Role of COX-2 in the Enhanced Vasoconstrictor Effect of Arachidonic Acid in the Diabetic Rat Kidney

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Abstract—In the rat isolated perfused kidney, arachidonic acid elicits cyclooxygenase-dependent vasoconstriction through activation of PGH/TxA2 receptors; responses are enhanced in kidneys from diabetic rats. This study examined the roles of cyclooxygenase-1/cyclooxygenase-2 in the enhanced renal vasoconstrictor effect of arachidonic acid in streptozotocin-diabetic rats. Release of 20-HETE was also determined, as this eicosanoid has been reported to elicit cyclooxygenase-dependent vasoconstriction. We confirmed that vasoconstrictor responses to arachidonic acid were enhanced in the diabetic rat kidney associated with a 2-fold–greater increase in the release of 6-ketoPGF1α, which was used as an index of cyclooxygenase activity. One and three micrograms of arachidonic acid increased perfusion pressure by $85 \pm 37$ and $186 \pm 6$ mm Hg, respectively, in diabetic rat kidneys compared with $3 \pm 1$ and $17 \pm 8$ mm Hg, respectively, in control rat kidneys. Inhibition of both cyclooxygenase isoforms with indomethacin (10 μmol/L) abolished the vasoconstrictor response to arachidonic acid in both diabetic and control rat kidneys, whereas inhibition of cyclooxygenase-2 with nimesulide (5 μmol/L) reduced perfusion pressure responses to 1 and 3 μg arachidonic acid only in the diabetic rat kidney to $15 \pm 8$ and $108 \pm 26$ mm Hg, respectively, consistent with a 3-fold increase in the renal cortical expression of cyclooxygenase-2. 20-HETE release from the diabetic rat kidney was reduced almost 6-fold and was not increased in response to arachidonic acid. These results demonstrate that the renal vasoconstrictor effect of arachidonic acid is solely dependent on cyclooxygenase activity, with no evidence for a contribution from 20-HETE; in the diabetic rat, cyclooxygenase-2 activity contributes to the renal vasoconstrictor effect of arachidonic acid. (Hypertension. 2003;42[part 2]:837-843.)

Key Words: diabetes mellitus • kidney • cyclooxygenase • vasculature • arachidonic acid

Diabetes results in changes in vascular responsiveness that have been implicated in the renal hemodynamic adaptations and the development of vascular disease. Vasoconstrictor responses to endothelium-dependent agents are reduced,1,2 whereas responses to vasoconstrictor agents are decreased, increased, or unchanged.3–8 In studies conducted more than a decade ago, we observed that vasoconstrictor responses to angiotensin II and vasopressin in the isolated perfused kidney obtained from diabetic rats were impaired compared with those in kidneys from age-matched nondiabetic rats, whereas no differences in responses to phenylephrine were noted.3 In contrast, we found that the vasoconstrictor responses to arachidonic acid (AA) in the perfused kidney of diabetic rats were markedly enhanced.9 We also showed that the renal vasoconstrictor response to AA was inhibited by indomethacin and a PGH/TxA2 receptor antagonist but not by a thromboxane synthase inhibitor, indicating that the response was mediated by an endoperoxide.10 The enhanced responsiveness to AA in the diabetic rat kidney was associated with increased conversion of AA to prostaglandins by cyclooxygenase (COX), and we concluded that in the diabetic rat kidney there was greater conversion of AA to endoperoxides.9 A study of the autoperfused hindquarters of the diabetic rat also revealed increased vasoconstrictor responsiveness to AA that was COX-dependent and mediated through stimulation of PGH/TxA2 receptors.11

At the time of these studies, only one type of COX was recognized, and it was assumed that this isoform was responsible for the increased conversion of AA in the diabetic rat kidney. However, the discovery of an inducible enzyme, COX-2, which is constitutively expressed in some parts of the kidney12 and may be expressed in endothelial cells,13 raises the possibility that the enhanced responsiveness to AA in the diabetic rat kidney may involve this COX isoform. This possibility was supported by the observations of Komers et al14 of increased renal expression of COX-2 in the diabetic rat and the ability of a COX-2 inhibitor to reduce glomerular filtration rate (GFR). Our preliminary studies also revealed an increase in renal COX-2 expression in the diabetic rat. Our earlier studies with the use of indomethacin to abolish the renal vasoconstrictor response to AA sheds no light on the role of COX-2 because indomethacin inhibits both COX isoforms. Thus, the present study was conducted to determine the role of COX-2 in the renal vasoconstrictor effect of AA.
and whether this COX isoform contributed to the enhanced response in the diabetic rat. We also addressed the release of 20-HETE, a potent renal vasoconstrictor eicosanoid, in response to AA, as earlier studies in the isolated perfused kidney indicated that the vasoconstrictor effect of 20-HETE was also dependent on COX activity and stimulation of PGH2/TxA2 receptors.15

