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Resistance to hypertension mediated by intercalated cells of the collecting duct

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The renal collecting duct (CD), as the terminal segment of the nephron, is responsible for the final adjustments to the amount of sodium excreted in urine. While angiotensin II modulates reabsorptive functions of the CD, the contribution of these actions to physiological homeostasis is not clear. To examine this question, we generated mice with cell-specific deletion of AT_{1A} receptors from the CD. Elimination of AT_{1A} receptors from both principal and intercalated cells (CDKO mice) had no effect on blood pressures at baseline or during successive feeding of low- or high-salt diets. In contrast, the severity of hypertension caused by chronic infusion of angiotensin II was paradoxically exaggerated in CDKO mice compared with controls. In wild-type mice, angiotensin II induced robust expression of cyclooxygenase-2 (COX-2) in renal medulla, primarily localized to intercalated cells. Upregulation of COX-2 was diminished in CDKO mice, resulting in reduced generation of vasodilator prostanoids. This impaired expression of COX-2 has physiological consequences, since administration of a specific COX-2 inhibitor to CDKO and control mice during angiotensin II infusion equalized their blood pressures. Stimulation of COX-2 was also triggered by exposure of isolated preparations of medullary CDs to angiotensin II. Deletion of AT_{1A} receptors from principal cells alone did not affect angiotensin II–dependent COX2 stimulation, implicating intercalated cells as the main source of COX2 in this setting. These findings suggest a novel paracrine role for the intercalated cell to attenuate the severity of hypertension. Strategies for preserving or augmenting this pathway may have value for improving the management of hypertension.

Introduction

Precise control of urinary sodium excretion is a critical component of blood pressure regulation. At the molecular level, pathways regulating sodium handling by the kidney affect the reabsorptive functions of renal epithelia by modulating abundance and/or activities of specific channels and transporters. However, there is substantial heterogeneity in properties of solute and water reabsorption along the nephron. Accordingly, the final concentration of sodium in urine is determined by the aggregate actions of several diverse compartments of tubular epithelial cells. Among the different nephron segments, the collecting duct (CD) has been suggested to play a preeminent role in regulation of blood pressure. Based on its position in the terminal portion of the nephron, the CD is responsible for fine-tuning the final ≈5% of overall renal sodium reabsorption. Since 25,000 mEq of sodium is filtered across the glomerulus each day, even small adjustments in the amount of sodium reclaimed by the CD can have a substantial impact on extracellular fluid volumes and, hence, on blood pressure. This is clearly illustrated by the severe hypertension seen in inherited disorders, such as Liddle's syndrome, caused by mutations affecting sodium reabsorption in the CD (1).

There are two main epithelial cell lineages in the CD: principal cells (PCs) and intercalated cells (ICs) (2). PCs are the most abundant and are responsible for the major portion of salt and water transport. While a key function of ICs is regulation of urinary acidification (3), more recent studies have shown that they also contribute to sodium and chloride flux (4, 5). Reabsorptive functions of CD epithelia are controlled by paracrine mediators including endothelin, aldosterone, and angiotensin II (6). Among these mediators, the influence of aldosterone on CD physiology and its impact on blood pressure have been most thoroughly characterized (7). On the other hand, the contribution of direct, aldosterone-independent effects of angiotensin II in the CD to blood pressure control has been difficult to establish.

The predominant actions of angiotensin II to influence blood pressure are mediated by the type 1 angiotensin (AT_1) receptor (8), a member of the large family of G protein–coupled receptors (GPCRs). AT_1 receptors are also the target of angiotensin receptor blockers (ARBs), effective and widely used antihypertensive agents (9). Recent studies from our laboratory have highlighted the crucial role of AT_1 receptors within the kidney to control blood pressure (10) and promote the pathogenesis of hypertension (11). While AT_1 receptors are expressed throughout the kidney in epithelial cells along the entire nephron (12), the specific cell lineages that are key targets for regulation of blood pressure by angiotensin II are not clear.
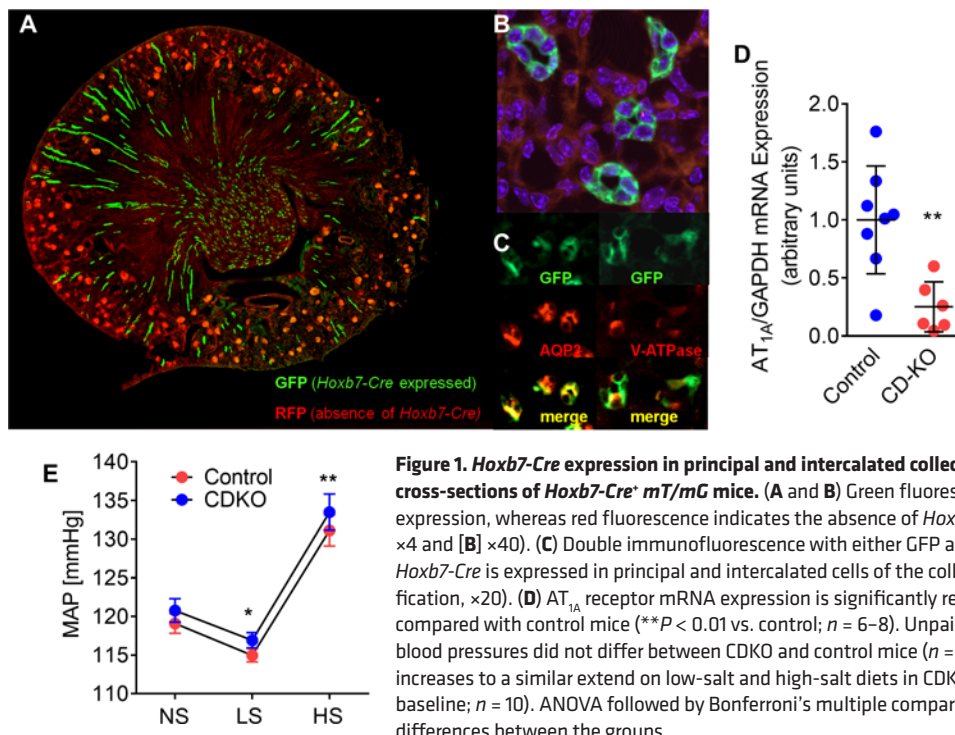
The purpose of the present studies was to define the physiological role of angiotensin II acting via AT_1 receptors in the CD. There is significant expression of AT_1 receptors in both PCs and ICs (13). Furthermore, studies in mice, cell culture, and isolated perfused tubules have shown that activation of AT_1 receptors on PCs in the CD promotes sodium reabsorption by enhancing expression of the epithelial sodium channel (ENaC) and its trafficking to the apical membrane and thereby contribute to the initiation of angiotensin II–dependent hypertension (14–16). In intercalated cells, AT_1 receptors also modulate chloride and bicarbonate transport (17–19). Accordingly, our original hypothesis was that AT_1 receptors in the CD would promote sodium reabsorption and thus would have prohypertensive actions. Yet in the course of our studies, we identified a dominant pathway linked to AT_1 receptors in epithelial cells of the CD triggering expression of cyclooxygenase-2 (COX-2), generating vasodilator prostaglandins with powerful actions to *attenuate* the severity of hypertension.

