Acute upregulation of COX-2 by renal artery stenosis

BIANCA MANN, ANDREAS HARTNER, BOYE L. JENSEN, KARL F. HILGERS, KLAUS HöCHERL, BERNHARD K. KRAMER, AND ARMIN KURTZ. Acute upregulation of COX-2 by renal artery stenosis. Am J Physiol Renal Physiol 280: F119–F125, 2001.—This study aimed to characterize the influence of acute renal artery stenosis on cyclooxygenase-2 (COX-2) and renin expression in the juxtaglomerular apparatus. For this purpose, male Sprague-Dawley rats received a left renal artery clip, and COX-2 mRNA, COX-2 immunoreactivity, plasma renin activity, and renin mRNA levels were determined. COX-2 mRNA and COX-2 immunoreactivity in the macula densa region in the clipped kidneys increased as early as 6 h after clipping and reached a maximal expression 1–2 days after clipping. Although values for plasma renin activity were elevated markedly at all time points examined, remaining renin mRNA levels were unchanged after 6 h and then increased to reach a maximum value 1–2 days after clipping. In the contralateral intact kidney, renin mRNA and COX-2 immunoreactivity decreased to ~50% of their normal values. To investigate a possible causal relationship between the changes of COX-2 and of renin expression, clipped rats were treated with the COX-2 blocker celecoxib (40 mg·kg⁻¹·day⁻¹). This treatment, however, did not change renin mRNA either in the clipped or in the contralateral intact kidney. Our findings indicate that renal artery stenosis causes ipsilaterally an acute upregulation and contralaterally a downregulation of juxtaglomerular COX-2 expression. The lacking effect of celecoxib on renin gene expression does not support the concept of a direct mediator function of COX-2-derived prostaglandins in the control of renin expression during renal hypoperfusion.

cyclooxygenase-1; cyclooxygenase-2; juxtaglomerular apparatus; renin; prostaglandins; celecoxib

BEING INVOLVED IN THE CONTROL of renal blood flow, glomerular filtration, and salt excretion, prostaglandins have a major impact for kidney function (28). As indicated by the expression of the cyclooxygenases (COX) as the key enzymes for prostanoid formation, there appears to be a significant regional heterogeneity of prostaglandin formation within the kidney. Thus the inner medulla has by far the highest expression level of COX among the kidney zones (15, 25, 30). In the renal cortex, COX are mainly associated with vascular structures and glomeruli (17, 25) with one striking exception, namely the high-level expression of COX-2 in cells of the late thick ascending limb of Henle, including the macula densa region (12, 13, 17, 27). As to the function of COX-2 in this region, there is emerging evidence for a possible regulator role of COX-2 activity in the control of renin synthesis and renin secretion. It is known that prostaglandins, in particular PGE₂ and prostacyclin, stimulate renin secretion and renin gene expression at the level of the juxtaglomerular epitheloid cells (1, 7, 16, 18), which are directly neighbored by the macula densa.

From experiments with COX inhibitors, it has been suggested that prostaglandins are relevant for the stimulation of renin synthesis and renin secretion by the macula densa (8, 10, 23, 24). More recent experiments now demonstrate that COX-2 expression in the macula densa increases during low salt intake (12) and that the well-known stimulation of renin synthesis and renin secretion by low salt at the macula densa site can be attenuated markedly by more selective COX-2 inhibitors (11, 26).

Prostaglandins appear also to be involved in the stimulation of renin synthesis and renin secretion during acute renal hypoperfusion, since COX inhibitors were found to lower renin secretion (2, 3, 5, 14) or renin mRNA levels (24) in this condition. Because a rather similar effect was recently reported for a selective COX-2 inhibitor in rats with acute aortic coarctation (29), one might speculate about a further role of macula densa-derived prostaglandins in the control of the renin system by the renal perfusion pressure. Supportive to this assumption is the observation that, in chronically hypoperfused rat kidneys, both juxtaglomerular COX-2 and renin expression were found to be increased (13, 29). The kinetics of changes of COX-2 expression in the macula densa and their temporal relation to changes of renin secretion and renin gene expression after acute renal artery stenosis, however, are un-

