inhibition of nitric oxide formation with nitro-L-arginine methyl ester or aldose reductase with zopolrestat failed to alter the release of 20-HETE from the diabetic rat kidney. Inasmuch as 20-HETE may be metabolized by cyclooxygenase (COX), the expression/activity of which is increased in diabetes, we included indomethacin in the perfusate of the isolated kidney to inhibit COX but found no effect on 20-HETE release. Diabetic rats were treated for 3 wk with fenofibrate to increase expression of cytochrome P-450 (CYP4A) in an attempt to find an intervention that would restore release of 20-HETE from the diabetic rat kidney. However, fenofibrate reduced 20-HETE release in diabetic and control rat kidneys but increased expression of CYP4A. Only insulin treatment of diabetic rats for 2 wk to reverse the hyperglycemia and maintain blood glucose levels at <200 mg/dl reversed the renal deficit in 20-HETE. We conclude that oxidative stress, increased aldose reductase activity, or increased COX activity does not contribute to the renal deficit of 20-HETE in diabetes, which may be directly related to insulin deficiency.

stereotaxic hypertension, insulin; fenofibrate

20-HYDROXYEICOSATETRAENOIC ACID (20-HETE) is a cytochrome P-450 (CYP4A)-derived eicosanoid that is the principal product of arachidonic acid (AA) metabolism in the kidney, where it exhibits pro- and antihypertensive actions, depending on the site of activity: vascular vs. tubular. Thus vascular 20-HETE contributes to the myogenic response and autoregulation of renal blood flow (14, 39, 40), whereas tubular 20-HETE promotes natriuresis by its ability to inhibit transport mechanisms (41). Consequently, excess production of vascular 20-HETE or diminished formation of tubular 20-HETE may lead to hypertension.

In experimental diabetes, there are many reports of derangements in renal AA metabolism that may contribute to hyperfiltration of early diabetes. Most studies have revealed increased cyclooxygenase (COX) activity (17, 24, 30); recently, increased expression of COX-2 has been demonstrated (16, 26), and such increased COX-2 expression has been linked to hyperfiltration (16). However, there is little information concerning renal CYP activity and the formation of 20-HETE in diabetes, where deficient renal vascular 20-HETE could contribute to hyperfiltration. Although there are several reports of increased renal and hepatic ω-hydroxylase expression/activity in experimental diabetes (1, 18, 31), our studies have shown reduced 20-HETE release from the perfused kidney of the streptozotocin (STZ)-diabetic rat (26).

The present studies were conducted to confirm the preliminary results and to investigate some possible mechanisms.

First, diabetes is a condition of oxidative stress (9, 22), and Kunert et al. (19) reported that increased oxidative stress may reduce the activity of CYP4A as superoxide reduced the production of 20-HETE by renal cortical microsomes, and Hoagland et al. (11) found that tempol treatment of Dahl S rats, which exhibit a renal deficit of 20-HETE, increased urinary 20-HETE excretion.

Second, NO synthase activity and the generation of NO are increased in diabetes, although the action of NO is reduced because of its interaction with superoxide (7, 22, 32, 36), which results in the formation of peroxynitrite. NO and peroxynitrite have been shown to have a negative influence on CYP4A (23, 35). We previously showed that NO can reduce CYP-dependent conversion of AA by renal microsomes (23), and Wang et al. (35) reported that peroxynitrite inhibited 20-HETE synthesis by renal microvessels.

Third, aldose reductase activity is increased in diabetes and may contribute to the oxidative stress, which, in turn, may reduce CYP activity via mechanisms already discussed.

Finally, oxidative stress increases COX-2 expression (15, 21), which could result in the metabolism of 20-HETE (6).

Consequently, we addressed the effect of tempol to reduce superoxide, which we have shown to prevent induction of renal COX-2 in diabetic rats (21), on release of 20-HETE from the isolated kidney of diabetic rats. Tempol should also reduce peroxynitrite formation and may, therefore, exhibit multiple effects. Similarly, we tested the effects of an inhibitor of NO synthesis, inasmuch as NO and peroxynitrite reduce CYP activity, and we showed that treatment of diabetic rats with nitro-L-arginine methyl ester (L-NAME) also prevented the induction of COX-2 (5). Also, we tested the effects of chronic inhibition of aldose reductase on renal 20-HETE release in diabetic rats. Finally, we tested the effects of acute inhibition of COX on release of 20-HETE. We confirmed our earlier results of decreased release of 20-HETE from the perfused kidney of diabetic rats and showed that renal microvessels also exhibited a defect in 20-HETE production, manifestations that were associated with reduced CYP4A expression in the kidney cortex and renal microvessels. Treatment of diabetic rats with

