Inhibition of the Renin-Angiotensin System Upregulates Cyclooxygenase-2 Expression in the Macula Densa

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Abstract—The expression of cyclooxygenase 2 (COX-2) in the late thick ascending limb, including the macula densa, is found to be upregulated in an activated renin-angiotensin system. How this upregulation is managed is not yet known. We therefore considered the possibility that the stimulation of COX-2 expression is triggered by the activation of the renin-angiotensin system. For this purpose, we treated male Sprague-Dawley rats with the angiotensin I–converting enzyme inhibitor ramipril (10 mg/kg per day), the angiotensin II type 1 (AT1) receptor blocker losartan (30 mg/kg per day), and the angiotensin II type 2 (AT2) receptor blocker PD123319 (6 mg/kg per day) for 4 days. We determined the expression of COX-2 mRNA and protein in the renal cortex. We found that ramipril and the AT1 receptor blocker losartan increased COX-2 mRNA and COX-2 immunoreactivity in the macula densa 4-fold, whereas the AT2 blocker PD123319 showed no effect. A low-salt diet (0.02% wt/wt) stimulated COX-2 expression in the kidney cortex 2-fold. The combination of a low-salt diet with ramipril led to a further increase of COX-2 mRNA and COX-2 immunoreactivity compared with low salt or ramipril alone. These data indicate that endogenous angiotensin II apparently inhibits COX-2 expression in the macula densa via AT1 receptors and can therefore not account for the stimulation of COX-2 expression associated with an activated renin-angiotensin system. Because macula densa–derived prostaglandins are considered stimulators of renin secretion and renin synthesis, inhibition of macula densa COX-2 by angiotensin II could form a novel indirect negative feedback control of the renin system. (Hypertension. 1999;34:503-507.)

Key Words: renin ■ prostaglandins ■ angiotensin II

Within the renal cortex, cyclooxygenase 2 (COX-2) is almost exclusively expressed in cells of the late thick ascending limb of Henle, including the macula densa cells.1–3 The macula densa cells are directly adjoined to the renin-producing cells of the afferent arterioles. Because prostaglandins in general4–6 and macula densa–derived prostaglandins in particular7,8 are well-known stimulators of renin synthesis and of renin secretion, it is possible to assume a major function of COX-2 in the control of the renin system. This assumption is supported by the observation that the stimulation of the renin system during low salt intake,9 renal artery stenosis,10 or furosemide treatment11 is attenuated by cyclooxygenase inhibitors. Moreover, it was found that the expression of COX-2 in the macula densa is markedly upregulated in the aforementioned conditions associated with an activated renin-angiotensin system (References 1 and 3 and K. Wolf, unpublished data, 1999). The signal pathways that lead to upregulation of COX-2 in the macula densa are not yet known. Because macula densa cells express angiotensin II (Ang II) receptors,12 it would be an obvious explanation that it is the activation of the renin-angiotensin system itself that induces and maintains COX-2 expression in the macula densa, thus generating a kind of positive feedback of renin secretion and renin gene expression during chronic challenges of the renin system. To the best of our knowledge, the influence of the renin-angiotensin system on macula densa COX-2 expression is not yet known. We therefore aimed to examine the effects of inhibition of Ang II formation on macula densa COX-2 expression in both a normal and an activated renin-angiotensin system.

Our results show that converting enzyme inhibition and Ang II type 1 (AT1) receptor blockade but not Ang II type 2 (AT2) receptor blockade potently stimulate COX-2 expression in the macula densa, which indicates that endogenous Ang II itself is an inhibitor of COX-2 expression in the macula densa. Consequently, Ang II cannot be the mediator for COX-2 expression in situations associated with an activated renin-angiotensin system. Conversely, it appears that Ang II could exert an indirect negative feedback effect on renin secretion and renin synthesis via inhibition of macula densa–derived prostaglandins.

Methods

Animal Experiments
Six groups of male Sprague-Dawley rats (weight, 220 to 250 g), each composed of 8 animals, were used for the experiments. Before the
experiments began, the drinking behavior of the animals was monitored to ensure that correct doses of the drugs were applied. Animals in group 1 remained untreated and served as controls. Animals in group 2 received the angiotensin-converting enzyme (ACE) inhibitor ramipril (10 mg/kg per day) via the drinking water for 4 days. Animals in group 3 received the AT1 receptor blocker losartan (30 mg/kg per day) via the drinking water for 4 days. In group 4, the AT1-specific blocker PD123319 was given daily by subcutaneous injection (6 mg/kg per day). Animals in group 5 were kept on a low-salt diet (0.02% wt/wt) for 10 days, and animals in group 6 were kept on a low-salt diet for 10 days and additionally received ramipril during the last 3 days (days 8 to 10). The rats were killed by decapitation.