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known. It was the aim of this study therefore to address this question in rats with acute unilateral renal artery clips. We found that renal artery stenosis caused ipsilaterally an acute upregulation and contralaterally a downregulation of juxtaplomerular COX-2 expression with time courses that preceded the respective changes of renin mRNA. These data would be compatible with a mediator function of macula densa-derived prostaglandins for renin synthesis during renal hypoperfusion. However, we also found that a putative selective COX-2 inhibitor did not attenuate the increase of renin mRNA upon renal artery clipping, suggesting that the parallel rises of renin and of COX-2 expression are not necessarily causally related.

MATERIALS AND METHODS

Animal experiments. Male Sprague-Dawley rats (220–250 g) were used for the experiments. Animals were kept at normal food (Altromin) and had free access to tap water. Left renal arteries were clipped (0.2 mm ID silver clips; Degussa) under pentobarbital sodium anesthesia. In controls, the left renal artery was touched only with forceps (sham clipped). Five rats each were killed by decapitation 6, 24, 48, and 96 h after (sham) clipping.

An additional set of experiments was performed with rats that were fed via gavage with the COX-2 inhibitor celecoxib (20 or 60 mg/kg two times a day) or with vehicle at 8:00 AM and 6:00 PM of each experimental day. For the treatment with celecoxib, commercially available capsules (100 mg Celebrex; Pfizer) were dissolves in propylene glycol. Three days after the start of the treatment regimen, animals received a 0.2-mm left renal artery clip, and treatment with celecoxib or with vehicle was continued for another 2 days until death of the animals.

Blood was collected, and EGTA plasma was kept frozen at −20°C until determination of plasma renin activity and free plasma concentration of celecoxib.

The kidneys were removed and were cut in longitudinal halves. One half was stored in fixation solution until COX-2 immunohistochemistry. From the remaining halves, the cortexes were dissected with a scalpel blade under a stereomicroscope. Cortex pieces were frozen in liquid nitrogen and stored at −80°C until isolation of total RNA.

COX-2 immunoreactivity. After fixation in methyl-Carnoy solution (60% methanol, 30% chloroform, 10% glacial acetic acid), tissues were dehydrated by bathing in increasing concentrations of methanol, followed by 100% isopropanol. The tissue was embedded in paraffin, and 4-μm sections were cut with a Leitz SM 2000R microtome (Leica instruments). After deparaffinization, endogenous peroxidase activity was blocked with 3% H2O2 in methanol for 20 min at room temperature. Sections were layered with the primary antibody and incubated at 4°C overnight. After addition of the secondary antibody (dilution 1:500; biotin-conjugated, rabbit anti-goat IgG), the sections were incubated with avidin D horse-radish peroxidase complex (Vectorstain DAB kit; Vector Laboratory) and exposed to 0.1% diaminobenzidine tetra-hydrochloride and 0.02% H2O2 as a source of peroxidase substrate. Each slide was counterstained with hematoxylin-eosin. As a negative control, we used equimolar concentrations of preimmune goat (for the primary antibody) or rabbit IgG (for the second antibody).

Antibodies. COX-2 was stained with a 1:500 dilution of a commercially available antiserum (M-19; Santa Cruz Biotechnology) raised in goat. Western blot experiments confirmed that the antisera detects only the inducible 72 kDa COX-2 but not COX-1 (data not shown).

Extraction of RNA. Total RNA was extracted from dissected kidney cortexes basically according to the acid-guadinium-phenol-chloroform protocol of Chomczynski and Sacchi (4). RNA pellets were dissolved in diethyl pyrocarbonate-treated water, the yield of RNA was quantified by spectrophotometry at 260 nm, and samples were separated into aliquots and stored at −80°C until further processing. The quality of extracted RNA was confirmed by the observation of intact 18 S and 28-S bands after gel electrophoresis in an ethidium bromide-stained agarose gel.