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tempol, l-NAME, or zopolrestat failed to restore 20-HETE release from the perfused kidney. Furthermore, acute inhibition of COX did not affect 20-HETE release. Therefore, we tested the effects of a peroxisome proliferator-activated receptor (PPAR)-α agonist that has been shown to induce CYP4A expression and increase renal microsomal conversion of AA to 20-HETE. However, treatment of diabetic rats with fenofibrate failed to restore renal 20-HETE release and, surprisingly, produced a further decline, whereas insulin restored renal 20-HETE in the diabetic rat.

METHODS

Diabetes was induced in male Wistar rats (150–175 g body wt) by treatment with STZ (70 mg/kg iv); control rats were given an equivalent volume of the vehicle (i.e., citrate buffer, pH 4.5). Experiments were conducted 3–4 wk later. Various different protocols were adopted, depending on the treatment, and each series of experiments had its own control groups: untreated diabetic and nondiabetic rats.

First, we confirmed that renal 20-HETE release was reduced in kidneys from diabetic rats, and we determined release from renal microvessels. The studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

Isolated perfused kidney. After pentobarbital sodium (65 mg/kg ip) anesthesia, the right renal artery was cannulated via the mesenteric artery to prevent interruption of blood flow. The kidney was removed and perfused with oxygenated Krebs buffer at 37°C at constant flow to obtain a perfusion pressure of 60–90 mmHg. Once a stable perfusion pressure was obtained, the combined venous and ureteral perfusate was collected for determination of 20-HETE by gas chromatography-mass spectrometry (GC-MS).

Renal microvessels. Kidneys from control and diabetic rats were flushed with ice-cold Krebs buffer via the aorta and excised. Microvessels were isolated by microdissection on ice in Krebs buffer and flushed with ice-cold Krebs buffer via the aorta and excised. Matography-mass spectrometry (GC-MS).

Renal microvessels. Kidneys from control and diabetic rats were flushed with ice-cold Krebs buffer via the aorta and excised. Microvessels were isolated by microdissection on ice in Krebs buffer and then incubated in 1 ml of oxygenated Krebs buffer at 37°C for 30 min. The incubate was removed, and d2-20-HETE was added as internal standard for measurement of 20-HETE by GC-MS. The vessels were incubated for a further 30 min in buffer containing 7 μM AA, and the incubate was used for the determination of 20-HETE. In some cases, renal microvascular preparations were used to determine CYP4A and nitrotyrosine expression (see below).

Oxidative stress. To address the role of oxidative stress, three groups of five rats were used: an untreated nondiabetic group, an untreated diabetic group, and a diabetic group given tempol in their drinking water (1 mmol/l) for ≥28 days, beginning 48 h after the induction of diabetes. Previously, we showed that, after 4 wk of diabetes, renal 20-HETE release from the isolated perfused kidney is reduced compared with age-matched control rats (26). Inasmuch as the water intake of diabetic rats is markedly increased, they received tempol at ~120 mg·kg⁻¹·day⁻¹, which is higher than the dose used in most studies. However, in the study of Dobrián et al. (8), tempol was administered at 90 mg·kg⁻¹·day⁻¹. Rats were anesthetized, the kidney was prepared for perfusion (see above), and the perfusate was collected for the determination of 20-HETE.

Role of NO. To address a role of NO in the reduction of renal 20-HETE release in diabetes, six diabetic and six control rats were given l-NAME (100 mg·kg⁻¹·day⁻¹) in their drinking water for 14–18 days, beginning 60 h after the administration of STZ or citrate buffer. Thus, by 2 wk of diabetes, we have shown that release of 20-HETE from the isolated perfused kidney is reduced. Six untreated diabetic and six untreated nondiabetic rats served as the respective controls. Administration of l-NAME (100 mg·kg⁻¹·day⁻¹) results in a moderate elevation of blood pressure and has been shown to influence CYP. Perfusates from the isolated kidney were collected for the determination of 20-HETE release (see above).