Results

Elimination of AT_{1A} receptors from the CD does not affect baseline blood pressure or salt sensitivity. In order to understand the impact of direct effects of angiotensin II in the CD on blood pressure regulation, we generated mice lacking the AT_{1A} receptor in all epithelial cells of CD using cell-specific gene targeting. To this end, inbred 129/SvEv mice with a conditional (floxed) *Agtr1a* allele were crossed with an inbred 129/SvEv *Hoxb7-Cre* transgenic mouse line expressing *Cre* in both PCs and ICs of the CD (CDKO mice) as described previously (20). In order to verify tissue specificity of *Hoxb7* promoter expression in the kidney, *Hoxb7-Cre* mice were crossed with a double-fluorescence reporter mouse (*mT/mG*) (Figure 1A), where GFP expression is triggered by *Cre* recombinase. As shown in Figure 1, B and C, GFP expression is confined to epithelial cells of the CD with circumferential GFP expression in tubules. GFP fluorescence colocalized with AQP2, a marker for principal cells, and V-ATPase, a marker of intercalated cells, consistent with expression in both PCs and ICs (Figure 1, B and D). After verifying the specificity of Cre expression in CD in the *Hoxb7-Cre* mice, we assessed the extent of deletion of AT_{1A} receptors from isolated medullary CDs (ICMDs). AT_{1A} mRNA expression levels were significantly reduced, by ≈75%, in ICMDs of CDKO mice compared with controls ($P < 0.01$) (Figure 1D).

To test the impact of AT_{1A} receptors in the CD on blood pressure regulation, we measured blood pressures of 129/SvEv-*Hoxb7-CreAgtr1a^{fl/fl}* (CDKO) and their *Cre*-negative littermates (control) by radiotelemetry. As shown in Figure 1E, baseline mean arterial blood pressures (MAPs) on a normal-salt (0.4% NaCl) diet were very similar between control and CDKO mice (119 ± 1 mmHg vs. 121 ± 2 mmHg; $n = 10$). To determine whether deletion of AT_{1A} receptors from CD would affect adaptation to changes in dietary salt intake, blood pressures were continuously measured while CDKO and control mice were fed normal, low- (<0.002% NaCl), and high-salt (6% NaCl) diets for 5 consecutive days each. Upon the transition from normal to low-salt diet, blood pressure decreased significantly in controls (119 ± 1 to 115 ± 1 mmHg; $P < 0.02$; $n = 10$) and then increased to 131 ± 5 mm Hg ($P < 0.01$; $n = 10$) after 5 days on the high-salt diet, consistent with sodium sensitivity typically seen in 129 strain mice (21). As shown in Figure 1E, the magnitude and pattern of blood pressure changes were virtually identical in CDKO mice. Thus, these results suggest that AT_{1A} receptors in the CD do not have a major impact on baseline blood pressure or responses to extremes of dietary sodium intake.

Deletion of AT_{1A} receptors from the CD exaggerates angiotensin II–dependent hypertension. To determine whether AT_{1A} receptors in the CD contribute to the pathogenesis of hypertension, we induced hypertension in CDKO and control mice by infusing angiotensin II by mini-osmotic pump at 1,000 ng/kg/min for 2 weeks. As shown in Figure 2A and Supplemental Figure 1 (supplemental material available online with this article; https://doi.org/10.1172/jci.insight.92720DS1), angiotensin II infusion increased blood pressures abruptly and significantly in control mice, and increases in MAPs and systolic pressures were sustained throughout the period of angiotensin II infusion. MAP in CDKO mice averaged over 14 days of angiotensin II infusion

Figure 1. *Hoxb7-Cre* expression in principal and intercalated collecting duct cells. Representative kidney cross-sections of *Hoxb7-Cre⁺ mT/mG* mice. (**A** and **B**) Green fluorescence indicates the presence of *Hoxb7-Cre* expression, whereas red fluorescence indicates the absence of *Hoxb7-Cre* expression (original magnification, [**A**] ×4 and [**B**] ×40). (**C**) Double immunofluorescence with either GFP and AQP2 or GFP and V-ATPase confirms that *Hoxb7-Cre* is expressed in principal and intercalated cells of the collecting duct in the kidney (original magnification, ×20). (**D**) AT_{1A} receptor mRNA expression is significantly reduced in isolated collecting ducts of CDKO compared with control mice (**$P < 0.01$ vs. control; $n = 6$–8). Unpaired Student's t test was used. (**E**) Baseline blood pressures did not differ between CDKO and control mice ($n = 10$). Blood pressures significantly decrease and increases to a similar extend on low-salt and high-salt diets in CDKO and control mice (*$P < 0.05$; **$P < 0.01$ vs. baseline; $n = 10$). ANOVA followed by Bonferroni's multiple comparisons post-hoc test was used to compare the differences between the groups.

was 163 ± 3 mmHg, compared with 151 ± 3 mmHg in controls ($P < 0.01$; $n = 16$). To determine whether this exaggerated hypertensive response to angiotensin II in CDKO mice was accompanied by changes in renal sodium handling, we measured urinary sodium excretion during the first 7 days of the angiotensin II infusion, while sodium intake was clamped at a constant level. As shown in Figure 2B, we found that the extent of the cumulative positive sodium balance was significantly increased in CDKO mice compared with controls during the first 5 days of angiotensin II infusion (0.75 ± 0.07 vs. 0.50 ± 0.04 mmol; *$P < 0.02$; $n = 6$). Thus, elimination of AT_{1A} receptors from PCs and ICs of the CD augmented hypertension in CDKO mice, and this was associated with reduced natriuresis, indicating enhanced renal sodium reabsorption and alteration in the pressure-natriuresis response.

The enhanced hypertensive response in CDKO mice is not due to impaired NO or aldosterone generation. Our observation that CDKO mice have an exaggerated response to chronic infusion of angiotensin II suggests that AT_1 receptors in epithelia of the CD trigger a pathway with powerful effects to oppose the development of hypertension, such that in their absence, the severity of hypertension is enhanced. Previous studies indicate that NO derived from renal medullary epithelial cells may modulate the development of hypertension (22, 23). Thus, we considered the possibility that AT_{1A} receptors within the CD might stimulate NO generation as a compensatory response, and elimination of this pathway might be responsible for the severe hypertension we observed in CDKO mice. To test this possibility, we first measured urinary nitrate/nitrite excretion as a measure of renal NO production in control and CDKO mice before and during angiotensin II infusion. As shown in Figure 3A, equivalent levels of urinary nitrate/nitrite were seen at baseline. During the first 5 days of angiotensin II infusion, urinary NO nitrate/nitrite levels tended to be higher in CDKO mice, but this difference did not achieve statistical significance.