RNase protection assays for COX-1, COX-2, renin, and β-actin. COX-1, COX-2, renin, and β-actin mRNA levels were measured by RNase protection assays basically as described (9, 15, 24). In brief, after linearization and phenol/chloroform purification, the plasmids yielded radiolabeled antisense cRNA transcripts by incubation with SP6 polymerase (Promega) and [α-32P]GTP (Amersham) according to the Promega riboprobe in vitro transcription protocol. RNA probes (5 × 105 counts/min (cpm)) were hybridized with 40 μg total RNA (COX-1 and COX-2), 20 μg total RNA (renin), 1 μg total RNA (β-actin), and 20 μg TRNA (negative control) at 60°C overnight and were then digested with RNase A/T1 (RT/30 min) and proteinase K (37°C/30 min). After phenol/chloroform extraction and ethanol precipitation, protected fragments were separated on a 8% polyacrylamide gel. The gel was dried for 2 h, bands were quantitated in a Phospholmage (Packard), and autoradiography was performed at −80°C for 1–3 days.

Determination of plasma renin activity. Plasma renin activity was determined with a commercially available RIA kit for ANG I (Sorin Biomedica, Dusseldorf, Germany).

Determination of free plasma concentrations of celecoxib. Free plasma concentrations of celecoxib were determined after deproteinization using HPLC separation with pure celecoxib as a standard.

Statistics. Data are presented as means ± SE. The level of significance were calculated by ANOVA followed by Bonferroni’s test for multiple comparisons. P < 0.05 was considered to be significant.

RESULTS

To induce an acute renal artery stenosis, rats received a unilateral 0.2-mm renal artery clip that caused a strong stimulation of renin secretion as indicated by the markedly elevated plasma renin activities. Plasma renin activity was already strongly increased 6 h after clipping, which was the first time point of examination in our study (Fig. 1). Renin mRNA levels in the clipped and in the contralateral kidney also displayed characteristic time courses in the way that renin mRNA increased to a maximum between 1 and 2 days in the clipped kidney (Fig. 2, top) and constantly decreased in the intact kidney (Fig. 3, top). Notably, renin mRNA in the hypoperfused kidney was not increased 6 h after clipping (Fig. 2, top), when plasma renin activity was already strongly elevated (Fig. 1).

COX-2 expression in the clipped and in the intact kidney was characterized by two complementary approaches, namely quantification of glomeruli with associated COX-2 immunoreactivity in the macula densa region and semiquantification of COX-2 mRNA in dissected kidney cortexes.
COX-2 immunoreactivity in the cortices of sham-clipped, clipped, and contralateral intact kidneys was almost exclusively restricted to tubular structures in the direct vicinity of glomeruli (Fig. 4). Higher magnification revealed that COX-2 was clearly expressed in macula densa cells (Fig. 4). Both COX-2 immunoreactivity and cortical COX-2 mRNA abundance increased in parallel in the clipped kidney (Fig. 2, middle and bottom). Already 6 h after placing the clips, both parameters had doubled their values (Fig. 2). Both reached maximal values 2 days after clipping, when they had increased three- to fourfold over control (Fig. 2).

In the contralateral intact kidney, renin mRNA abundance had decreased significantly 1 day after clipping and remained suppressed afterward (Fig. 3, top). COX-2 immunoreactivity in the intact kidney paralleled the decrease of renin mRNA (Fig. 3, bottom). COX-2 mRNA abundance in the intact kidney, however, was not significantly changed after clipping (Fig. 3, middle).

For comparison, we also assayed COX-1 mRNA abundance in the cortex of clipped kidneys and their contralaterals. The COX-1 mRNA/β-actin mRNA ratio, however, was not different between sham clipped, clipped, and contralateral intact kidneys (data not shown) at any of the times examined in this study.