Renal vasoconstrictor responses to AA were compared in control and diabetic rat kidneys in the presence of indomethacin to inhibit both COX isoforms and nimesulide to inhibit COX-2. As an index of COX activity, release of 6-ketoPGF1α was determined because the effects of AA were presumed to be dependent on endothelial conversion, and prostacyclin is the major endothelial prostanoïd. Responses to phenylephrine were tested to ensure that nimesulide did not affect vasoconstrictor mechanisms. The results indicate that part of the renal vasoconstrictor effect of AA in the diabetic rat resulted from its conversion by COX-2, the renal expression of which was increased in the diabetic rat. It is unlikely that 20-HETE contributes to the response as release of 20-HETE was greatly reduced in the diabetic rat and unaffected by AA.

Methods

Diabetes was induced in male Wistar rats (weight, 160 to 180 g), with streptozotocin (70 mg/kg IV) in citrate buffer (pH 4.5). Age- and weight-matched control rats were given an equivalent volume of the vehicle, citrate buffer; 4 to 8 weeks later, 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 through 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 to 90 mm Hg. Glucose levels in tail vein blood were determined with a glucometer (Ames). The procedures followed were in accordance with institutional guidelines.

After at least 10 minutes of perfusion and once a stable perfusion pressure was obtained, vasoconstrictor responses to 1, 3, and 10 μg AA were determined in the absence and presence of nimesulide (5 μmol/L) and indomethacin (10μmol/L) for both the diabetic and nondiabetic rats. Nimesulide and indomethacin were included in the buffer from the beginning of the perfusion, and the concentration of nimesulide was chosen to inhibit COX-2 without affecting COX-1.16

Thus, for human recombinant COX-1 and COX-2, the IC50 of nimesulide was 70 μmol/L and 1.27 μmol/L, respectively. However, another report lists the IC50 of nimesulide as 9.2 μmol/L for COX-1 and 0.52 μmol/L for COX-2.17 Consequently, we also used a different COX-2 selective inhibitor, NS398 (1 μmol/L), in some additional experiments with diabetic rats. Responses to phenylephrine (100 and 300 ng) were also determined as a negative control for nimesulide, as our previous studies showed no difference in responsiveness between diabetic and nondiabetic rat kidneys.5,9 We did not test the effects of indomethacin on vasoconstrictor responses to phenylephrine, as earlier studies revealed no effect of indomethacin on vasoconstrictor responses to either phenylephrine or angiotensin II in control or diabetic rats (unpublished observations).

Prostaglandin Measurements

One-minute perfusate collections were made 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 prostanoïds in the renal perfusates were determined by enzyme immunoassay with kits obtained from Cayman Chemical Company. 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. Thus, an increase in vasodilator prostaglandin levels in response to AA would also indicate an increase in the formation of endoperoxides, which mediate the vasoconstrictor effect of AA.

20-HETE

Part of the renal perfusates from untreated control and diabetic rat kidneys were also used to determine 20-HETE. After addition of d2-20-HETE (100 pg/mL) as an internal standard, eicosanoids were extracted with ethyl acetate after acidification of the sample to pH 4 with acetic acid and 20-HETE separated by reverse-phase HPLC as described.18 Samples were derivatized, and 20-HETE was determined with the use of GC-MS as reported.18

Western Blot

The cortex was homogenized in RIPA buffer and subjected to centrifugation at 10 000 and 14 000 rpm. The protein in the supernatant was determined with the use of a Bio-Rad assay kit, and 50 μg was mixed with 5× SDS-PAGE sample buffer (500 mmol/L DTT, 0.2% bromophenol blue, and 50% glycerol) and boiled for 3 minutes. Proteins were separated on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, and immunoblotted with a rabbit antimouse COX-2 polyclonal antibody (1:1000 dilution; Caymen Chemical Company). Membranes were washed with Tris-buffered saline containing Tween 20 and incubated with horseradish peroxidase-conjugated antisera. COX-2 protein was then detected by enhanced chemiluminescence.

Analysis of Data

All data are expressed as mean±SEM and were compared by means of an unpaired t test or ANOVA in which individual points were compared by means of a modified t statistic (Bonferroni). A value of P<0.05 was considered statistically significant.

