Upregulation of cortical COX-2 in salt-sensitive hypertension: role of angiotensin II and reactive oxygen species

Edgar A. Jaimes,¹,²,⁴ Ming-Sheng Zhou,¹,² Damien D. Pearse,³ Leopold Puzis,³ and Leopoldo Raij¹,²,⁴

¹Department of Veterans Affairs Medical Center, ²Vascular Biology Institute, ³The Miami Project to Cure Paralysis, and ⁴Renal Division, Miller School of Medicine, University of Miami, Miami, Florida

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Jaimes EA, Zhou MS, Pearse DD, Puzis L, Raij L. Upregulation of cortical COX-2 in salt-sensitive hypertension: role of angiotensin II and reactive oxygen species. Am J Physiol Renal Physiol 294: F385–F392, 2008. First published December 19, 2007; doi:10.1152/ajprenal.00302.2007.—Salt-sensitive (SS) hypertension is a vascular diathesis characterized by reduced cardiovascular and renal nitric oxide bioavailability and local upregulation of ANG II. We have demonstrated that rats infused with ANG II manifest increased cortical cyclooxygenase (COX)-2 expression and activity via NADPH oxidase-derived reactive oxygen species (ROS). In the present studies we used Dahl salt-sensitive (DS) rats to test the hypothesis that hypertensive SS rats have increased cortical COX-2 upregulation, which is mediated by ANG II and ROS. DS rats were placed on either a normal-salt diet (0.5% NaCl) or a high-salt diet (4% NaCl) for 6 wk and treated with either the ANG II type 1 (AT₁) receptor blocker candesartan (Can, 10 mg·kg⁻¹·day⁻¹) or the SOD mimetic tempol (1 mmol/l). Hypertensive SS rats had a twofold increase in the cortical expression of COX-2 as assessed by Western blot. These changes in COX-2 expression were accompanied by a 10-fold increase in COX-2 mRNA expression and a 2-fold increase in the urinary excretion of PGE₂. Treatment with either the AT₁ receptor blocker Can or the SOD mimetic tempol did not reduce blood pressure but resulted in significant reductions in the cortical expression of COX-2 and the urinary excretion of PGE₂. In conclusion, we have demonstrated that local activation of the renin-angiotensin system, via increased ROS generation, mediates COX-2 upregulation in hypertensive SS rats. These studies unveil novel mechanistic pathways that may play a role in the pathogenesis of hypertensive renal injury.

METHODS

Experimental groups. Six-week-old male DS rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and maintained under controlled conditions of light, temperature, and humidity. The animals were housed in facilities accredited by the American Association for Accreditation of Laboratory Animal Care. These studies were approved by the Institutional Animal Care and Use Subcommittee at the Miami Department of Veterans Affairs Medical Center. After 2-wk accommodation to the new environment, the rats were divided into four groups and treated for 6 wk as follows: NS, fed 0.5% NaCl diet (n = 6); HS, fed 4% NaCl diet (n = 6); HS/Can, fed 4% NaCl diet plus the AT₁ receptor blocker candesartan (10 mg·kg⁻¹·day⁻¹ by gavage; n = 6); HS/tempol, fed 4% NaCl diet plus the superoxide dismutase (SOD) mimic tempol (1 mmol/l in the drinking water; n = 6). Mean blood pressure was measured weekly by the tail cuff method (Visitech Systems, Apex, NC). A separate group of rats were divided into three groups and treated for 6 wk as follows: NS, fed 0.5% NaCl diet (n = 6); HS, fed 4% NaCl diet (n = 6); HS/AmI, fed 4% NaCl diet plus the calcium channel blocker amiodipine (10 mg·kg⁻¹·day⁻¹ by gavage; n = 6). Systolic blood pressure (SBP) was measured in conscious rats by the tail cuff method after the 6 wk of treatment.

COX protein expression. COX-2 and COX-1 protein expression in renal cortex and renal medulla were determined by Western blot as

blockade or direct NADPH oxidase inhibition with Gp91-ds-tat peptide normalizes endothelial dysfunction and ROS independently of blood pressure.