Role of aldose reductase. To address any contribution of aldose reductase activity to the reduction of renal 20-HETE in diabetes, either by increasing oxidative stress or possibly by reducing NADPH, we used an inhibitor of aldose reductase, zopolrestat. This agent was added to the drinking water of a group of diabetic (n = 6) and control rats (n = 6) so that they received 50 mg·kg⁻¹·day⁻¹ beginning 72 h after administration of STZ or citrate buffer, and treatment was continued for up to 4 wk. Untreated diabetic and nondiabetic rats (n = 6 in each group) were used as controls. The IC₅₀ for zopolrestat in an acute study was 3.6 mg/kg; in an earlier study, we found that administration of zopolrestat at 100 mg·kg⁻¹·day⁻¹ to diabetic rats for 4–6 wk restored the elevated sorbitol levels in the kidney to values not different from those in nondiabetic rats. Renal sorbitol levels of 13.1 ± 3.8 and 2.3 ± 1 μg/g in diabetic and control rats, respectively, were reduced to 4.9 ± 1 μg/g after zopolrestat. Moreover, it has been reported that zopolrestat at 40 mg/kg normalized nerve sorbitol levels of diabetic rats. On the basis of these considerations, we opted for a dose of 50 mg·kg⁻¹·day⁻¹.

Role of COX. In a separate study, we measured 20-HETE release from control and diabetic rat kidneys that were untreated or treated with indomethacin (2.8 μM) to inhibit COX. Indomethacin was included in the buffer from the beginning of the perfusion, and the kidneys were exposed to indomethacin for ≥15 min before the samples were collected. This concentration of indomethacin was effective in inhibiting COX, inasmuch as the endoperoxide-mediated vasoconstrictor effect of AA was abolished.

PPARα stimulation with fenofibrate. Diabetic and age-matched control rats were treated with fenofibrate (100 mg/day added to the food) for 28 days beginning 48 h after the administration of STZ or citrate buffer. Untreated diabetic and nondiabetic rats (n = 5) served as controls. At the end of the treatment period, the kidney was isolated and perfused as described previously, and release of 20-HETE into the perfusate was determined. In kidneys from two treated and untreated diabetic rats, expression of CYP4A was determined to verify that fenofibrate did induce expression of this protein.

Effect of insulin. We determined 20-HETE release from the perfused kidney of diabetic rats (n = 6) that were treated with insulin for 12 days, starting 2 wk after the induction of diabetes, to maintain blood glucose levels below 200 mg/dl. Protamine zinc insulin was administered subcutaneously twice per day, and the dose was adjusted according to daily blood glucose measurements; the dose ranged from 3 to 5 U/day for each rat. The treated rats were compared with untreated diabetic rats (n = 5) and age-matched nondiabetic rats (n = 6).

Renal conversion of AA. Inasmuch as increased renal microsomal CYP4A activity has been reported in diabetic rats (31), we also addressed conversion of AA by kidney homogenates. Kidneys were homogenized in phosphate-buffered saline containing 250 mM sucrose, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 7.7 μM/ml protease inhibitor cocktail (Pierce). Samples were centrifuged at 3,000 g for 15 min, the supernates were decanted and centrifuged at 11,000 g for 30 min, and the supernate was used for the conversion experiments after determination of protein concentration. One milligram of protein was incubated in 100 mM potassium phosphate buffer containing 10 mM MgCl₂, 10 μM indomethacin, 1 mM NADPH (reduced), and 20 μM AA for 15 min at 37°C. The reaction was stopped by addition of 2 M formic acid to adjust pH to 3.5–4, and 1 ng of d₂-20-HETE was added as an internal standard before extraction of the eicosanoids with ethyl acetate. 20-HETE in the samples was measured by GC-MS (see below).