To further test the role of NO in this response, control and CDKO mice were infused with angiotensin II as above while their blood pressures were monitored by radiotelemetry. After 1 week of angiotensin II infusion (Figure 3B and Supplemental Figure 2), MAPs and systolic arterial blood pressures in CDKO mice was significantly higher than in controls (183 ± 3 mmHg vs. 169 ± 4 mmHg; $P < 0.05$; $n = 6$), as we had seen previously. At this point, N^G-nitro-L-arginine methyl ester (L-NAME; 20 mg/kg/d) was added to their drinking water to inhibit NO generation. As depicted in Figure 3B, administration of L-NAME caused blood pressures to significantly rise further in both groups. After 1 week of L-NAME plus angiotensin II, the absolute difference in blood pressure between the controls and CDKO mice was further enhanced, suggesting that impaired NO generation was *not* the cause of exaggerated hypertension in the CDKO mice.
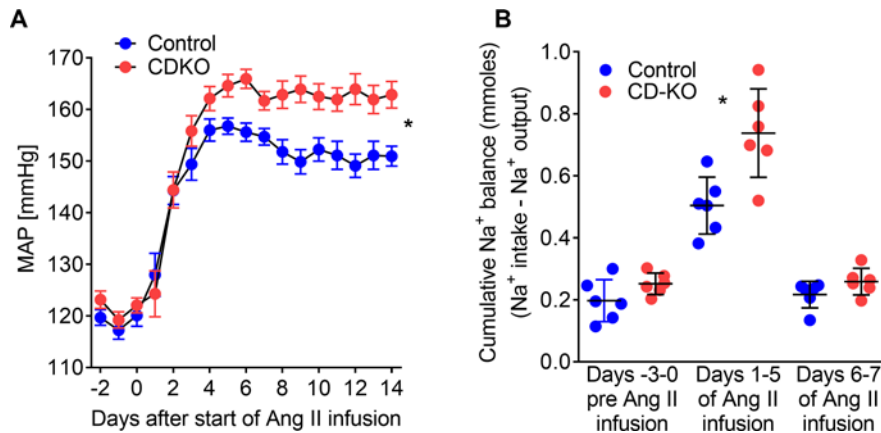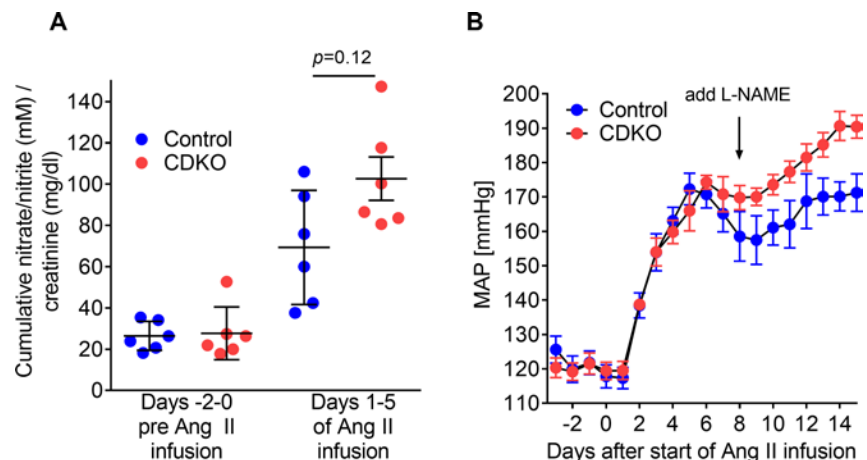
Figure 2. Exaggerated blood pressure response to angiotensin II in mice lacking AT1 receptors from the collecting duct (CDKO mice). (**A**) Chronic angiotensin II infusion (1,000 ng/kg/min) caused a greater hypertensive blood pressure response in CDKO compared with control mice (MAP: 163 ± 3 vs. 151 ± 3 mmHg, *P < 0.001 vs. angiotensin II control; n = 16). (**B**) In accordance with the exaggerated blood pressure response, cumulative positive sodium balance was significantly increased in CDKO compared with control mice during the first 5 days of angiotensin II infusion (0.75 ± 0.07 vs. 0.50 ± 0.04 mmol; *P < 0.02 vs. angiotensin II control; n = 6). ANOVA followed by Bonferroni's multiple comparison post-hoc test was used to compare the differences between the groups.

As aldosterone also may contribute to the pathogenesis of angiotensin II–dependent hypertension, we also compared aldosterone responses in CDKO and control mice. Accordingly, we measured urinary aldosterone excretion rates, which were similar in angiotensin II–infused CDKO and control mice (14,556 ± 1,571 vs. 12,306 ± 1,428 pg/24-hour urine; P = 0.30, n = 12).

AT$_{1A}$ receptors in the CD trigger expression of COX-2. In exploring other possible mechanisms to explain the more severe hypertension seen in CDKO mice, we considered previous studies suggesting that COX-2 in the kidney may modify effects of angiotensin II upon blood pressure (24). Accordingly, we measured expression of COX-2 in kidneys from CDKO and control mice at baseline and after 2 weeks of chronic angiotensin II infusion. In the renal medulla, COX-2 mRNA l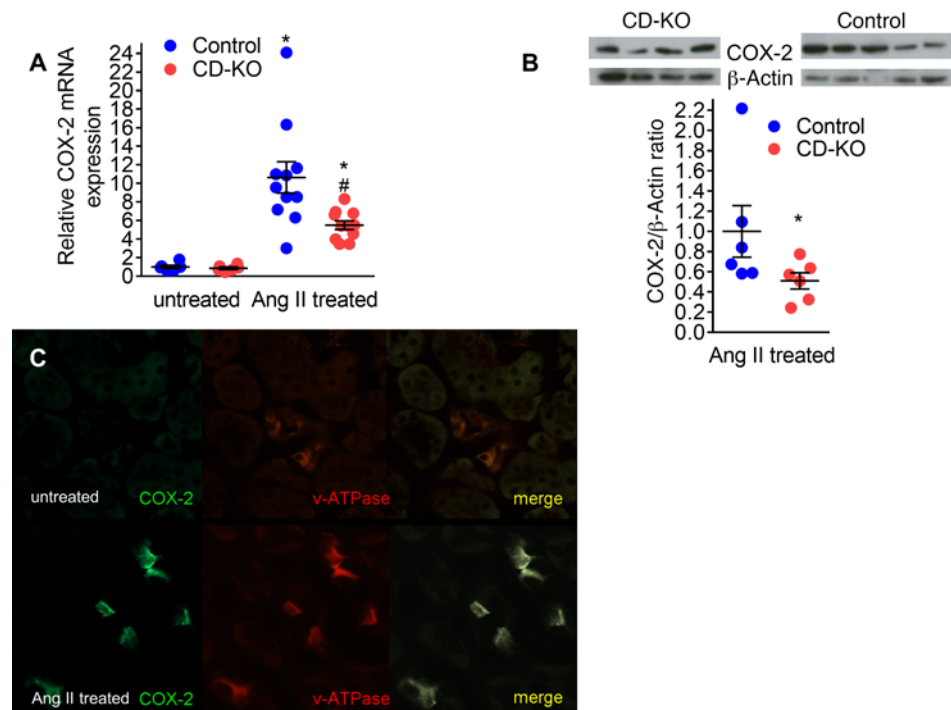evels were similar in control and CDKO mice at baseline (Figure 4A). With angiotensin II infusion, levels of COX-2 mRNA increased almost 10-fold in controls. However, as shown in Figure 4A, while COX-2 expression was also enhanced by angiotensin II in the CDKO mice, COX-2 levels were attenuated by ≈50% compared with controls (10.7 ± 1.8 vs. 5.5 ± 0.5 arbitrary units; P < 0.01; n = 11). Similarly, inner medullary COX-2 protein levels in CDKO mice were diminished after chronic angiotensin II infusion compared with controls as shown in Figure 4B. On the other hand, in renal cortex, levels of COX-2 mRNA were similar in controls and CDKO mice at baseline and with angiotensin II infusion (data not shown).