In previous studies in Sprague-Dawley rats we demonstrated (11) that ANG II infusion increases glomerular cyclooxygenase (COX)-2 expression and activity via NADPH oxidase-derived ROS. Moreover, studies by others have shown that renal cortical COX-2 is increased in several models of renal disease that are characterized by activation of the RAS, such as renal ablation (34), renovascular hypertension (17), diabetes (15), and the stroke-prone spontaneously hypertensive rat (30). The two enzymes that are rate limiting in the synthesis of prostaglandins are phospholipase A₂, which releases arachidonic acid from phospholipids in cell membranes, and COX, which catalyzes the conversion of arachidonic acid to PGG₂ and to PGH₂ (6). COX-1 is constitutively expressed in most cell types (24, 28). COX-2 is induced in response to a variety of stimuli including proinflammatory cytokines (5) and growth factors (25, 27) and in the kidney is constitutively expressed in the macula densa and thick ascending limb (3). In the present studies we used DS rats to test the hypothesis that hypertensive SS rats have increased renal COX-2 upregulation, which is mediated by ANG II and ROS.

THE DAHL SALT-SENSITIVE (DS) RAT is a paradigm of human salt-sensitive (SS) hypertension, a type of hypertension that affects over 50% of hypertensive patients (35). Patients with SS hypertension are at a significantly higher risk for the development of hypertensive end-organ damage including atherosclerosis, endothelial dysfunction, and renal injury (36). We (36) and others have shown that SS hypertension is a vascular diathesis characterized by reduced cardiovascular and renal nitric oxide (NO) bioavailability and local upregulation of the renin-angiotensin system (RAS). Indeed, when measured directly in rats with SS hypertension, there is elevated intrarenal angiotensinogen, accompanied by increased expression of the angiotensin II (ANG II) type 1 (AT₁) receptor and sustained ANG II levels (13). Endothelial dysfunction in SS hypertension is associated with upregulation of NADPH oxidase and an increased production of reactive oxygen species (ROS) (36). We have shown (37) that either AT₁ receptor
previously described (11). Briefly, after death, the renal cortex was dissected and homogenized and protein was separated on 6% SDS gels under reducing conditions and transferred to a nitrocellulose membrane (Hybond ECL, Amersham Biosciences, Piscataway, NJ). The blots were incubated overnight with rabbit anti-murine polyclonal antibody to COX-2 or COX-1 (catalog no. 160126, Cayman, Ann Arbor, MI) or actin (Santa Cruz Biotechnology, Santa Cruz, CA). After the blots were washed they were incubated with goat anti-rabbit antibody (Santa Cruz Biotechnology) for 1 h at a 1:2,000 dilution, and the signal was detected by luminol chemiluminescence.

**Immunohistochemistry.** After death, kidneys were harvested and fixed in Tissue-Tek Express Molecular Fixative (Sakura, Torrence, CA). For antigen retrieval, slides were immersed in target retrieval solution (Dako Cytomation, Carpinteria, CA) for 30 min at 90°C. Immunoreactivity for specific proteins was localized with polyclonal rabbit anti-murine COX-2 antibody (Cayman) or polyclonal rabbit anti-murine COX-1 antibody (Cayman). As secondary antibody we used goat anti-rabbit IgG (Santa Cruz Biotechnology). Visualization was accomplished by subsequent use of an alkaline phosphatase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) followed by development with the Vectastain ABC-Elite Kit (Vector, Burlingame, CA). COX-2 and COX-1 expression were quantitated in at least three different areas per slide by measuring stain intensity (NIH Image). Macula densa COX-2 expression was quantitated by counting the number of stained macula densa cells and normalizing for the total number of glomeruli.

**Real-time PCR.** COX-2 mRNA expression was determined by real-time PCR. Total RNA was isolated with the RNeasy mini kit (Qiagen, Valencia, CA). A 5-μg aliquot of total RNA was used for cDNA synthesis with the Superscript preamplification system (Life Technologies). Primers and probes for COX-2 were designed with Primer Express software 101 (ABI). As an active reference, endogenous 18S ribosomal RNA was amplified with specific primers and probes labeled with VIC (ABI). The comparative threshold cycle (CT) method was used for relative quantification and statistical analysis. A unit difference in cycle value represents a twofold change in mRNA abundance.

**PGE2 measurements.** Urinary PGE2 was measured by enzyme immunoassay (Cayman) according to the manufacturer’s instructions and adjusted for 24-h urine volume.

