Inhibition of Cyclooxygenase-2 in the Rat Renal Medulla Leads to Sodium-Sensitive Hypertension

Tewabech Zewde, David L. Mattson

Abstract—Cyclooxygenase-2 expression in the renal medulla is regulated by dietary salt intake. The present study was performed to determine the influence of chronic inhibition of medullary cyclooxygenase-2 on arterial blood pressure in conscious Sprague-Dawley rats maintained on a high-salt (4% NaCl) or a low-salt (0.4% NaCl) diet. Rats were uninephrectomized and instrumented with femoral arterial and femoral vein or renal medullary interstitial catheters. Each rat received a continuous medullary or intravenous infusion of saline (0.5 mL per hour) for 3 control days, followed by infusion of the cyclooxygenase-2 inhibitor NS-398 (10 mg/kg per day) for 5 days. Medullary interstitial infusion of NS-398 significantly increased mean arterial pressure in the 4% NaCl group from 126±2 to 146±2 mm Hg (n=6) but did not alter blood pressure in the 0.4% NaCl group (n=6). Intravenous infusion of NS-398 to rats on the 4.0% NaCl diet also failed to alter mean arterial pressure (n=5). To test the blood pressure effect of a mechanistically different inhibitor of cyclooxygenase-2, an antisense oligonucleotide against cyclooxygenase-2 (18-mer; 8 nmol per hour) was infused into the renal medulla of rats maintained on a high-salt diet. Administration of the antisense oligonucleotide reduced cyclooxygenase-2 immunoreactive protein by 36% and significantly increased mean arterial pressure from 127±2 to 147±2 mm Hg (n=6). Renal medullary interstitial infusion of a scrambled oligonucleotide did not alter arterial pressure (n=5). These results demonstrate the importance of cyclooxygenase-2 in the renal medulla in maintaining blood pressure during high-salt intake. (Hypertension. 2004;44:424-428.)

Key Words: kidney ▪ prostaglandins ▪ cyclooxygenase ▪ rats ▪ blood pressure

Prostaglandins are derived from arachidonic acid by the catalytic activity of cyclooxygenase (COX) and have potent effects in the kidney to alter hemodynamics and sodium and water excretion.1–4 The 2 COX isoforms, COX-1 and COX-2, have similar biochemical activity but differ with respect to cellular expression pattern and regulation.1,5,6 COX-1 is constitutively expressed in a variety of tissues, whereas COX-2 is induced by inflammatory stimuli and growth factors.7–9 COX-2 is also reported to be constitutively expressed in the kidney, lung, and brain.6,10–12 Both isoforms of COX have been shown to be expressed in the rat renal medulla, a major site of prostaglandin synthesis,6,11,13 but the isoforms are differentially localized. Although COX-1 is predominantly expressed in the collecting duct, COX-2 has been localized to medullary interstitial cells.11,14–16 Because the medullary interstitial cells span the area between the vasa recta and the medullary tubules,17 products of COX-2 may have important effects on renal medullary function. Indeed, it has been demonstrated recently that COX-2 inhibition leads to a significant reduction in medullary blood flow in mice.18 Renal expression of COX-2 also appears to be physiologically regulated; a high-salt diet has been demonstrated to increase COX-2 in the renal medulla, whereas a low-salt diet increased COX-2 in the renal cortex.19 Together, these studies suggest an important role of COX-2–derived prostaglandins in the renal handling of sodium and water.

The therapeutic use of nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with major side effects, particularly in the gastrointestinal tract. These effects are attributed to inhibition of COX-1–derived prostaglandins because products of COX-1 are considered to have a variety of functions including maintenance of gastric epithelial integrity. The introduction of COX-2 inhibitors has attracted a great deal of interest because these drugs reduce major gastrointestinal problems while providing analgesic and anti-inflammatory effects similar to that of NSAIDs.20,21 However, there is growing evidence for a physiological role of COX-2 in renal function suggesting that selective COX-2 inhibitors will result in adverse effects on kidney function. Selective COX-2 inhibitors have been shown to decrease prostacyclin production in humans22,23 and renal excretion of prostaglandins in rats.24 It has also been reported that COX-2 inhibition induced sodium retention and decreased renal blood flow.25,26 Prostaglandins derived from COX-2 have been reported to have potent effects on renal medullary blood flow and sodium and water excretion,3,18 and modulate the pressor response to angiotensin II18 and NO synthase inhibition.27 However, the influence of COX-2 inhibitors on blood pressure is not yet clear. In rats, prolonged treatment with COX-2 inhibitors led to an increase18,28 or no change29 in mean arterial blood pressure (MAP), whereas COX-2 inhibition in dogs increased...
blood pressure only during a low-salt diet.\textsuperscript{25} Similarly, the reports from human studies are also controversial, indicating an increase\textsuperscript{19} or no change\textsuperscript{22,26} in blood pressure in association with COX-2 inhibition. Because COX-2 is localized in the renal medulla and its expression has been shown to be regulated by dietary salt intake,\textsuperscript{13} we investigated the effect of chronic blockade of medullary COX-2 on blood pressure regulation in conscious Sprague-Dawley rats that received a low- or high-NaCl diet.