Results

Body weights of the diabetic rats were significantly less than those of the age-matched nondiabetic control rats. Thus, for the streptozotocin-treated rats, mean body weight at the time of the experiments was 260±18 g versus 390±18 g for the citrate-treated rats. Blood glucose levels for the diabetic and control rats were 24.6±0.8 mmol/L and 7.0±0.7 mmol/L, respectively. Per fusate flow rates, which generated perfusion pressures of 60 to 90 mm Hg in the diabetic and control groups, were 16.2±1.2 and 15.2±1.2 mL/min, respectively, and were unaltered by nimesulide or indomethacin. Perfusion pressures in the diabetic and control groups were 76±4 and 68±4 mm Hg, respectively.

Responses to Arachidonic Acid

Figure 1 shows the dose-dependent vasoconstrictor responses to AA in untreated kidneys from diabetic (n=5) and nondiabetic (n=7) rat kidneys. At all doses tested (1, 3, and 10 μg), AA elicited much greater increases in perfusion pressure in the diabetic rat kidneys (P<0.05) but did not influence the response to 10 μg AA (Figure 2). The greatest inhibitory
effect of nimesulide was observed for the lowest dose of AA, consistent with the preferential conversion of low concentrations of AA by COX-2.20,21 In contrast, nimesulide was without effect on the vasoconstrictor responses to AA in kidneys obtained from nondiabetic rats (Figure 3; n=5). The vasoconstrictor responses to phenylephrine were unaffected by nimesulide in either the diabetic or nondiabetic rat kidneys (Figures 2 and 3).

We also used a different COX-2–selective inhibitor, NS398, in another group of diabetic rats (duration, 3 to 4 weeks), which exhibited greater sensitivity to the vasoconstrictor effects of AA. Thus, 0.3, 1, and 3 μg AA were tested and increased perfusion pressure by 21±14, 202±40, and 267±18 mm Hg, respectively, in the untreated diabetic group (n=3) versus 2±1, 67±15, and 234±34 mm Hg, respectively, in the diabetic rat kidney treated with NS398 (n=3).

In contrast to nimesulide and NS398, indomethacin abolished the vasoconstrictor activity of AA in both control and diabetic rat kidneys (P<0.05) (Figures 2 and 3), confirming that the response was COX-dependent.

Prostaglandin Efflux
Release of PGE_2 was variable in samples obtained from diabetic and control rat kidneys. There was a tendency for reduced basal release of PGE_2 from the diabetic rat kidney (0.51±0.10 ng/min versus 1.54±0.85 ng/min; Figure 4), but the difference did not achieve significance. The release of PGE_2 from the diabetic and control rat kidneys was not different after stimulation with 1 μg AA, but the increase in release from the diabetic rat kidney (from 0.51±0.10 ng/min to 2.1±0.50 ng/min; P<0.05) was greater than that of the control (from 1.54±0.85 ng/min to 2.25±0.05 ng/min; NS). Nimesulide significantly reduced basal release of PGE_2 to 0.24±0.04 ng/min in the diabetic rats kidneys (P<0.05), but the change in nondiabetic rats kidneys to 0.42±0.20 ng/min did not achieve significance. Nimesulide did not influence the increase in PGE_2 release from diabetic rat and nondiabetic rat kidneys in response to 1 μg AA (Figure 4). In the presence of indomethacin, basal release of PGE_2 was reduced to 0.23±0.04 ng/min in the citrate group (NS) and 0.26±0.03 ng/min (P<0.05) in the diabetic group, and the increase in response to 1 μg AA was prevented.

Basal release of 6-ketoPGF_1α, determined in samples obtained before the administration of AA, was not different in diabetic and control rat kidneys (Figure 5). The increase in the release of 6-ketoPGF_1α after 1 μg AA was greater (P<0.05) in the diabetic group (3.37±0.59 ng/min) than the control group (1.46±0.50 ng/min). Nimesulide did not affect basal release of 6-ketoPGF_1α in either the diabetic (0.85±0.22 ng/min) or the control group (0.65±0.06 ng/min). However, the release of 6-ketoPGF_1α after 1 μg AA was reduced in the diabetic group (P<0.05), but the change in the control group
did not achieve significance (Figure 5). Indomethacin reduced basal release of 6-ketoPGF \(_{1\alpha}\) to 0.36 ± 0.01 ng/min and 0.40 ± 0.07 ng/min from control (NS) and diabetic (\(P<0.05\)) kidneys, respectively, and prevented the increase in response to 1 \(\mu\)g AA.