The kidneys were removed and were cut in longitudinal halves. One half was placed and stored in fixation solution until COX-2 immunohistochemistry was determined. From the remaining halves, the cortices 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, and 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 minutes at room temperature. Sections were layered with the primary antibody and incubated at 4°C overnight. After the addition of the second antibody (dilution 1:500; biotin-conjugated, goat anti-rabbit immunoglobulin G or rabbit anti-goat immunoglobulin G, respectively), the sections were incubated with avidin D horseradish-peroxidase complex (Vectorstain DAB kit, Vector Laboratory) and exposed to 0.1% diaminobenzidine tetrahydrochloride and 0.02% H2O2 as a source of peroxidase substrate. Each slide was counterstained with hematoxylin. As a negative control, we used equimolar concentrations of preimmune rabbit or goat immunoglobulin G.

**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 antisem 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 cortices, basically according to the acid-guanidinium-phenol-chloroform protocol of Chomczynski and Sacchi.13 RNA pellets were dissolved in diethylpyrocarbonate-treated water, the yield of RNA was quantified by spectroscopy at 260 nm, and aliquot samples were stored at −80°C.

**Ribonuclease Protection Assays for COX-1, COX-2, Renin, and Cytoplasmic β-Actin**

COX-1, COX-2, renin, and β-actin mRNA levels were measured by RNase protection assays, basically as described.11,14,15 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-Pharmacia) according to the Promega riboprobe in vitro transcription protocol. cRNA probes (5×106 cpm) were hybridized with 20 μg of total RNA (COX-1 and COX-2), 20 μg of total RNA (renin), 1 μg of total RNA (β-actin), and 20 μg of tRNA (negative control) at 60°C overnight and then digested with RNase A/T1 (room temperature/30 minutes) and proteinase K (37°C/30 minutes). After phenol/chloroform extraction and ethanol precipitation, protected fragments were separated on an 8% polyacrylamide gel. The gel was dried for 2 hours, and bands were quantified in a Phosphoimager (Instant Imager 2024, Packard). Autoradiography was performed at −80°C for 1 to 3 days. The abundance of renin, COX-1, and COX-2 is presented relative to the level of β-actin mRNA as a standard.

**Statistical Analysis**

Data are presented as mean±SEM. Level of significance was calculated by ANOVA followed by Bonferroni’s test for multiple comparisons. *P*<0.05 was considered significant.

**Results**

Figure 1 shows a representative autoradiograph of RNase protection assays for renin, COX-1, and COX-2 mRNA with RNA from kidney cortex of 2 control and 2 ramipril-treated animals. The figure demonstrates that not only renin mRNA but also COX-2 mRNA is upregulated by treatment with the ACE inhibitor ramipril. Semi-quantification revealed that in animals treated with the ACE inhibitor, renocortical renin mRNA was 4-fold increased, which suggests effective interruption of the negative feedback control of renin synthesis by Ang II (Figure 2, top). In addition, COX-2 mRNA in the kidney cortex was increased ∼4-fold in these animals (Figure 2, middle), whereas the abundance of COX-1 mRNA was not different from that found in control rats (Figure 2, bottom). The COX-2 expression within the renal cortex was localized by immunohistochemistry. In both control and ramipril-treated rats, COX-2 immunoreactivity was confined to the late thick ascending limb and to the macula densa (Figure 3). In ramipril-treated animals, COX-2 immunoreactivity was increased ∼4-fold as semiquantified by the percentage of glomeruli that displayed COX-2 immunoreactivity in the macula densa region (Figure 4). Because these data suggested that endogenous Ang II is an inhibitor rather than a stimulator of macula densa COX-2 expression, it appeared of interest to us to further study the influence of converting enzyme inhibition during a stimulated renin-angiotensin system. Such stimulation was induced by a low-salt diet, as indicated by the increased levels of renal renin mRNA (Figure 2, top). A low-salt diet also increased cortical COX-2 mRNA levels (Figure 2, middle), although COX-1 mRNA remained unchanged (Figure 2,
The combination of the ACE inhibitor ramipril with a low-salt diet tended to further increase cortical COX-2 expression (Figure 2, middle), although this increase did not reach the defined level of significance because of the rather broad scatter of COX-2 mRNA in these animals. Considering the renin mRNA levels in the same groups, one can see that renin levels are also elevated in the low-salt diet plus ramipril group compared with the group with ramipril alone (Figure 2, top). To elucidate whether the COX-2 upregulation after converting enzyme blockade is mediated by angiotensin receptors, we performed additional experiments with the AT$_1$ receptor blocker losartan and the AT$_2$ receptor blocker PD123319. Ramipril treatment and losartan treatment for 4 days produced similar results of the increase of renin (Figure 5, top) and COX-2 mRNAs (Figure 5, bottom). PD123319, a highly specific blocker of AT$_2$ receptors, showed no influence on renin or COX-2 levels.