**Urinary protein excretion.** Urinary protein excretion was measured by Bio-Rad assay and adjusted for urine volume in 24 h.

**RESULTS**

**Cortical COX expression in hypertensive SS rats.** First we determined by Western blot whether COX-2 is upregulated in a SS model of hypertension that is associated with significant end-organ injury and increased intrarenal RAS activation. Six-week-old DS rats were fed either a normal-salt diet (0.5% NaCl diet, NS) or a high-salt diet (4% NaCl, HS) for 6 wk. Blood pressure was measured by the tail cuff method. As expected, a high-salt diet resulted in significant increases in SBP: NS 149 ± 3 vs. HS 206 ± 5 mmHg (n = 6, P < 0.05). Hypertensive SS rats had a significant increase in the cortical expression of COX-2 as assessed by Western blot (Fig. 1), accompanied by concomitant increases in the urinary excretion of PGE2 (Fig. 2). These findings therefore demonstrate that hypertension in SS rats is accompanied by significant increases in cortical COX-2 expression and activity.

Using immunohistochemistry (Fig. 3, A and B), we observed COX-2 expression in scattered single cells or small groups of cells in the macula densa region and thick ascending limb of both normotensive and hypertensive SS rats. We did not observe a significant increase in the number of cells expressing COX-2 or changes in COX-2 distribution in hypertensive SS rats compared with their normotensive counterparts, suggesting that the increases in COX-2 as assessed by Western blot are likely due to increases in the amount of COX-2 per cell (NS 22.5 ± 3 vs. HS 24.3 ± 4 positive cells/100 glomeruli; P = not significant).

To determine whether transcriptional mechanisms are involved, we measured COX-2 mRNA expression by real-time PCR. Hypertensive SS rats had a significant increase in COX-2 mRNA expression compared with their normotensive counterparts: NS ΔCT 17 ± 0.9 vs. HS ΔCT 11.98 ± 1.95 (P < 0.05;
a 1-point reduction in ΔC_T equals a 2-fold increase in mRNA expression). These findings suggest that the observed increase in cortical COX-2 protein expression in hypertensive SS rats is mediated via transcriptional mechanisms.

To determine whether hypertension in SS rats is also linked to increases in COX-1 expression, COX-1 protein expression was measured by Western blot. As shown in Fig. 4, hypertensive SS rats had significant increases in cortical COX-1 expression compared with their normotensive counterparts. Using immunohistochemistry, we observed COX-1 expression in occasional glomeruli (Fig. 5, A and B) as well as in the distal convoluted tubule and cortical collecting duct of both normotensive and hypertensive DS rats (Fig. 5, D and E). Although we did not observe changes in distribution, we observed occasional groups of cells that displayed significantly greater intensity of COX-1 immunoreactivity in hypertensive SS rats compared with their normotensive counterparts [NS 65.4 ± 7 vs. HS 146.9 ± 8 optical density (OD) units; P < 0.05].

Medullary COX expression in salt-sensitive hypertensive rats. To determine whether COX-2 is upregulated in the renal medulla of hypertensive DS rats we measured medullary COX-2 expression in DS rats on either a normal-salt or a high-salt diet. As shown in Fig. 6, hypertensive SS rats on the high-salt diet had a significant increase in medullary COX-2 expression compared with their normotensive counterparts. Immunohistochemistry studies showed similar COX-2 distribution in the outer and inner medulla of both normotensive and hypertensive SS rats (Fig. 3, D–I), although there was a significant increase in the intensity of COX-2 staining in the inner medulla (NS 107.7 ± 12 vs. HS 157.4 ± 9 OD units; P < 0.05) but not in the outer medulla (NS 107.9 ± 8 vs. HS 111.4 ± 11 OD units; P = not significant). In contrast to our findings in the renal cortex, COX-1 expression was significantly reduced in the medulla of hypertensive DS rats as assessed by Western blot compared with their normotensive counterparts (Fig. 7). By immunohistochemistry we observed significant COX-1 expression in the collecting ducts.
from both outer and inner medulla (Fig. 5, G and H). In hypertensive SS rats, the intensity of COX-1 staining as assessed by immunohistochemistry was diminished compared with their normotensive counterparts (NS 95.7 ± 5 vs. HS 48 ± 8 OD units; \( P < 0.05 \)).