### Methods

Experiments were performed on male Sprague-Dawley rats (weighing 250 to 300 g) obtained from Harlan Laboratories (Madison, WI). Rats were housed in the animal resource center at the Medical College of Wisconsin with rat chow and tap water provided ad libitum. All animal procedures were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

#### Western Blotting Protocol

Rats were maintained on a low- or high-salt diet for 2 weeks before the study. The animals were euthanized with an overdose of sodium pentobarbital intraperitoneally; pieces of the renal medulla were removed rapidly and frozen on dry ice, and the protein was extracted as described previously.\textsuperscript{30} Protein samples were electrophoretically size separated using a discontinuous system consisting of a 12% polyacrylamide resolving gel and a 5% polyacrylamide stacking gel. High-range molecular weight markers (\textasciitilde 40 to 200 kDa) were loaded into 1 lane as a size standard. Equivalent amounts of total protein from rats on a low- and high-salt diet were added to adjacent lanes, and the samples were run at 200 V for 60 minutes on an 8 \times 10 cm electrophoresis cell (Bio-Rad). After separation, proteins were electrophoretically transferred to a nitrocellulose membrane at 100 V for 1 hour. These membranes were washed in Tris-buffered saline (TBS), blocked with 5% nonfat dried milk (NFM) in TBS (NFM/ TBS) for 2 hours, and incubated with a 1:1000 dilution of affinity-purified rabbit antiserum to COX-2 (Cayman Chemical) for 2 hours at room temperature. After washing with TBS, blots were incubated with horseradish peroxidase–labeled goat anti-rabbit IgG in 2% NFM/TBS for 2 hours. The bound antibody was detected by enhanced chemiluminescence (ECL; Amersham) on x-ray film. A monoclonal mouse antibody raised against the structural protein \(\beta\)-actin (Sigma) was used as a loading control. Membranes were stripped between incubations with the different antibodies in a Tris-buffered solution containing 2% sodium dodecyl sulfate and 100 mmol/L \(\beta\)-mercaptoethanol at 50°C.

#### Surgical Preparation and Studies in Conscious Rats

Rats were anesthetized with a mixture of ketamine (50 mg/kg IM) and acepromazine (5 mg/kg IM), and the right kidney was removed. The nephrectomy was performed so that the remaining kidney was the sole determinant of renal function. After 10 days of recovery, catheters were implanted in the femoral artery, femoral vein, or renal medullary interstitium as described previously.\textsuperscript{30} After surgical instrumentation, each rat was housed individually in a specially designed stainless steel cage and received a continuous medullary interstitial infusion of the vehicle in saline (0.5 mL per hour) while infused intravenously at the same rate (0.5 mL per hour).

To examine the influence of COX-2 inhibition using a mechanistically different inhibitor, additional experiments were performed in which an antisense oligonucleotide against COX-2 was infused into the medullary interstitial space. In this experiment, rats were maintained on the 4.0% NaCl diet. Saline was infused into the medullary interstitial catheter for 3 control days followed by a 5-day intramedullary infusion of a phosphorothioated antisense oligonucleotide for COX-2 (5'-CTCGGAAGAGCATCAGCAG-3'; 8 nmol per hour). A phosphorothioated scrambled oligonucleotide (5'-CATGCGTAGGGACGCAG-3'; 8 nmol per hour) was infused into an identically prepared group of rats as a control. The dose of antisense oligonucleotide used is the same as that used in our previous studies.\textsuperscript{30} After 5 days of infusion of the antisense or scrambled oligonucleotides, animals were euthanized with an overdose of sodium pentobarbital intraperitoneally, the kidney was removed, and pieces of the renal medulla were rapidly removed and prepared for Western blotting to determine COX-1 and COX-2 protein as described above.

#### Statistics

Data are presented as means\(\pm\)SE. The comparison of MAP between groups on different diets and between intravenous and intramedullary infusion was made with a 2-way ANOVA and a Tukey post hoc test. The differences in Western blot densitometry were evaluated using an unpaired \(t\) test. A confidence level of \(P<0.05\) was considered significant.