**Discussion**

COX-2 is generally considered an inducible enzyme. In the kidney cortex, however, it is constitutively expressed in the late thick ascending limb and macula densa. Within the macula densa, COX-2 expression can be further enhanced by...
low salt intake, renal hypoperfusion, and pharmacological inhibition of salt transport (K. Wolf, unpublished data, 1999). The cellular pathways within the macula densa that activate COX-2 gene expression are not yet known. A common feature of the aforementioned conditions that cause upregulation of COX-2 in the macula densa is that they are associated with enhanced renin secretion and consequently with enhanced formation of Ang II. Because macula densa cells express Ang II receptors, it is conceivable that Ang II stimulates COX-2 expression in the macula densa. Our data, however, are not consistent with this assumption, because we found that inhibition of Ang II formation in fact strongly enhanced COX-2 mRNA and protein expression in the macula densa. By inhibition of the AT₁ and the AT₂ receptors with specific drugs, we demonstrated that COX-2 upregulation in states of an inhibited renin-angiotensin system is mediated via AT₁ receptors. This excludes the hypothesis that elevated bradykinin levels might be responsible for the increase of COX-2 in the macula densa. A potential role for kinins in context with COX-2 should not be neglected per se because several studies found bradykinin to be a stimulator for COX-2 or prostaglandin E₂. Bradykinin-mediated prostaglandin E₂ release coupled with COX-2 upregulation has been demonstrated in human airway smooth muscle cells and in cultured fibroblasts. The degree of stimulation exerted by the converting enzyme inhibitor and the AT₁ receptor blocker losartan was much stronger than that induced by a low-salt diet, which produced a 2-fold stimulation, in agreement with previous reports. Because a low-salt diet per se stimulates renin secretion and Ang II formation, we wondered what would occur if the formation of Ang II was blocked during a low-salt diet. Our data show that in combination, both stimulators produce an additional increase of COX-2 expression, which would fit with the assumption that elevated levels of Ang II cause a feedback inhibition of COX-2 in the macula densa. Provided that Ang II directly acts on macula densa cells, it is reasonable to speculate on a common final step in the regulation of COX-2 in the macula densa, in which the rate of salt intake, salt transport activity, perfusion, and Ang II cooperate. The effects of low salt intake, salt transport inhibition, and renal hypoperfusion, all of which stimulate macula densa COX-2 expression, could lead to reduction of salt transport of the macula densa. The possibility that reduced salt transport could stimulate macula densa COX-2 expression was already postulated by Schnermann. Whether or not Ang II directly modulates salt transport in the macula densa is unknown.

Our data suggest that endogenous Ang II is a potent inhibitor of macula densa COX-2 expression. Because prostaglandins are stimulators of renin secretion and in consequence of Ang II formation, downregulation of macula densa COX-2 could represent a novel indirect feedback mechanism that contributes to the well-known inhibition of the renin system by Ang II (Figure 6).

Taken together, the results of our study indicate that endogenous Ang II does not mediate the stimulation of COX-2 expression in the macula densa that is seen in conditions associated with a stimulated renin-angiotensin system. Conversely, Ang II appears to be a potent physiological inhibitor of macula densa COX-2 expression. The cellular mechanisms by which Ang II inhibits COX-2 expression should be elucidated in further experiments.

Acknowledgments

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Ku 859/2-3). The expert technical assistance provided by Karl-Heinz Götz is gratefully acknowledged.

References


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Hypertension. 1999;34:503-507
doi: 10.1161/01.HYP.34.3.503

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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