**COX-2 Expression**

Renal cortical homogenates from diabetic rat kidneys (n = 4) had greater COX-2 protein expression than those from nondiabetic control rats (n = 5), consistent with the increased release of prostaglandins after challenge with AA. Densitometric analysis with \(\beta\)-actin used as a reference revealed a 3-fold increase in renal cortical COX-2 protein expression in the diabetic rat (Figure 6; \(P<0.05\)).

**20-HETE Release**

Basal 20-HETE release from untreated kidneys was much lower (0.34 ± 0.06 ng/min; \(P<0.05\)) for the diabetic rat (n = 5) than for the control rat (2.00 ± 0.66 ng/min; n = 6) and did not significantly increase after challenge with AA (Figure 7). Thus, the levels were 0.44 ± 0.10 ng/min for the diabetic versus 2.30 ± 0.74 ng/min for the control after administration of 1 \(\mu\)g AA.

**Discussion**

The results of this study confirm earlier findings of increased COX-dependent vasoconstrictor responsiveness to AA in the diabetic rat.\(^9,11\) Because the earlier studies were performed before the discovery of COX-2, the contribution of this isozyme to the vasoconstrictor effect of AA could not be addressed. Because of recent observations of increased renal expression of COX-2 in the diabetic rat,\(^14\) we addressed the role of COX-2 in the enhanced renal vasoconstrictor response to AA that was associated with increased release of prostanooids. The results of this study suggest that increased expression or activity of COX-2 contributes to the enhanced renal vasoconstrictor effect of AA in the diabetic rat. This conclusion is based on the observations that in the diabetic rat kidney in which expression of COX-2 protein is increased, the vasoconstrictor responses to AA were greatly enhanced, particularly at lower doses, and that two selective inhibitors of COX-2 reduced the vasoconstrictor response to the lower doses of AA. Furthermore, the enhanced release of prostanoids from the diabetic rat kidney in response to AA was also reduced by inhibition of COX-2. Based on our earlier studies in diabetic and nondiabetic rats, we concluded that the mediator of the renal vasoconstrictor response to AA was a COX-derived endoperoxide that stimulated PGH\(_{2}\)/TxA\(_{2}\) re-
Because prostaglandin release in response to AA was increased in the diabetic rat kidney compared with the control, we infer that increased generation of endoperoxides is primarily responsible for the enhanced vasoconstrictor response to AA. However, we cannot exclude a contribution of increased sensitivity of the renal vasculature to agonists of PGH2/TxA2 receptors in diabetes, which we had previously reported.9 The results of the present study are consistent with increased receptor sensitivity as nimesulide reduced AA-stimulated 6-ketoPGF1α release to the level observed in the control but the vasoconstrictor response to AA, although reduced compared with untreated diabetic rat kidneys, was still greater than that observed in the control kidneys. Thus, despite similar levels of prostanoid production, presumably including the mediator, PGH2, in the presence of a COX-2 inhibitor, vasoconstrictor responses to AA were not normalized. We can, however, exclude a generalized increase in renal vasoconstrictor responsiveness in the diabetic rat because responses to phenylephrine were not significantly different from those obtained in control rats.

A major finding of this study is the contribution of COX-2 to the conversion of AA to vasoconstrictor metabolites in the diabetic rat kidney in contrast to nondiabetic rat kidneys, in which inhibition of COX-2 failed to alter the vasoconstrictor effect of AA. Nonetheless, COX-2 does appear to contribute to the release of PGE2 because nimesulide reduced basal release, whereas that stimulated by AA does not appear to involve COX-2, as nimesulide did not reduce the release. It is possible that PGE2 is of tubular rather than vascular origin, as COX-2 has been reported to be constitutively expressed in the macula densa and tubule.12,22 Consistent with the results of an earlier study showing decreased urinary excretion of PGE2 in the diabetic rat, the renal release of PGE2 tended to be lower in the diabetic rat kidney.

PGI2 is considered the principal prostanoid of vascular tissue, and its synthesis has been attributed, in part, to the activity of COX-2.24,25 However, basal release of 6-ketoPGF1α, the hydrolysis product of PGI2, which was not different between diabetic and control rat kidneys, was unaffected by nimesulide indicating little contribution of

**Figure 5.** Release of 6-ketoPGF1α from untreated (n=4), nimesulide-treated (n=5), and indomethacin-treated (n=3) kidneys from diabetic (A) and control (B) rats before and after challenge with 1 μg AA. *P<0.05 vs corresponding control; &P<0.05 vs corresponding citrate group; #P<0.05.

**Figure 6.** Western blot analysis showing expression of COX-2 protein in samples of renal cortex obtained from diabetic (STZ) and control (citrate) rats. *P<0.05 vs control.