Measurement of 20-HETE. Ten milliliters of the renal perfusate from treated and untreated control and diabetic rat kidneys were used to determine 20-HETE release by GC-MS. After addition of d₂-20-HETE (100 pg/ml) as an internal standard, eicosanoids were extracted from the perfusate with ethyl acetate after acidification to pH 4 with acetic acid. The organic phase was decanted, dried under nitrogen, and...
reconstituted into 100 μl of methanol for purification by reverse-phase HPLC using an Ultrasphere C-18 column (250 × 4.6 mm; Beckman) and a linear solvent gradient of water in acetonitrile (37.5% to 0%) containing 0.01% acetic acid at 1.875%/min with a flow rate of 1 ml/min. The fractions corresponding to authentic 20-HETE were collected, the solvent was evaporated under nitrogen, and the residue was reconstituted in 30 μl of acetonitrile. After addition of 30 μl of 10% disopropylethylamine in acetonitrile and 30 μl of 10% pentafluorobenzylbromide, the mixture was incubated for 30 min at room temperature. The mixture was dried, and the residue was dissolved in 60 μl of N,O-bis(trimethylsilyl)-trifluoroacetamide and reacted with 20 μl of pyridine for 30 min at room temperature. After the samples were dried, they were reconstituted in 35 μl of isooctane, and 1 μl was injected into a fused silica capillary tube (15 m, 0.25 mm ID, 0.2 μm film thickness) and eluted with a temperature gradient from 180 to 300°C at 25°C/min and a 40 cm/s flow of helium. 20-HETE was detected with a mass spectrometer operating in the ionization mode with methane (2 Torr source pressure) as a moderating gas with detection of negative ions (electron capture ionization). Selective ion monitoring was used to record ion abundance at mass-to-charge ratios of 391 and 393, which correspond to endogenous 20-HETE and internal standard, respectively. The amount of 20-HETE in the sample was then determined by comparison with a standard curve.

Nitrotyrosine and CYP4A expression. Renal microvessels were sonicated in RIPA buffer, and the cortex was homogenized in RIPA buffer. After centrifugation at 17,000 g, protein in the supernatant was determined, and 10 μg from microvessels and 25 μg from cortex were mixed with 5× SDS-PAGE sample buffer and boiled for 4 min. Proteins were separated as described above and immunoblotted with a rabbit anti-nitrotyrosine antibody (1:5,000 dilution; Cayman Chemical) or a rabbit anti-CYP4A antibody (Affinity Bioreagents). Membranes were washed with Tris-buffered saline containing Tween 20 and incubated with horseradish peroxidase-conjugated antiserum. Nitrotyrosine and CYP4A expression was then detected by enhanced chemiluminescence or infrared fluorescence.

Analysis of data. Values are means ± SE and were compared using an unpaired t-test or ANOVA in which individual points were compared using a modified t-statistic (Bonferroni’s test). *P < 0.05 was considered statistically significant.

RESULTS

Renal microvessels. To support our earlier observation of reduced 20-HETE formation by the kidney of diabetic rats, we measured 20-HETE from renal microvessels of control and diabetic rats. As shown in Fig. 1, 20-HETE release from microvessels of diabetic rats is reduced compared with control rats and associated with reduced expression of CYP4A.

Expression of nitrotyrosine. Using expression of nitrotyrosine as an index, we verified that peroxynitrite is increased in diabetic rats. Figure 2 shows Western blot analysis of nitrotyrosine expression in diabetic rat renal microvessels compared with age-matched control rats. Densitometry revealed a twofold increase in nitrotyrosine expression in diabetic rats.

Effect of tempol. At the time of experiments to measure 20-HETE release from the kidney, mean body weight in the citrate group was 387 ± 14 g compared with 295 ± 14 g for the STZ group and 291 ± 6 g for the STZ group treated with tempol. Blood glucose levels for the respective groups were 126 ± 10, 489 ± 33, and 553 ± 25 mg/dl. Basal perfusion pressures were not different among the groups. As shown in Fig. 3, the release of 20-HETE from the perfused kidney of the diabetic rat was significantly less, approximately one-quarter, than that of the control group and was not modified by tempol treatment, which reduced renal cortical expression of nitrotyrosine in diabetic rats (Fig. 4). Administration of AA did not increase 20-HETE release from the isolated kidney of control or diabetic rats.

Effect of l-NAME. Body weights for the citrate, citrate + l-NAME, STZ, and STZ + l-NAME groups were 347 ± 14, 310 ± 9, 268 ± 20, and 246 ± 6 g, respectively. Blood glucose levels of diabetic rats were not affected by l-NAME: 442 ± 29 and 390 ± 26 mg/dl for the untreated and treated groups, respectively. l-NAME elevated blood pressure in the diabetic and control groups. In the citrate group, systolic blood pressure was 130 mmHg compared with 150 ± 9 mmHg in the citrate group treated with l-NAME; in the STZ group, blood pressure was 126 ± 3 mmHg and increased to 140 ± 3 mmHg in the STZ group treated with l-NAME. Basal perfusion pressure was higher in the l-NAME-treated groups than in their respective control groups. Figure 5 shows the release of 20-HETE from the isolated perfused kidney from the various groups. As mentioned above, 20-HETE release from the diabetic group was greatly reduced compared with that from the citrate-treated group and was not affected by l-NAME. In contrast, l-NAME reduced 20-HETE release from kidneys of the nondiabetic rats. As mentioned above, challenge with AA did not affect 20-HETE release.