In view of the parallel increases of COX-2 and of renin expression after renal artery clipping, it was obvious to consider a possible causal role of COX-2-related prostaglandins for the stimulation of renin secretion and renin gene expression. For this purpose, we treated 2K-1C rats with the commercially available COX-2 inhibitor celecoxib at a dose of 20 mg/kg body wt two times per day, starting the treatment 3 days before placing the left renal artery clips. The animals were then analyzed 2 days after placing the clips. At the time of death, we measured free plasma concentrations of celecoxib of 1.67 ± 0.27 (SE) μg/ml (n = 5) in the treated rats. Plasma renin activities and renin mRNA levels both in clipped and in contralateral intact kidneys, however, were not different between celecoxib and vehicle-treated 2K-1C rats (Fig. 5). To rule out that the daily dose of 40 mg/kg of celecoxib might have been too low to inhibit renal COX-2 activity, we performed additional experiments with a threefold higher dose (2 × 60 mg·kg⁻¹·day⁻¹) of celecoxib. This treatment did not change basal plasma renin activity [11 ± 3 and 10 ± 2 ng ANG I/ml for vehicle (n = 5) and celecoxib (n = 5)-treated rats, respectively] nor the renin mRNA- to-β-actin mRNA ratio [0.013 ± 0.005 and 0.011 ± 0.004 cpm/cpm for vehicle (n = 5) and celecoxib (n = 5)-treated rats, respectively]. The higher dose of celecoxib...
coxib also did not change plasma renin activity in rats with the left renal artery clip \[46 \pm 10 \text{ and } 49 \pm 9 \text{ ng ANG I/ml for vehicle (n = 5) and celecoxib (n = 5)}\)-treated rats, respectively] nor the renin mRNA-to-β-actin mRNA ratio in the clipped kidney \[0.030 \pm 0.005 \text{ and } 0.028 \pm 0.003 \text{ cpm/cpm for vehicle (n = 5) and celecoxib (n = 5)}\)-treated rats, respectively].

**DISCUSSION**

In the rat kidney cortex, COX-2 immunoreactivity shows a very distinct distribution by being restricted to cells of the late thick ascending loop of Henle, including the macula densa region (12, 13, 18, 28). In previous studies, we and others found that chronic renal artery stenosis is associated with an increased expression of COX-2 in the macula densa region and an elevation of renin expression in cells of the juxtaglomerular apparatus (13, 29). These data gave rise to the speculation of a relationship between the upregulation of COX-2 in the macula densa and renin expression. A temporal relationship between COX-2 and renin expression, however, could not be deduced from those studies.

**Fig. 4.** Distribution of COX-2 immunoreactivity in rat kidney cortex from a rat bearing a renal artery clip for 2 days. A: incubation with COX-2 antiserum. B: incubation with preimmune serum (magnification \(\times 100\)). C: localization of COX-2 immunoreactivity in the macula densa (magnification \(\times 400\)). *COX-2 immunoreactivity.
Therefore, juxtaglomerular COX-2 and renin expression were also investigated in the model of acute renal artery stenosis.

After unilateral renal artery clipping, both cortical COX-2 mRNA and COX-2 immunoreactivity increased in parallel, suggesting that the increase of COX-2 in the macula densa is a rapid process that is due to enhanced de novo synthesis of COX-2 protein rather than to impaired degradation. The increase of COX-2 mRNA and protein expression in the macula densa preceded the increase of renin mRNA in the clipped kidney. Therefore, an enhanced formation of prostaglandins through COX-2 in the macula densa could in principle contribute to the increase of renin mRNA and consequently to the increase of renin synthesis.

In the intact contralateral kidney, both COX-2 immunoreactivity and renin mRNA decreased in parallel, providing the possibility that a reduced formation of prostaglandins could eventually contribute to the downregulation of renin expression in the contralaterals of stenosed kidneys.