**Figure 7.** Release of 20-HETE from kidneys of nondiabetic (n=6) and diabetic (n=5) rats before and after administration of 1 μg AA. *P<0.05 vs citrate.
COX-2 to release from unstimulated kidneys. In contrast, AA-stimulated release of 6-ketoPGF1α from kidneys of both groups was reduced by nimesulide indicating a role for COX-2 in the production of renal PGI2. A contribution of COX-2 to increased conversion of AA and the enhanced vasoconstrictor response is consistent with recent reports of increased renal expression of COX-2 in the diabetic rat. Komers et al14 demonstrated increased renal cortical expression of COX-2 and associated this with the renal hemodynamic changes of diabetes by showing that a COX-2 inhibitor reduced GFR. The results of the present study also revealed increased renal expression of COX-2, but we have not localized this change to specific sites within the kidney, notably blood vessels. Consequently, we cannot speculate on whether the metabolism of AA by COX-2 (or for that matter, by COX-1) occurs in the endothelium or vascular smooth muscle or both. However, most studies of the vascular effects of AA demonstrate an endothelium-dependent effect,26-30 and preliminary studies indicate that removal of the endothelium reduces the renal vasoconstrictor effect of AA. It is of interest to note that cultured vascular smooth muscle cells exposed to levels of glucose that mimic those seen in diabetes exhibit increased expression of COX-2 when challenged with interleukin.31

In these studies, we used nimesulide as the inhibitor of COX-2 at a concentration (5 μmol/L) that is considered to be devoid of any significant effect on COX-1.16 We cannot exclude some effect on COX-1, but our results are consistent with effects on COX-2 as nimesulide, in control rat kidneys, was without effect on the vasoconstrictor action of AA that was abolished by indomethacin, a combined COX-1/COX-2 inhibitor.10 These results indicate that in control rat kidneys, the vasoconstrictor effect of AA is dependent on COX-1 activity. We do not know why the inhibitory effect of nimesulide on the vasoconstrictor activity of AA was restricted to the lower two doses in the diabetic rat kidney, although at low concentrations AA is preferentially metabolized by COX-2.20,21

The ability of nimesulide to reduce the renal vasoconstrictor effect of AA in the diabetic rat cannot be attributed to a generalized reduction in vascular responsiveness, as nimesulide did not affect the response to AA in the nondiabetic rat, nor did it reduce the renal vasoconstrictor effect of phenylephrine in either control or diabetic rats. Similarly, in the anesthetized dog, nimesulide enhanced the action of norepinephrine to reduce GFR without affecting the increases in renal vascular resistance.32 Blockade of PGH2/TxA2 receptors by nimesulide cannot explain the inhibition of the vasoconstrictor response to AA in the diabetic rat kidney, as the response to AA in the control rat was not reduced. In retrospect, U46619 rather than phenylephrine would have been the better choice of agonist to address effects on vasoconstrictor responsiveness, as it should mimic the effects of the endoperoxides. Thus, any effect of nimesulide on vasoconstrictor responses to AA in the absence of an effect on responses to U46619 could be attributed to an effect on conversion of AA by COX-2.

Another important finding of the present study was the marked reduction in renal 20-HETE release from the diabetic rat. We addressed a possible role of increased 20-HETE formation in the vasoconstrictor effect of AA, as 20-HETE is a renal vasoconstrictor agent, and earlier studies showed that in the isolated perfused kidney, the vasoconstrictor effect of 20-HETE was abolished by indomethacin or antagonism of PGH2/TxA2 receptors.15 Consequently, the use of these pharmacological agents cannot distinguish a contribution of endoperoxides versus 20-HETE as mediators of the renal vasoconstrictor effect of AA. However, the results of the present study indicate that 20-HETE does not contribute to the enhanced renal vasoconstrictor effect of AA in the diabetic rat, as release of 20-HETE was less than one fifth of that released from control rat kidneys and 20-HETE was not increased in response to challenge with AA.

Perspectives
We suggest that the enhanced COX-dependent renal vasoconstrictor effect of AA in the diabetic rat is partially attributable to the activity of the COX-2 isoform, the renal expression of which is increased in diabetes. There is little evidence for a contribution of 20-HETE to the vasoconstrictor effect of AA, which failed to stimulate release of 20-HETE. The expression of vascular COX-2 in diabetes and increased formation of vasoactive prostanoids, particularly those producing vasodilatation, could contribute to the renal hemodynamic changes associated with this condition, that is, increased renal blood flow and GFR. Similarly, a deficit of vascular 20-HETE would be expected to promote vasodilatation.

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