Effect of inhibition of aldose reductase. Body weights of the citrate, citrate + zopolrestat, STZ, and STZ + zopolrestat groups were 384 ± 18, 376 ± 11, 247 ± 28, and 271 ± 8 g, respectively. Treatment with zopolrestat did not affect blood glucose levels in the diabetic rats: 436 ± 41 and 437 ± 29 mg/dl for the untreated and treated groups, respectively. Zopolrestat did not affect basal perfusion pressure in isolated kidneys of diabetic and nondiabetic rats. Figure 6 shows the renal release of 20-HETE before and after AA administration. As mentioned above, 20-HETE release was greatly reduced in the diabetic group, approximately one-fourth of that of the
control group, and was unaffected by zopolrestat, which re-
duced 20-HETE in the nondiabetic rats. As mentioned above,
20-HETE release was not increased in response to administra-
tion of AA.

Effect of indomethacin. As in the previous studies, 20-HETE
release from the isolated perfused kidneys of diabetic rats was
approximately one-fourth that of control rat kidneys. Inclusion
of indomethacin in the perfusate to inhibit COX did not
influence the levels of 20-HETE in the perfusates of control or
diabetic rat kidneys (Fig. 7) but abolished the COX-dependent
vasoconstrictor response to AA that is mediated by endoper-
oxides and reduced basal and AA-stimulated release of PGE2
and 6-keto-PGF1α, confirming that COX was inhibited. For
example, basal release of 6-keto-PGF1α from control and
diabetic kidneys was 0.97 ± 0.21 and 1.07 ± 0.18 ng/min,
respectively, and was reduced to 0.35 ± 0.13 and 0.40 ± 0.07
ng/min, respectively, in the presence of indomethacin. Simi-
larly, AA-stimulated release of 6-keto-PGF1α was 2.4 ± 0.68
and 4.43 ± 0.5 ng/min for control and diabetic kidneys,
respectively; these values were reduced to 0.54 ± 0.11 and
0.48 ± 0.17 ng/min, respectively, in the presence of indometh-
acin. However, AA failed to stimulate an increase in 20-HETE
release (data not shown).

Effect of fenofibrate. Body weights tended to be lower in
diabetic and control rats treated with fenofibrate (267 ± 12 and
342 ± 10 g, respectively) than their untreated counterparts
(312 ± 15 and 414 ± 9 g, respectively). However, fenofibrate
had no effect on blood glucose levels in diabetic (459 ± 19 vs.
455 ± 24 mg/dl) or control (124 ± 6 vs. 132 ± 9 mg/dl) rats.
However, kidneys from fenofibrate-treated control rats exhibited higher basal perfusion pressure for a lower flow rate than untreated control rat kidneys.

Fenofibrate increased expression of CYP4A in diabetic and control rat kidneys relative to untreated rats: a 2- and 1.5-fold increase in diabetic and nondiabetic rats, respectively. Surprisingly, however, release of 20-HETE from the perfused kidney of the diabetic rat was not increased by fenofibrate; rather, it was further reduced from 0.66 ± 0.24 to 0.24 ± 0.03 ng/min. Release of 20-HETE from untreated control rat kidneys was 2.33 ± 0.27 ng/min, which was reduced to 0.12 ± 0.01 ng/min in the fenofibrate-treated group (Fig. 8).

Effect of insulin. Insulin significantly increased body weight compared with untreated diabetic rats (308 ± 13 vs. 252 ± 12 g); body weight in the control group was 383 ± 14 g. Blood glucose levels at the time of kidney perfusion were 430 ± 14, 130 ± 26, and 111 ± 6 mg/dl for the untreated diabetic, insulin-treated diabetic, and nondiabetic groups, respectively (Fig. 9). Insulin did not affect basal renal perfusion pressure. As previously observed, release of 20-HETE from the perfused kidney was much lower for the diabetic than the nondiabetic group: 0.18 ± 0.03 vs. 1.63 ± 0.59. This defect was corrected by insulin treatment, and release of 20-HETE in this group was 1.45 ± 0.33 ng/min.