**Effects of AT\(_1\) receptor blockade on COX expression in hypertensive SS rats.** To determine the role of AT\(_1\) activation on COX-2 expression in hypertensive SS rats, a separate group of SS rats was put on a 4% NaCl diet and treated with the AT\(_1\) receptor blocker Can (10 mg kg\(^{-1}\) day\(^{-1}\) by gavage) for 6 wk.
Fig. 6. Kidney medulla COX-2 protein expression in salt-sensitive rats. Kidney medulla COX-2 protein expression is increased in hypertensive salt-sensitive rats compared with their normotensive counterparts. Treatment with the AT1R blocker Can or the SOD mimetic tempol increases COX-2 expression.

A: representative Western blots for COX-2 and actin, which was used to control for unequal loading. Lane 1, NS; lane 2, HS; lane 3, HS/Can; lane 4, HS/tempol. B: densitometry data analysis performed after reprobing the blot with an α-actin antibody (n = 6; *P < 0.05 vs. all other conditions).

Treatment with Can did not significantly reduce SBP: HS + Can 196 ± 7 vs. HS 206 ± 6 mmHg (n = 6, P = not significant) or proteinuria (Fig. 8) but significantly reduced COX-2 protein expression in the renal cortex as assessed by Western blot (Fig. 1). This change in COX-2 expression was accompanied by concomitant reductions in urinary excretion of PGE2 (Fig. 2). As shown in Fig. 6, and in contrast with our findings in the renal cortex, AT1 receptor blockade with Can resulted in further increases in medullary COX-2 expression. COX-1 expression was not modified by treatment with Can, suggesting that ANG II does not mediate cortical COX-1 expression in hypertensive SS rats (Fig. 4). In the renal medulla, AT1 receptor blockade in hypertensive SS rats increased COX-1 expression to the levels of their normotensive counterparts (Fig. 7), suggesting that in hypertensive SS rats ANG II downregulates COX-1 expression in the renal medulla.

Role of ROS as mediators of COX expression in SS rats. In a model of ANG II-induced hypertension we established the mechanistic association among ANG II, the AT1 receptor, ROS, and COX-2 (11). To determine the role of ROS on COX-2 expression in vivo in hypertensive SS rats a group of rats was placed on a high-salt diet (4% NaCl) and treated with the SOD mimetic tempol (1 mmol/l) in the drinking water for 6 wk. Treatment with tempol resulted in a small but nonsignificant reduction in blood pressure: HS 206 ± 6 vs. HS + tempol 193 ± 3 mmHg (n = 6, P = not significant vs. HS). Renal cortical COX-2 expression was measured by Western blot. Tempol treatment resulted in a significant reduction in cortical COX-2 expression (Fig. 1) and urinary PGE2 excretion (Fig. 2), suggesting that ROS mediate COX-2 expression in hypertensive SS rats. In contrast, treatment with tempol further increased COX-2 expression in the medulla of hypertensive DS rats (Fig. 6). Tempol did not significantly modify cortical COX-1 expression (Fig. 4), suggesting that ROS do not play a major role as modulators of cortical COX-1 expression and increased medullary COX-1 expression to the levels of their normotensive counterparts (Fig. 7).

Effects of blood pressure reduction on COX-2 expression in hypertensive Dahl salt-sensitive rats. To determine the effects of blood pressure reduction on COX-2 expression we studied a separate cohort of SS rats divided into three groups: NS (0.5% NaCl; n = 6), HS (4% NaCl; n = 6), and HS/Aml (10 mg·kg⁻¹·day⁻¹ by gavage; n = 6). Blood pressure was measured by the tail cuff method. SBP was significantly higher in the HS group (187 ± 6 mmHg) compared with the NS group (143 ± 4 mmHg; P < 0.05 vs. HS). Treatment with Aml resulted in significant reductions in SBP (159 ± 2 mmHg; P < 0.05 vs. HS). In contrast to the experiments utilizing Can or tempol, treatment with Aml did not reduce cortical COX-2 expression (Fig. 9) and resulted in a reduction, albeit not significant, in medullary expression of COX-2 (Fig. 10). Treatment with Aml also did not reduce, but significantly increased, the urinary excretion of PGE2 (Fig. 11). These findings suggest that the increased expression of COX-2 observed in these rats is not related to the