To determine the cellular source of angiotensin II–stimulated COX-2 expression, we examined medullas from kidneys of wild-type mice by immunocytochemistry and confocal microscopy. At baseline, we found minimal COX-2 staining in renal medullae of untreated wild-type mice (Figure 4C, top row). By contrast, as shown in the bottom row of Figure 4C, distinct cellular expression of COX-2 was readily apparent after chronic infusion of angiotensin II. COX-2 staining colocalized almost exclusively with V-ATPase, a marker for ICs, indicating that angiotensin II causes potent upregulation of COX-2 in ICs in the renal medulla (bottom row). Moreover, aquaporin 2 (AQP2), a specific marker for PCs, did not colocalize with COX-2 (Supplemental Figure 3) suggesting that the upregulation of COX-2 occurs in ICs and not PCs of the CD.

Figure 3. NO does not modify the exaggerated blood pressure response to angiotensin II in mice lacking AT$_1$ receptors from the collecting duct (CDKO mice). (**A**) During the first 5 days of angiotensin II infusion, cumulative urinary nitrate/nitrite levels did not differ significantly between CDKO and control mice (102.7 ± 26.30 vs. 69.34 ± 27.68 mM/mg/dl creatinine; P = 0.12; n = 6). Unpaired Student's t test was used to compare the various groups. (**B**) The exaggerated blood pressure response to angiotensin II is not due to impaired NO generation, as chronic administration of L-NAME (20 mg/kg/d) did not decrease the blood pressure difference between CDKO and control mice (n = 6). ANOVA followed by Bonferroni's multiple comparison post-hoc test was used to compare the differences between the groups.

Figure 4. Deletion of collecting duct–specific AT$_{1A}$ receptors modifies medullary COX-2 expression. (**A**) After 14 days of angiotensin infusion, medullary COX-2 mRNA expression was significantly increased in CDKO (1.0 ± 0.1 vs. 5.5 ± 0.5 AU; *$P < 0.001$; n = 6–11) and control mice (1.0 ± 0.2 vs. 10.7 ± 1.8 AU; *$P < 0.001$; n = 6–11) compared with untreated mice. Moreover, the increase in medullary COX-2 mRNA expression was significantly attenuated in CDKO compared with control mice (10.7 ± 1.8 vs. 5.5 ± 0.5 AU; #$P < 0.01$; n = 11). Unpaired Student's t test was used to compare the various groups. (**B**) Likewise, medullary COX-2 protein levels were significantly reduced in angiotensin II– treated CDKO compared with control mice (0.5 ± 0.1 vs. 1.0 ± 0.3; *$P < 0.05$; n = 6). Unpaired Student's t test was used. Lanes were run on the same gel but were noncontiguous. See complete unedited blots in the supplemental material. Results are representative of 3 independent experiments. (**C**) In order to determine the cellular source of COX-2, immunocytochemistry was performed in the medulla of untreated and angiotensin II–treated control mice. In untreated mice, representative confocal laser scanning microscopy showed minimal COX-2 staining. By contrast, in angiotensin II–treated control mice, COX-2 staining was apparent and colocalized almost completely with V-ATPase, a marker for intercalated cells (original magnification, ×20).

Angiotensin II stimulates COX-2 expression in isolated inner medullary CDs and MDCK cells. To verify the capacity of angiotensin II to regulate COX-2 expression in CD epithelia, we next carried out a series of studies in IMCDs in vitro. Suspensions enriched for CD segments were prepared as described previously (25, 26) with slight modifications. To test whether we could recapitulate the stimulation of COX-2 by angiotensin II that we observed in vivo, we incubated suspensions of IMCD with angiotensin II in vitro and quantitated COX-2 protein levels in protein homogenates by Western blot analysis using a specific anti–COX-2 antibody. As shown in Figure 5A, following incubation with angiotensin II, there was a concentration-dependent increase in COX-2 protein levels, with a maximal >2-fold increase at concentrations of 10^{-7} M angiotensin II. By contrast, angiotensin II did not affect COX-2 levels in IMCDs isolated from *Agtr1a$^{-/-}$* mice lacking AT$_{1A}$ receptors (Figure 5B), confirming an absolute requirement for the AT$_{1A}$ receptor to mediate this response. Yet the response was completely preserved in mice lacking AT$_{1A}$ receptors only in PCs of the CD (*AQP2-Cre Agtr1a$^{fl/fl}$*), and significantly greater than in CDs from CDKO mice, as shown in Figure 5, C and D. These studies in IMCD suspensions indicate that angiotensin II directly stimulates COX-2 expression in ICs of the CD via activation of AT$_{1A}$ receptors. Finally, we also performed studies in Madin-Darby canine kidney clone 11 (MDCK C-11) cells, which resemble ICs of the CD (Supplemental Figure 4) (27). In MDCK C-11 cells permanently transfected with AT$_1$ receptors, angiotensin increased COX-2 expression in a concentration-dependent manner (Figure 5E). This increase was also accompanied by a significant increase in the generation of PEG$_2$ (Figure 5F).