#### Results

#### Effects of Dietary Salt Intake on COX-2 Expression in the Renal Medulla

As shown in Figure 1, administration of a high-salt diet (8.0% NaCl; \(n=6\)) to Sprague-Dawley rats for 2 weeks led to significantly greater immunoreactive COX-2 protein in the renal medulla compared with rats maintained on a low-sodium diet (0.4% NaCl; \(n=6\)) for 2 weeks. Densitometry revealed that the amount of COX-2 was increased by 40% in the high-salt rats. The \(\beta\)-actin signal was unaltered in the renal medulla between rats on the low- and high-salt diets. In a separate experiment, no differences were detected in
COX-2 immunoreactive protein in the renal medulla of rats maintained on a 4.0% NaCl diet compared with rats maintained on a 0.4% NaCl diet. Effect of Chronic Infusion of a COX-2 Inhibitor on MAP of Conscious Rats

As shown in Figure 2, in rats that received medullary infusion, the control MAP in the 4.0% NaCl diet group was greater than that of the low-salt (0.4% NaCl) diet group. Renal medullary interstitial infusion of NS-398 to the rats maintained on the 4.0% NaCl diet led to a significant increase in MAP. The control MAP averaged 126/110 mm Hg and increased to 146/120 mm Hg after a 5-day medullary interstitial infusion of the COX-2 inhibitor (n=6). Rats maintained on the low-salt diet had a similar level of arterial blood pressure during the control period (116/100 mm Hg) as was observed after the 5-day medullary interstitial infusion of NS-398 (n=6). In contrast to the effect of intramedullary infusion of the COX-2 inhibitor to rats on a 4.0% NaCl high-salt diet, intravenous infusion of the same dose of NS-398 to rats on a high-salt diet did not change the MAP from the control value of 118/2 mm Hg (n=5). Interestingly, MAP was significantly decreased after 1 day of NS-398 infusion into the renal medulla of rats on the 0.4% NaCl diet and after a single day of intravenous infusion to rats maintained on the 4.0% NaCl diet.

Effect of Chronic Renal Medullary Infusion of an Antisense Oligonucleotide for COX-2 on MAP and COX-2 Protein Expression in the Renal Medulla

To test the blood pressure effect of a mechanistically different inhibitor of COX-2, an antisense oligonucleotide against COX-2 or a scrambled oligonucleotide (18-mer; 8 nmol per hour) was infused into the renal medullary interstitial space of rats maintained on the high-salt diet (4.0% NaCl). Administration of the antisense oligonucleotide led to a significant increase in MAP from 127±2 to 147±2 mm Hg (n=6; Figure 3). As a control for the antisense infusion, renal medullary interstitial infusion of a scrambled oligonucleotide did not alter MAP from control levels (124±2 mm Hg; n=5). To document the specificity of the treatment with the antisense oligonucleotide, the amount of immunoreactive COX-2 and COX-1 protein in the renal medulla of rats infused with the antisense and scrambled oligonucleotides was compared by Western blotting. As illustrated in Figure 4, infusion of the antisense oligonucleotide for COX-2 for 5 days significantly decreased COX-2 immunoreactive protein in the renal medulla by 36% compared with rats that were infused with the scrambled oligonucleotide. However, the antisense oligonucleotide did not affect the COX-1 immunoreactive protein in the renal medulla.

Discussion

COX-2 is localized predominantly in the medullary interstitial cells, and its expression has been shown to be regulated physiologically. The present data indicate that a high-salt diet significantly increased COX-2 immunoreactive protein in the renal medulla when compared with the medulla of rats maintained on a low-salt diet. This observation is consistent with previous studies that demonstrated increased COX-2 protein in the renal medulla of rats maintained on a high-salt diet. Although no difference was detected in COX-2 protein between rats that received 4% NaCl and 0.4% NaCl diet, these rats showed a difference in the response to COX-2 inhibition. Thus, selective infusion of the COX-2 inhibitor NS-398 into the renal medulla led to a sustained increase in...
Our data showed that the same intramedullary infusion concentrates the infused compounds after a single day of intramedullary infusion of NS-398 at a concentration of NS-398 that increased MAP when administered into the renal medulla did not significantly affect MAP when given intravenously. Although there was a tendency for MAP to increase during intravenous administration of NS-398, there were no statistically significant changes in blood pressure, thus indicating that the observed increase in MAP during intrastitial infusion of NS-398 is likely attributable to the direct inhibitory effect of this drug on COX-2 in the renal medulla rather than systemic effects.