Fig. 7. Kidney medulla COX-1 protein expression in salt-sensitive rats. Kidney medulla COX-1 protein expression is reduced in hypertensive salt-sensitive rats compared with their normotensive counterparts. Treatment with the AT1R blocker Can or the SOD mimetic tempol increases COX-1 expression. A: representative Western blots for COX-2 and actin, which was used to control for unequal loading. Lane 1, NS; lane 2, HS; lane 3, HS/Can; lane 4, HS/tempol. B: densitometry data analysis performed after reprobing the blot with an α-actin antibody (n = 6; *P < 0.05 vs. all other conditions).
hemodynamic effects of hypertension in this model of SS hypertension.

DISCUSSION

In these studies we have demonstrated for the first time that hypertensive SS rats have an increase in cortical COX-2 expression that is mediated by ANG II and ROS. We also found increases in COX-2 expression in the medulla of hypertensive SS rats that, in contrast with the renal cortex, are not mediated by ANG II or ROS. Importantly, our studies also suggest that COX-2 upregulation in these animals is not dependent on the hemodynamic stress of hypertension.

COX-2 is induced in response to different stimulants including ANG II (11), growth factors such as PDGF (33) and transforming growth factor-β, and proinflammatory cytokines such as IL-1 and TNF-α (5). Renal COX-2 expression is also increased in conditions associated with activation of the RAS including renal ablation (34), diabetes (15), ureteral obstruction (22), diuretic administration, and low-salt diet (7). Although COX-2 is considered the inducible isofrom, there is constitutive expression of COX-2 in several segments of the nephron such as the macula densa and the medullary interstitium (7). COX-1 on the other hand is constitutively expressed in most cell types, although its expression can also be regulated (28).

In our studies we have demonstrated a significant increase in the cortical expression of COX-2 in hypertensive SS rats that was accompanied by concomitant increases in the urinary excretion of PGE2. This increase in COX-2 was prevented by treatment with an AT1 receptor blocker, suggesting that these effects are mediated via activation of the ANG II AT1 receptor. Our findings support our previous studies (11) in hypertensive ANG II-infused rats, as well as studies by others (23) in ANG II-infused mice, suggesting a role for ANG II as a mediator of COX-2 activity in the kidney. Moreover, maneuvers that are associated with activation of the RAS such as diuretic administration and low-salt diet (7) have been shown to increase COX-2 expression at the macula densa, supporting the view that activation of the RAS increases COX-2 expression and activity. Importantly, in the present studies AT1 receptor blockade reduced COX-2 expression without significantly lowering blood pressure or proteinuria, suggesting that the increase in COX-2 expression in SS rats is independent from the hemodynamic stress of hypertension. We also observed a significant increase in COX-1 cortical expression that was not modified by AT1 blockade, suggesting that, in contrast to COX-2, the expression of COX-1 in hypertensive SS rats is independent from ANG II. We speculate that, in contrast to COX-2, the expression of COX-1 is more dependent on the hemodynamic stress of hypertension. In the renal medulla we also observed significant increases in COX-2 expression that were further increased by ANG II and that, on the contrary, ANG II may have a downregulatory effect on medullary COX-2 expression. In addition, and in contrast with our findings in the renal cortex, SS hypertension resulted in reductions in medullary COX-1 expression that were normalized by treatment with an AT1 receptor blocker, suggesting that ANG II downregulates COX-1 expression in the renal medulla of hypertensive SS rats. Approximately 50% of all hypertensive patients can be classified as salt sensitive (35). Clinically they are characterized by an increased risk for the development of cardiovascular complications including atherosclerosis, endothelial dysfunction, and proteinuria (2). SS hypertension has been considered a volume-dependent hypertension because these subjects have low plasma levels of renin. However, previous studies in the DS rat, a paradigm of SS hypertension in humans, have demonstrated that in SS hypertension there is increased local activation of the RAS, reduced NO bioavailability, and increased ROS production (13, 20, 32). In support of this notion, treatment with AT1 receptor blockers reduces cardiac and/or renal dysfunction in hypertensive SS rats (8, 14, 21), suggesting that the local RAS may be inappropriately activated and contributes to the development of end-organ injury in SS hypertension. Administration of a high-salt diet to salt-resistant rats has been shown to result in significant suppression of the intrarenal levels of ANG II (13). In contrast, a high-salt diet in SS rats results in maintained kidney levels of ANG II, increased levels of angiotensinogen, and increased expression of
the AT₁ receptor (13), which in combination likely contribute to the enhanced activity of the renal RAS in SS hypertension. ROS have also been shown to be required for COX-2 induction in response to proinflammatory cytokines in different cell types (5) and, as we have recently shown (11), in response to ANG II in mesangial cells. To determine the role of ROS in cortical COX-2 expression in hypertensive SS rats we took advantage of the availability of the SOD mimic tempol. Treatment with tempol has been shown to significantly reduce the production of ROS in different animal models of hypertension including the DS rat (12, 19). In our studies the administration of tempol resulted in a significant reduction in the cortical expression of COX-2, suggesting that ROS mediate renal cortex expression of COX-2 in the hypertensive DS rat. In support of these findings, treatment with tempol has been shown to reduce the increases in renal COX-2 expression associated with diabetes (16). Tempol, however, increased medullary COX-2 expression, suggesting that ROS do not mediate COX-2 expression in the medulla of hypertensive SS rats. We (10) and others (26) have shown that NO is an important mediator of COX-2 expression and activity. We speculate that increased NO bioavailability as the result of ROS inhibition may be responsible for the increase in COX-2 expression in the renal medulla of hypertensive SS rats treated with tempol. These findings also suggest the existence of important differences in the modulation of COX-2 expression between the cortex and medulla in SS hypertension.