Stimulation of vasodilator prostanoids by angiotensin II is diminished in CDKO mice. To determine whether the observed difference in expression of COX-2 in the medullary CD impacts the generation of
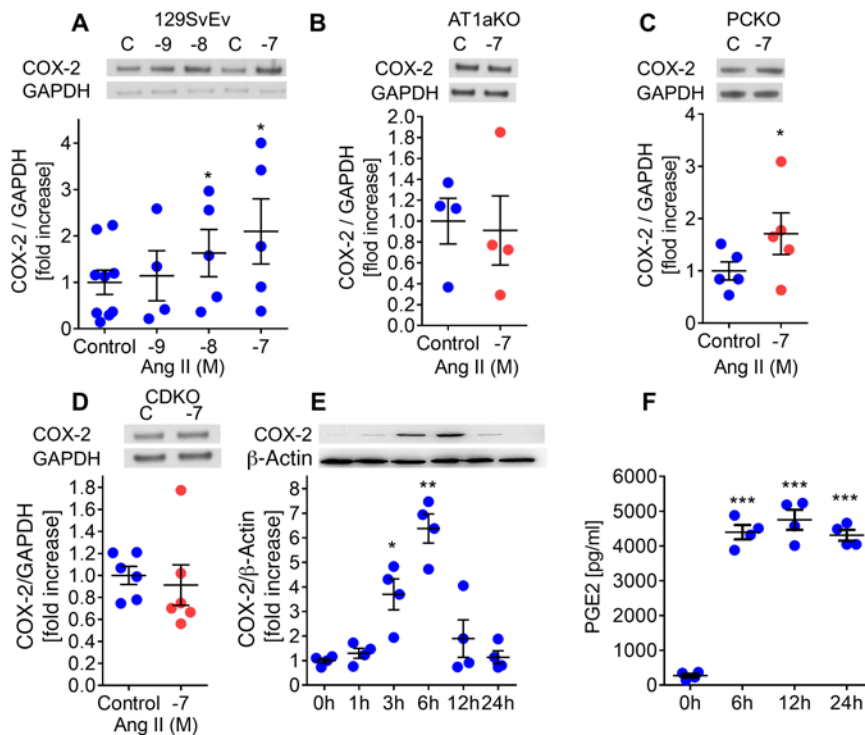
Figure 5. Densitometric analysis of COX-2 in isolated inner medullary collecting ducts of 129/SvEv, *Agtr1a−/−*, PCKO, and CDKO mice. (**A**) Angiotensin II (Ang II) increases COX-2 expression in isolated collecting ducts of 129/SvEv control mice in a dose-dependent manner (10^{-8} M: 1.8 ± 0.5 COX-2/GAPDH [fold increase], *P < 0.05 vs. control; n =5; 10^{-7} M: 2.3 ± 0.7 COX-2/GAPDH [fold increase], P < 0.05 vs. control; n =5). Paired Student's *t* test was used to compare the different groups. (**B**) Angiotensin II (10^{-7} M) failed to increase COX-2 protein in isolated collecting ducts of *Agtr1a−/−* mice. (**C** and **D**) Angiotensin II (10^{-7} M) increases COX-2 protein levels by about 2-fold in isolated collecting ducts of mice lacking the AT_{1A} receptor only in principal cells (1.7 ± 0.2 vs. 1.0 ± 0.4; *P < 0.05; n = 5) but failed to induce COX-2 expression in CDKO mice. Unpaired Student's *t* test was performed to compare the various groups. (**E** and **F**) Angiotensin II (10^{-7} M) significantly induced COX-2 expression in MDCK cells (1.00 ± 0.10 [0 hours]; 1.30 ± 0.20 [1 hour]; 3.70 ± 0.63 [3 hours]; 6.38 ± 0.60 [6 hours]; 1.90 ± 0.76 [12 hours]; 1.14 ± 0.26 [24 hours]; *P < 0.05, **P < 0.01; n = 4) and significantly increased prostaglandin E_2 (PGE_2) generation measured in the supernatant of the stimulated MDCK cells (272 ± 53 [0 hours]; 4,397 ± 288 [6 hours]; 4,752 ± 209 [12 hours]; 4,310 ± 157 [24 hours]; ***P < 0.001; n = 4). ANOVA followed by Bonferroni's multiple comparison post-hoc test was used to compare the differences between the groups. See complete unedited blots in the supplemental material.

prostanoids by the kidney, we measured urinary excretion of metabolites of key vasodilator prostanoids, PGE_2 and prostacyclin, along with TXB_2, the stable metabolite of the potent vasoconstrictor eicosanoid thromboxane A_2 (TXA_2). As shown in Figure 6, A–C, there were no differences between control and CDKO mice in urinary excretion of any of these prostanoids at baseline. Infusion of angiotensin II caused significant increases in urinary excretion of all 3 prostanoids in the control mice. Although urinary excretion of TXB_2 increased to a similar extent in controls and CDKO mice (3,233 ± 839 vs. 4,451 ± 1,011 pg/24-hour urine with angiotensin II; P = 0.37; n = 10–11), there was a significant attenuation of angiotensin II–stimulated excretion of PGE_2 (2,395 ± 441 vs. 5,852 ± 1,726 pg/24-hour urine; P < 0.01; n = 10–11) and 6-keto-PGF1a (1,889 ± 386 vs. 5,125 ± 1,374 pg/24-hour urine; P < 0.05; n = 10–11) in CDKO mice compared with controls (Figure 6, A–C). Thus, in the CDKO mice, the failure to fully upregulate medullary COX-2 expression during angiotensin II infusion was associated with impaired generation of vasodilator prostanoids, resulting in a skewing of the ratio of vasodilator to vasoconstrictor prostanoids in the kidney.

COX-2 inhibition abolishes the difference in blood pressure between CDKO mice and controls during angiotensin II infusion. To test whether reduced expression of COX-2 and production of vasodilator prostanoids is responsible for the augmented blood pressure response in CDKO mice, we treated both groups with a specific COX-2 inhibitor (rofecoxib) during chronic angiotensin II infusion. As shown in

Figure 7 and Supplemental Figure 5, after 1 week of angiotensin II infusion, systolic blood pressure and MAP were significantly higher in CDKO mice (156 ± 4 mmHg) compared with controls (142 ± 6 mmHg; P < 0.05; n = 10), as noted previously. At that point, treatment with rofecoxib (10 mg/kg) was initiated, which caused blood pressures to increase significantly (P < 0.05 vs. pre-treatment) in both groups to 163 ± 6 mmHg in the CDKO mice and 161 ± 5 mmHg in controls to nearly identical levels. Thus, the COX-2 inhibitor completely abolished the blood pressure difference between the two groups.

Discussion

The major objective of these studies was to define the contribution of AT_{1A} receptors in CD epithelium to blood pressure homeostasis. AT_1 receptors are expressed throughout the distal nephron (12), and relatively high concentrations of angiotensin II can be detected in the distal tubular fluid (28). Previous studies from other laboratories have shown that AT_1 receptors can modulate functions of epithelial cells in the CD. For example, on the apical membrane of the PCs in the cortical CD (CCD), luminal angiotensin II stimulates amiloride-sensitive sodium transport by increasing activity of the ENaC through an AT_1 receptor–dependent mechanism influencing the initial phase of angiotensin II–dependent hypertension (14, 16, 29). Angiotensin II also increases chloride absorption in the CCD through a
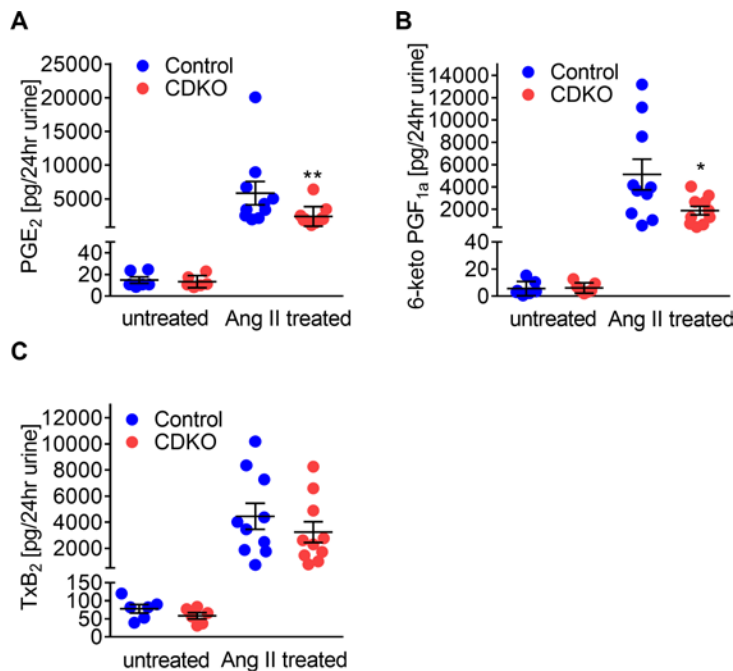
Figure 6. AT$_{1A}$ receptors in the collecting duct stimulate vasodilator prostanoids. After chronic angiotensin II infusion, urinary metabolites of the vasodilator prostanoids (**A**) PGE$_2$ and (**B**) prostacyclin (6-keto PGF$_{1a}$) were significantly reduced in CDKO mice compared with control mice (PEG$_2$: 2,395 ± 441 vs. 5,852 ± 1,726 pg/24-hour urine; **P < 0.01 vs. angiotensin II–treated controls, n = 10–11; prostacyclin 6-keto PGF$_{1a}$: 1,889 ± 386 vs. 5,125 ± 1,374 pg/24-hour urine; *P < 0.05 vs. angiotensin II–treated controls, n = 10–11). (**C**) In contrast, urinary thromboxane (TXB$_2$) excretion did not differ between CDKO and control mice (3,233 ± 839 vs. 4,451 ± 1,011 pg/24-hour urine, n = 10–11). Unpaired Student's t test was performed to compare the different groups.