The present data indicate that the hypertensive effect of NS-398 was attributable to blockade of production of COX-2 products in the renal medulla. The importance of prostaglandins to maintain medullary blood flow and promote sodium excretion is well documented. Studies in anesthetized mice demonstrated that COX-2 inhibition decreased medullary blood flow as well as urine volume and sodium excretion, whereas a COX-1 inhibitor had no such effect, thus indicating the role of COX-2 in modulating medullary blood flow and salt excretion.33 Both isoforms of COX are expressed abundantly in the renal medulla; however, they are localized differentially, with COX-1 expressed primarily in collecting duct and COX-2 predominantly in interstitial cells of the renal medulla.1 Cultured medullary interstitial cells have been shown to synthesize COX-2-derived prostaglandin E2 (PGE2), which has been shown to dilute the vasa recta and hence modulate the renal medullary microcirculation and sodium excretion.35 PGE2 is also shown to inhibit NaCl absorption in the thick ascending limb and medullary collecting duct.36 Moreover, targeted disruption of EP3 prostaglandin receptor, the dilatory PGE2 receptor, led to hypertension in response to an elevated salt intake in mice.37 These studies demonstrate an important role of PGE2 in enhancing sodium and water excretion that protects systemic blood pressure from hypertension during salt intake. COX-2 is also suggested as a major source of vascular endothelial PGH2, a potent vasodilator.22,23 In addition, salt intake has been shown to increase plasma level of 6-keto PGF1α (prostaglandin F), which is attenuated by administration of COX-2 inhibitors.28 The hypertensive effect of COX-2 blockade in our studies is therefore likely attributable to inhibition of COX-2-derived prostanoids that mediate the renal tubular and vascular response to salt intake.

In the present experiments, infusion of 2 mechanismcally different inhibitors of COX-2 (NS-398 or an antisense oligonucleotide) into the renal medullary interstitial space led to a similar magnitude increase in arterial blood pressure. Selectivity of the COX-2 inhibitor NS-398 has been demonstrated previously in both in vitro and in vivo preparations.36,37 Despite these previous data, there is a possibility that NS-398 had an effect to inhibit COX-1 and COX-2 when infused into the renal medullary interstitial space. To address the possibility that COX-1 could be mediating the observed effects, we used an antisense oligonucleotide to inhibit COX-2 protein. The selectivity of the antisense was documented by demonstrating that treatment with the antisense oligonucleotide significantly decreased COX-2 protein without affecting COX-1 protein in the renal medulla of treated rats. Thus, the results from the present study indicate that the effects observed were attributable to alterations in COX-2.

It was mildly surprising to observe that arterial blood pressure increased fairly rapidly during the initial 24-hour infusion of NS-398. It is possible that the increase in MAP is arterial blood pressure in rats maintained on a high-salt (4% NaCl) diet. In contrast, medullary interstitial infusion of NS-398 failed to alter blood pressure when administered to rats maintained on a low-salt (0.4% NaCl) diet. Therefore, we conclude that COX-2 products in the renal medulla are important in the maintenance of a constant blood pressure during changes in sodium intake. Because we did not detect a change in COX-2 protein between the rats on the 0.4% and 4.0% NaCl diet, it is presently unclear how important an increase in COX-2 protein is to this response.

In support of the importance of COX-2 in the renal medulla is the observation that systemic administration of NS-398 to rats on a high-salt diet did not lead to a change in blood pressure. Moreover, chronic administration of a mechanismcally different inhibitor of COX-2, an antisense oligonucleotide, into the renal medullary interstitial space also led to an increase in arterial pressure in rats on a high-sodium diet. Together, these results indicate that COX-2 in the renal medulla is important in the maintenance of arterial blood pressure when rats are placed on a high-sodium diet.

Chronic oral administration of the COX-2 inhibitors celecoxib or rofecoxib at doses of 10 to 30 mg/kg per day caused an increase in blood pressure after 1 week, whereas in the present study, an increase in blood pressure was achieved after a single day of intramedullary infusion of NS-398 at a dose of 10 mg/kg per day. Previous studies have shown that intramedullary infusion concentrates the infused compounds in the renal medulla.31 Our data showed that the same
attributable to sodium retention. Alternatively, there could have been an alteration in the release of lipid-like factors from the renal medulla or alterations in renal afferent nerve activity, which could be mediating the changes observed in the present study. We can only speculate in regard to the mechanism of hypertension in this model at the present time.

In summary, the present studies demonstrate an upregulation of COX-2 immunoreactive protein in the renal medulla in response to dietary salt intake. Chronic infusion of the COX-2 selective inhibitor NS-398 or antisense oligonucleotide for COX-2 into the renal medullary interstitial space led to hypertension during high-salt intake. Similar hypertensive effect was not observed during low-sodium intake. These results indicate the importance of COX-2 in the renal medulla in homeostasis of fluid and electrolyte balance and arterial blood pressure after an increase in dietary salt intake.

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References
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