Conversion of AA. The transformation of AA to 20-HETE by the 11,000-g supernate of whole kidney homogenates did not differ between diabetic and control rats. Thus 10.94 ± 0.13 ng of 20-HETE were generated by the preparation from diabetic rats compared with 10.43 ± 0.22 ng by the preparation from the control rat.

**DISCUSSION**

The present studies confirmed an earlier preliminary finding that 20-HETE release from diabetic rat kidneys was reduced compared with nondiabetic rats (26). The reductions in 20-HETE release were associated with decreased expression of renal microvessel CYP4A, the major isoform that converts AA to 20-HETE. This finding is in contrast to reports from Kroetz et al. (18) indicating that hepatic 20-HETE formation is increased in diabetes and from Shimojo et al. (31) that renal and hepatic microsomal CYP4A activity is increased. We addressed this discrepancy, in part, by examining conversion of AA by renal homogenates but found no difference between diabetic and control rats. Indeed, we found that renal microvessel 20-HETE formation was reduced by diabetes. Also, in contrast to our findings, other studies have suggested that insulin directly inhibits CYP activity (20). Thus, reduced insulin levels resulting from the effects of STZ would be expected to lead to an increase in the activity of CYP and, thereby, the formation of 20-HETE. We have no simple explanation for these differences. Indeed, we found that insulin
treatment of diabetes reversed the deficit in renal 20-HETE release.

The present studies were extended to address potential mechanisms for the reduction of 20-HETE release in diabetic rats. Thus CYP4A is under diverse regulatory influences, and in the present studies we examined the potential role of oxidative stress, which is a feature of diabetes. We previously showed increased excretion of isoprostanes (21), an index of oxidative stress, in diabetes, whereas other investigators reported increased renal and vascular formation of superoxide (9, 22). Inasmuch as superoxide has been shown to inhibit CYP4A activity (19), we used an SOD mimetic to reduce superoxide levels; tempol was used at a dose that reduced renal superoxide production and prevented the induction of renal COX-2 in the diabetic rat (21), which has also been linked to oxidative stress (15). Moreover, superoxide can interact with NO to form peroxynitrite, which is increased in diabetes. Several studies have shown that nitrotyrosine, a marker for peroxynitrite, is increased in diabetes (4, 33), a finding we have confirmed here using renal microvessels. NO and peroxynitrite reduce CYP4A expression and activity (23, 35) and could contribute to reduced renal 20-HETE in the diabetic rat. Scavenging of superoxide by tempol or inhibition of NO synthesis should reduce the formation of peroxynitrite, and we report here that tempol and L-NAME reduced renal cortical expression of nitrotyrosine. However, neither treatment modified release of 20-HETE from the perfused kidney of diabetic rats, suggesting that oxidative stress, in the form of superoxide or peroxynitrite, was not responsible for the deficit. These results contrast with those from other studies where tempol treatment of Dahl salt-sensitive rats or L-NAME treatment of Sprague-Dawley rats for 10 days increased the renal expression of CYP4A and conversion of AA to 20-HETE by renal microsomes (11, 23).

Similarly, we found no effect of an inhibitor of aldose reductase on 20-HETE release. We used zopolrestat to inhibit aldose reductase, the activity of which is increased in diabetes and may be a source of oxidative stress by reducing antioxidant systems such as glutathione (3). Thus, aldose reductase converts glucose to sorbitol when glucose levels are elevated in diabetes. The reduction of glucose to sorbitol by aldose reductase results in reduced levels of NADPH, which is needed to regenerate reduced glutathione. Therefore, decreased glutathione levels may predispose to oxidative stress. However, the lack of effect of zopolrestat supports the concept that oxidative stress is not the cause of decreased renal expression and activity of CYP4A in the diabetic rat. In contrast, our previous studies indicate that oxidative stress is responsible for the induction of renal COX-2 in the diabetic rat, inasmuch as tempol or L-NAME reduced the enhanced expression of COX-2; those results suggested a role of superoxide and peroxynitrite (5, 21).