mechanism that requires pendrin (30). Furthermore, activation of AT$_1$ receptors on the basolateral membrane of CCD cells stimulates the activity of potassium channels via an NO-dependent pathway (31).

Since the terminal portions of the nephron including the CD make the final adjustments in salt and water excretion ultimately determining the characteristics of urine flow and composition, we anticipated that direct, aldosterone-independent actions of AT$_1$ receptors to modulate sodium handling at these sites would impact blood pressure homeostasis and modify susceptibility to hypertension. Instead, we find that eliminating AT$_{1A}$ receptors from CD has negligible effects on baseline blood pressure or homeostatic responses to large alterations in dietary sodium intake. This finding is consistent with our recently published work showing that specific deletion of AT$_{1A}$ receptors from PCs does not affect baseline blood pressure and is associated with a modest attenuation of angiotensin II–dependent hypertension (16). Compared with this apparently dispensable role of AT$_1$ receptors in the CD at baseline, we found that elimination of AT$_{1A}$ receptors from the proximal tubule lowers baseline blood pressure and significantly impacts proximal sodium handling (21). Thus, within the renin-angiotensin system (RAS), there appears to be a division of control mechanisms between the proximal tubule and CD. Direct actions of AT$_1$ receptors have profound effects in the proximal nephron, whereas aldosterone seems to have a dominant role in the CD. This compartmentalization of regulatory effects of angiotensin II and aldosterone between nephron segments likely provides an expanded capacity for more precise control of sodium and potassium homeostasis (32).

Although deletion of AT$_{1A}$ receptors from CD epithelium had no apparent effect on baseline blood pressure, this caused a marked and unexpected exaggeration in the severity of angiotensin II–dependent hypertension. We show here that worsening of hypertension caused by deletion of AT$_{1A}$ receptors from CD is due to a failure to upregulate COX-2 in ICs and consequent impaired generation of vasodilator prostanoids. The potent role of COX-2 to influence blood pressure regulation is highlighted by consistent clinical associations between administration of specific COX-2 inhibitors and development of hypertension (33). However, definition of the critical mechanisms of blood pressure control by COX-2 has been incomplete. In animal studies, COX-2–derived prostanoids promote sodium excretion (34). Moreover, the capacity for COX-2 in the renal medulla to modulate blood pressure is suggested by observations that COX-2 expression in the medulla is enhanced with high-salt feeding (35) and selective intramedullary infusion of COX-2 inhibitors induces hypertension in rats fed a high-salt diet (36). Similarly,

Figure 7. Effects of COX-2 inhibition on angiotensin II–dependent hypertension. After 1 week of chronic angiotensin II treatment, CDKO and control mice were co-treated with a specific COX-2 inhibitor (rofecoxib 10 mg/kg/d). After 1 week of angiotensin II infusion, MAP was significantly higher in CDKO compared with control mice (156 ± 4 vs. 142 ± 6 mmHg; *P < 0.05; n = 10). Co-treatment with rofecoxib (10 mg/kg) caused a significant increase in blood pressure in both groups and abolished the difference in blood pressure between CDKO and control mice (163 ± 6 vs. 161 ± 5 mmHg; P = 0.70; n = 10). ANOVA followed by Bonferroni's multiple comparisons post-hoc test was used to compare the differences between the groups.
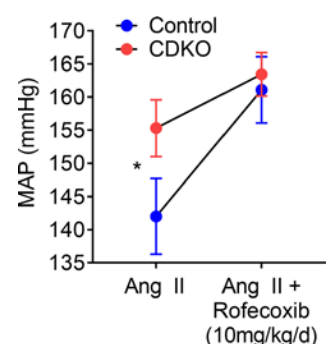
COX-2 inhibition selectively reduces medullary blood flow in mice and induces an exaggerated pressor response to angiotensin II (24). Such alterations in renal blood flow can affect blood pressure homeostasis, since changes in medullary blood flow significantly impact sodium excretion (37–39). On the other hand, prostanoids generated by COX-2 may also directly impact sodium and fluid reabsorption by renal epithelial cells (40). In addition, Zhang and colleagues recently showed that COX-2 activation in macrophages attenuates sodium sensitivity by enhancing natriuresis (41).

While a role for COX-2 in renal medulla to protect against hypertension has been suggested previously (24), the source of COX-2 had been attributed to renal medullary interstitial cells, an unusual cell lineage of uncertain origin and function. Here, we provide genetic evidence that ICs of the CD are an important source of COX-2, which is induced by activation of AT_1 receptors during hypertension. Although a previous study has reported little or no expression of COX-2 in CD epithelium (42), most of these experiments were carried out in normotensive, unperturbed animals. As in other tissues, expression of COX-2 in the kidney can be highly induced after various physiological stresses, and our studies show that such upregulation is induced in ICs by angiotensin II. This activation of COX-2 expression by angiotensin II in medullary CD contrasts with renal cortex, where AT_1 receptors suppress expression of COX-2 as part of the short-loop feedback pathway for renin release. (43), perhaps suggesting distinct signals linking AT_1 receptors and COX-2 in these two cell lineages.

Historically, the primary role of the IC was presumed to be regulation of acid-base balance. However, in recent years, it has become apparent that the IC participates in regulating extracellular fluid volume and plasma potassium levels through expression of a unique repertoire of transporters (4) and through paracrine communication with the PCs (5). For example, in the kidney, the anion exchanger pendrin is expressed predominantly in ICs and modulation of pendrin by AT_1 receptors contributes to the pathogenesis of hypertension (17). Moreover, PGE_2 generated in ICs regulate ENaC expression and thereby sodium balance in PCs during renal tubular acidosis (5). With ischemic kidney injury, ICs are among the first cell populations to express neutrophil gelatinase-associated lipocalin (NGAL), a marker of renal epithelial injury (44), perhaps indicating a role for these cells in sensing stress. Our studies assign an unexpected function to the IC as a source of mediators with a powerful capacity to modify responses to hypertension. As discussed above, these prostanoid mediators may act in a paracrine fashion to influence blood flow to the renal medulla or to directly modulate sodium reabsorption by the surrounding epithelium.