We attempted to prove such a causal link between COX-2 expression and renin expression by the use of the COX-2 inhibitor celecoxib (19). In spite of a significant plasma concentration of ~6 \( \mu \text{mol/l} \), however, we did not observe an effect of celecoxib on plasma renin activity nor on renin mRNA expression in the 2K-1C rats. This lacking effect of celecoxib (SC-58635) is at odds with a previous study demonstrating that the structurally closely related COX-2 inhibitor SC-58236 (19) almost blunts the rises of plasma renin activity and of renin mRNA in rats with aortic coarctation (29). Although we cannot yet provide a convincing explanation for the different effects of celecoxib and SC-58236 on renin secretion and renin mRNA expression in hypoperfused kidneys, it should be noted that SC-58236 has a biological half-life time of several days in rats, whereas the half-life time for celecoxib is in the range of several hours (19). Daily administration of SC-58236 is therefore expected to cause accumulation of SC-58236 and to achieve markedly higher systemic and intracellular levels of SC-58236 than of celecoxib. The inhibitory effect of SC-58236 and the lacking effect of celecoxib on the renin system might therefore be explained by a rather low sensitivity of COX-2 in rat thick ascending limb of Henle (TALH) and macula densa toward systemic COX-2 blockers; this requires rather high plasma concentrations of the blockers, which were not achieved with a dose of 20 mg/kg two times daily, although this dose has already been shown to potently inhibit COX-2-mediated PGE\(_2\) formation in the rat in vivo (31). After oral administration, celecoxib reaches its maximal concentration in the kidney after 1 h and then declines with a half-life of 4.1 h (18a). Because we administered celecoxib in 12-h intervals, the average renal tissue concentration of celecoxib in our experiments should have been ~25% of the peak concentration. A threefold higher dose of celecoxib (120 mg/kg.day\(^{-1}\) ) was also without any effect on plasma renin activity and on renin mRNA abundance. We consider it therefore less likely from our findings that COX-2-derived prostaglandins are relevantly involved in the stimulation of renin secretion and of renin synthesis under conditions of normal or reduced renal blood flow. This conclusion is in accordance with a recent study with salt-depleted humans, which also did not reveal an influence of celecoxib on elevated plasma renin activity (21).

Another possibility to explain the striking effect of SC-58236 on the renin system is to assume a blockade of COX-1 activity by SC-58236, because both SC-58236 and celecoxib also block COX-1 activity at high concentrations (19). Previous studies have in fact shown that preferential COX-1 inhibitors are capable of attenuating the stimulation of renin secretion and of renin gene expression in response to renal hypoperfusion (2, 3, 5, 21).
14, 24). We have tried to assess the potential role of COX-1 in mediating the effects of preferential COX-2 blockers by treating rats with a combination of 20 mg/kg celecoxib and 5 mg/kg indomethacin two times daily. This treatment regimen, however, was detrimental for the rats, which suffered from massive gastrointestinal bleeding, causing a fall of the hematocrit value below 20%. We can therefore not rule from our findings that a partial inhibition of COX-1 by SC-58236 might contribute to the inhibition of the renin system by this particular drug.

Irrespective of the detailed role of COX-2-derived prostaglandins in the control of the renin system, the question arises about the mechanisms causing the up- and downregulation of COX-2 in the thick ascending limb, including the macula densa region in response to unilateral renal artery clipping. Our findings show that the induction of COX-2 in the clipped kidney takes <6 h, indicating that acute rather than chronic processes cause the induction of COX-2. Considering that, apart from renal artery clipping, a low-salt diet also causes an upregulation of COX-2 in the macula densa (12), an altered salt transport, in particular, a decrease of salt transport at the macula densa site, could be a common denominator for the induction of COX-2. A similar conclusion has been reached previously by Schnermann (22). The molecular mechanism, however, by which a reduced salt transport by the thick ascending limb of Henle and macula densa should activate the COX-2 gene remains unclear. Because unilateral renal artery stenosis causes hyperfiltration in the contralateral kidney (20), one could further speculate that an increased rate of salt transport at the macula densa site causes the downregulation of COX-2 there.

Taken together, our findings show that unilateral artery clipping leads to an acute and marked increase of COX-2 expression in the macula densa and to an upregulation of renin secretion and renin synthesis. Our data, however, do not support the concept that COX-2-derived prostaglandins are of major importance for the stimulation of the renin system by renal hypoperfusion.

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