There are several other potential mechanisms whereby 20-HETE formation could be reduced in the diabetic rat. It has been reported that 20-HETE is a good substrate for COX, and the increase in COX-2 expression could result in metabolism of 20-HETE to 20-hydroxyprostaglandins (6); however, in the present study, where indomethacin was added to the renal perfusate to inhibit COX-1 and COX-2, we show that release of 20-HETE was not modified, thereby excluding a role for COX-dependent metabolism of 20-HETE. Incomplete inhibition of COX by indomethacin is unlikely to be a factor, since the COX-dependent vasoconstrictor effect of AA that is mediated by endoperoxides (25) was abolished and associated with reduced basal release of prostaglandins and abolition of AA-stimulated release.

Another potential mechanism involves increased expression/activity of heme oxygenase, which catabolizes heme and, thereby, influences the level of heme-containing proteins, including CYP enzymes. Previous studies have shown that induction of heme oxygenase results in reduced expression of CYP4A with an associated reduction of 20-HETE production (29). Inasmuch as renal heme oxygenase may be induced in the diabetic rat (10), it is possible that this mechanism may contribute to the reduced expression of CYP4A and the resultant decrease in 20-HETE. However, this possibility remains to be tested.

Finally, glycosylation of the CYP4A is a possibility in a diabetic environment. Roman (28) reported the possibility of a glycosylated form of CYP4A, which was much larger, 85 kDa, than the native protein, 51 kDa. However, we do not see a much larger form of CYP4A with the antibody that we used. Inasmuch as none of the interventions affected 20-HETE release from the perfused kidney of the diabetic rat, we examined the effects of fenofibrate, a PPARα agonist, which increases CYP expression and 20-HETE generation. Thus we have shown that renal microvesSEL CYP4A expression is reduced in the diabetic rat, which could be the consequence of the reduced PPARα expression that was reported by Kanie et al. (13). However, treatment of diabetic rats with fenofibrate for 3 wk did not increase 20-HETE release, despite an increase in the expression of CYP4A. Surprisingly, fenofibrate caused a further reduction in the release of 20-HETE from the diabetic rat kidney and caused a substantial reduction from kidneys of control rats. These observations are in an apparent contradiction to other studies showing that fibrate treatment increased microsomal conversion of AA to CYP metabolites (27, 38). However, fibrates increase peroxisomal beta-oxidation (2), which could account for reduced release of 20-HETE from the intact kidney but would not be detected using microsomes.

The only intervention that increased renal 20-HETE release in the diabetic rat was insulin treatment, which reversed the decrease and might be expected, inasmuch as insulin corrects all the abnormalities associated with insulin deficiency. However, we can only speculate regarding the derangement arising from insulin deficiency that results in the reduction of 20-HETE. Hyperglycemia itself or its sequelae appear not to contribute to the deficiency of 20-HETE; this idea is based on the results of studies in fructose-fed rats, a model of insulin resistance where plasma insulin levels are increased with only a slight increase in blood glucose levels (34). In this model, 20-HETE release is increased and associated with increased expression of CYP4A. However, when insulin is removed by inducing diabetes with STZ, 20-HETE release and CYP4A expression were reduced (34). These results suggest that CYP4A is regulated in some unknown way by insulin, and it would be worthwhile to examine the effects of insulin on CYP4A expression and activity in a freshly isolated single-cell system such as vascular smooth muscle. It is of interest that insulin treatment of diabetic rats, starting 2 wk after the induction of diabetes, reversed the deficiency in renal 20-HETE; whether insulin is capable of reversing the deficit in long-term diabetes remains to be determined.
We do not know the functional implications of decreased renal 20-HETE formation in the diabetic rat. Inasmuch as 20-HETE contributes to vasoconstrictor mechanisms (12) and has been implicated in renal autoregulation (39, 40), it is tempting to speculate that a renal vascular deficiency may contribute to the renal hyperfiltration, which is a characteristic of diabetes and should be prevented or reversed by insulin treatment to maintain normoglycemia. Further studies are required to examine this possibility and to determine the functional consequences of interventions that reverse the deficit in 20-HETE in the diabetic rat.

In summary, we have shown reduced renal 20-HETE release in diabetes, a condition of oxidative stress. However, oxidative stress does not appear to contribute to deficient 20-HETE in the diabetic rat. We suggest that insulin levels are the major determinant in the diabetic rat.

GRANTS

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