Our previous studies using kidney cross-transplants have highlighted the essential role of AT_1 receptors within the kidney for blood pressure regulation (11) and angiotensin II–dependent hypertension (10), consistent with Guyton's hypothesis (45). As we have discussed, direct actions of AT_1 receptors inside the kidney may affect fluid and solute excretion by controlling renal epithelial function and modulating renal blood flow and thereby influencing tubular fluid reabsorption. While the actions of AT_1 receptors typically increase blood pressure, we describe here what we believe to be a novel pathway linked to AT_1 receptors in the renal CD opposing the development of hypertension. This observation demonstrates unexpected complexity of the renal epithelial actions of angiotensin II. Furthermore, understanding the mechanisms of this pathway with powerful actions to resist the development of hypertension may have utility in optimizing antihypertensive treatments.

Methods

Experimental mice. Mice were bred and maintained in the Association for Assessment and Accreditation of Laboratory Animal Care–accredited animal facilities at the Durham Veterans' Affairs Medical Center (DVAMC) according to NIH guidelines. All the animal studies were approved by both the Duke University (IACUC no. A186-13-07) and DVAMC (IACUC no. 1506-002) Institutional Animal Care and Use Committees and conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (National Academies Press, 2011). CDKO mice were generated as described previously (20). In brief, in order to delete the AT_{1A} receptors from the CD, we crossed an inbred 129SvEv transgenic mouse line expressing *Cre* recombinase specifically in the CD under the control of a *Hoxb7* promoter (*Hoxb7-Cre*) with *Agtr1a^{fl/fl}* animals to generate *Hoxb7-Cre^+Agtr1a^{fl/fl}* (CDKO) mice and their corresponding control (Hoxb7 Cre^– *Agtr1a^{fl/fl}*) littermates for experiments. Additionally, in order to delete the AT_{1A} receptors only from PCs, we crossed an inbred 129/SvEv transgenic mouse line expressing *Cre* recombinase under the control of an *AQP2* promoter (*AQP2-Cre*) with *Agtr1a^{fl/fl}* mice as described previously (20). Global Agtr1a (AT1aKO) mice on a 129/SvEv background were generated as described previously (11). Membrane-targeted *tdTomato (mT)/*membrane-targeted *EGFP*

(*mG*) mice with *loxP* sites flanking the membrane-targeted *dtTomato* cassette followed by an N-terminal membrane-tagged version of *EGFP* were purchased from the Jackson Laboratory and crossed with the two *Cre* recombinase transgenic lines. *mTmG* mice normally express red fluorescence protein in all tissues. When *Cre* is present, the *mT* cassette is deleted, triggering expression of *mG* (46).

Intra-arterial blood pressure measurement by radiotelemetry. Blood pressures were measured in conscious, unrestrained mice after the implantation of a pressure-sensing catheter (TA11PA-C10, Transoma Medical) via the left common carotid artery as described previously (21). In separate experiments, baseline blood pressures were measured on 5 consecutive days while the animals ingested a conventional diet containing 0.4% sodium chloride. After baseline blood pressure levels were measured, mice were placed on a low-salt diet (<0.002% NaCl) for 5 days and then on a high-salt (6% NaCl) diet (Harlan Teklad) for another 5 days while blood pressures were continuously monitored.

Angiotensin II–induced hypertension and administration of L-NAME or rofecoxib. Baseline blood pressure was recorded for 3 consecutive days, and then an osmotic minipump (Alzet model 2004, DURECT) infusing angiotensin II (Sigma Aldrich) at a rate of 1,000 ng/kg per minute was implanted subcutaneously, as described previously (10). In separate groups of experiments, 1 week after beginning of angiotensin II infusion, we added either L-NAME (20 mg/kg/d) to the drinking water or rofecoxib (10 mg/kg/d; Sequoia Research) to the chow, and blood pressure measurements continued for another week.

Sodium balance studies. Measurements of sodium balance were carried out daily prior to and during chronic angiotensin II infusion with individual metabolic cages and a gel diet containing nutrients, water, and 0.1% w/w sodium (Nutra-Gel; Bio-Serv) as described previously (21).

Measurement of urinary nitrite and nitrate levels. Urinary nitrite and nitrate levels were measured daily prior to and during chronic angiotensin II infusion using an ELISA kit (Cayman Chemical, no. 780001) per the manufacturer's instructions (47). Urinary creatinine levels were assessed using an alkaline picrate assay (Creatinine Companion, Exocell Inc.).

Quantification of COX-2 mRNA levels in the kidney. Renal cortex and inner medulla were microscopically dissected, and total RNA was isolated by using an RNeasy Mini Kit according to the manufacturer's instructions. COX-2 and 18S expression levels were determined using TaqMan primers (Applied Biosystems) by real-time PCR, as described previously (48).

Immunofluorescence staining. Kidneys were fixed in 4% paraformaldehyde and incubated in 30% sucrose overnight. 5-μm longitudinal cryostat sections of kidney from CDKO and control mice were hydrated through graded ethanol solutions (100%–50%), equilibrated to water, and subjected to microwave antigen retrieval as described previously (20). For COX-2 staining, kidneys were fixed with GPAS (2.5% glutaraldehyde, 10 mM sodium *m*-periodate, 40 mM phosphate buffer, and 1% acetic acid) as described in ref. 49. Sections were blocked with 5% skim milk in PBS, 0.1% Triton X-100 for 60 minutes and incubated with a polyclonal rabbit anti-GFP antibody (1:1,000, Invitrogen), polyclonal goat anti-AQP2 antibody (1:500; C-17; Santa Cruz Biotechnology Inc.), polyclonal rabbit anti–V-ATPase (1:500; H-140; Santa Cruz Biotechnology Inc.), polyclonal goat anti–V-ATPase (1:500, N-20; Santa Cruz Biotechnology Inc.), or polyclonal goat anti–COX-2 antibody (1:500; M-19; Santa Cruz Biotechnology Inc.) diluted in 5% skim milk in PBS overnight at 4°C. Adjacent sections were washed extensively in 1× PBS and subsequently incubated with Alexa Fluor 568 and Alexa Fluor 568 as secondary antibodies (1:1,000) (Invitrogen) for 1 hour at room temperature. After being washed in PBS, the sections were mounted using Fluoromount-G (SouthernBiotech) containing 2 μg/ml DAPI (Sigma-Aldrich). Confocal fluorescence images were taken using a confocal Zeiss LSM 510 microscope and analyzed with AxioVision LE software (Carl Zeiss MicroImaging Solutions GmbH).