To determine the role of blood pressure reductions on COX-2 expression we performed additional experiments in a second group of SS rats placed on a high-salt diet and treated with the calcium channel blocker Aml. Treatment with Aml resulted in significant reductions in blood pressure. In contrast to our findings with Can and tempol, treatment with Aml did not significantly reduce COX-2 expression in these animals, suggesting that COX-2 upregulation is independent from the hemodynamic stress of hypertension in the hypertensive DS rat.

Studies in vivo in a mouse model of renal ablation associated with increased COX-2 expression demonstrated that COX-2 inhibition ameliorates the degree of renal injury in these animals, suggesting that COX-2-derived prostaglandins play an important role in the pathogenesis of chronic renal disease (4). Recent studies have reported that treatment with the COX-2 inhibitor celecoxib reduces renal injury in hypertensive DS rats (9).

Although in the aggregate these studies would suggest a role for COX-2 as mediator of renal injury, other studies have suggested that COX-2-derived prostaglandins antagonize the vasoconstrictor effects of ANG II in the glomerular microcirculation and therefore the upregulation of COX-2 when the RAS is activated would play a beneficial role (1, 29). Indeed, COX-2 inhibition in humans results in significant reductions in glomerular filtration rate (GFR) and is associated with blunting the production of vasodilatory prostaglandins (31). In addition, studies performed with renal biopsies have shown increased renal COX-2 expression with aging (18), suggesting a higher dependence on COX-2-derived prostaglandins in the elderly to maintain a normal GFR.

In conclusion, we have demonstrated that local activation of the RAS, via increased generation of ROS, mediates cortical COX-2 upregulation in hypertensive SS rats. These studies unveil novel mechanistic pathways that may play a role in the pathogenesis of hypertensive renal injury. However, like the Roman god Janus, COX-2 may have two faces: 1) the vasoconstrictive one, in which prostaglandins may bind to PG1₂ receptors or PGE₂ EP₂ receptors and antagonize the vasoconstrictive effects of ANG II brought about by volume depletion and renal hypoperfusion in the context of heart failure or liver cirrhosis; and 2) the vasoconstrictive and proproliferative one, mediated via binding of prostaglandins to EP₂ or EP₄ receptors. This may explain the existence of extensive contradictory data regarding the benefits and/or risks of COX-2 inhibition in renal disease. Very likely, blockade of specific prostaglandin receptors, when available, will be a suitable strategy to selectively unravel the pathological and/or beneficial renal effects of COX-2-derived prostaglandins under various pathophysiological conditions.
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