Immunoblotting for COX-2 expression levels in IMCD suspensions. Suspensions enriched for CD segments from control, CDKO, PCKO, and global $AT_{1A}KO$ mice were prepared as described previously (25) with slight modifications. Inner medullas were dissected from kidneys of 3 mice and then incubated in an enzymatic solution containing collagenase, hyaluronidase, and DNAse at 37°C for 40 minutes at 650 mOsm/kg with urea/NaCl to approximate the osmolality of the inner medulla. The suspension was passed through 100 μm mesh, followed by 3 rinses, pelleting at 60 *g*, and resuspending tubules in fresh buffer. A photomicrograph of a typical IMCD suspension is shown in Supplemental Figure 6. To test the purity of these suspensions, we carried out Western blot analysis for markers specific for distinct cell compartments in the kidney: (a) aquaporin-2 (AQP2) for CD, (b) NKCC2 for medullary thick ascending limb of the loop of Henle (mTAL), and (c) smooth muscle α-actin (smα-actin) for vascular structures

such as vasa recta. For these studies IMCD suspensions were homogenized and total protein was measured by the Bradford method. Equal amounts of protein (5 μg) were loaded onto a polyacrylamide gel, and proteins were resolved by electrophoresis. Target proteins were identified using specific antibodies, and levels of expression were quantified by Western blotting followed by densitometric analysis. As shown in Supplemental Figure 7, A–C, these preparations were highly enriched for IMCD markers without evidence for significant contamination by mTAL or vascular structures. For COX-2 expression, IMCD suspensions are homogenized in a buffer containing 20 mM HEPES (pH 7.4), 2 mM EDTA, 300 mM sucrose, 1.0% IGEPAL, 0.1% SDS, and a 1:100 dilution of a protease inhibitor cocktail (Sigma Aldrich) The membrane was incubated in blocking buffer containing 20 mmol/l Tris, 137 mmol/l NaCl, 5% nonfat dry milk, and 0.1% Tween 20 for 30 minutes and then with a 1:2,000 dilution of a COX-2–specific antibody (Santa Cruz Biotechnology Inc., 1747) in blocking buffer overnight at 4°C. After washing, the membrane was incubated with a secondary antibody (dilution 1:3,000) against the appropriate IgG conjugated to horseradish peroxidase (Amersham Biosciences). The reaction products were detected by enhanced chemiluminescence. To control for loading, detection of GAPDH was conducted using a 1:20,000 dilution of a monoclonal antibody (Millipore MAB374). Bands were semiquantified by densitometry, and the data are expressed as percent change in COX-2/GAPDH.

Immunoblotting for renal COX-2 expression levels. For assessment of renal medullary COX-2 expression levels after chronic angiotensin II treatment, renal inner medulla was dissected and placed into 30 mM Tris-HCl (pH 8.0) with 100 μM PMSF adapted from previous studies (50). After centrifugation at 10,000 *g* for 10 minutes, the supernatant was centrifuged for 60 minutes at 110,000 *g* to prepare microsomes. The microsomal pellet was solubulized in 50 mM Tris-HCl, 150 mM sodium chloride, 2 mM EDTA, 0.2% SDS, and 0.2% Triton X-100 (pH 7.4). A total of 15 μg of total protein was loaded onto 12% SDS-PAGE gels and then transferred to nitrocellulose membranes according to the manufacturer's instructions (X-Cell Blot Module, Invitrogen). Membranes were blocked in blocking buffer (5% nonfat dry milk and 0.1% Tween 20 in PBS) for 1 hour at room temperature and then incubated with primary rabbit polyclonal anti–COX-2 antibody (1:2,000; batch 160126, Cayman Chemical) and mouse anti–β-actin (1:10,000) (Sigma-Aldrich, A5316) overnight at 4°C. Bound primary antibody was detected with either anti-rabbit or anti-mouse HRP-conjugated secondary antibodies (1:2000) (Bio-Rad). Proteins were detected by enhanced chemiluminescence according to the manufacturer's directions (GE HealthCare). For densitometric quantification of COX-2 proteins, the immunoblots were converted into a digital format and analyzed using ImageJ (NIH). All densitometric values were normalized to an actin loading control. Immunoblots from each tissue sample were performed in triplicate.

Urinary excretion of prostanoid metabolites. Urinary excretion of prostanoids were measured as described previously (51). In brief, 24-hour urine samples were collected by metabolic cage at baseline and after 2 weeks of angiotensin II infusion. To remove particulate matter, urine samples were centrifuged and then immediately aliquoted and frozen at −80°C until assay. Stable urinary prostanoid metabolites (13,14-dihydro-15-keto-PGE$_1$/PGE$_2$ [prostaglandin E$_2$], thromboxane TxB$_2$ [TxA$_2$], and 6-keto-PGF$_{1\alpha}$ [prostaglandin I$_2$]) were measured using specific competitive enzyme immunoassays (Cayman Chemical, nos. 514010, 501020, 515211). Creatinine levels in urine were assessed using an alkaline picrate assay (Creatinine Companion, Exocell Inc.).

Cell culture experiments in MDCK C-11 cells. MDCK C-11 cells, a gift from Hans Oberleithner (Institute of Physiology II, University of Münster, Münster, Germany), were grown in Earle's medium supplemented with FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Biochrom). AT$_1$ receptor–expressing MDCK C-11 cells were generated by retroviral transduction. The GFP-tagged AT$_1$ receptor was kindly provided by László Hunyady (Semmelweis University, Budapest, Hungary). The AT$_1$ receptor was cloned into the pLE-GFP-C1 vector *HindIII/NotI* via PCR with the following primers: 5′-CGCGGGAAGCTTATGGCCCT-TAACTCTTC and 5′-GCGGGGGCGGCCGCCTCACTCCACCTCAAAAC. Briefly, infectious viral supernatants — containing the sequence verified AT$_1$ receptor cDNA or the GFP control — were produced by transient transfection of HEK293T cells. MDCK C-11 cells were infected with viral supernatants containing either the AT$_1$ receptor or a GFP control for 48 hours. Selection of transduced cells was performed by G418 (800 μg/ml). After selection, the maintenance dose of G418 was 400 μg/ml. AT$_1$ receptor expression was confirmed by quantitative real-time PCR (qPCR), and activation of the AT$_1$ receptor signaling pathway p42/44 by angiotensin II was confirmed by Western blot analysis (data not shown). After 6 hours of angiotensin II (0.1 μM) stimulation, MDCK C-11 cells were lysed with heated Laemmli buffer and

centrifuged (20000 g, 30 minutes, 4°C). Equal amounts of protein were loaded onto 10% SDS-PAGE gels and then transferred to nitrocellulose membranes according to manufacturer's instructions (X-Cell Blot Module, Invitrogen). Membranes were blocked and incubated with primary rabbit polyclonal anti–COX-2 antibody (1:2,000; batch 160126) Cayman Chemical) and mouse anti–β-actin (1:20,000) (Sigma-Aldrich, A5316) overnight at 4°C as described above.

For measurement of the prostaglandin E_2 secretion rate, the supernatants from angiotensin II–stimulated (0.1 μM) MDCK C-11 cells were collected and measured with a commercially available competitive enzyme immunoassay (Cayman Chemical) as described above.

Statistics. The values for each parameter within a group are expressed as mean ± SEM. All data were analyzed by either 1- or 2-way ANOVA, followed by Bonferroni's multiple comparison post-hoc test, Student's t test, or Mann-Whitney U test after testing for normal distribution. P values less than 0.05 were considered significant.

Author contributions

JS, SBG, and TMC designed the research; JS, DC, MH, MAS, TY, EK, and SBG performed the research; JS, DC, MH, MAS, TY, and EK analyzed the data; and JS and TMC wrote the manuscript.

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