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AT₁ receptor-mediated enhancement of collecting duct renin in angiotensin II-dependent hypertensive rats

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Abstract

Angiotensin II (ANG II)-infused rats exhibit increases in distal nephron renin expressed in principal cells of connecting tubules and collecting ducts. This study was performed to determine whether the augmentation of distal nephron renin involves ANG II type 1 (AT₁) receptor activation. Male Sprague-Dawley rats (200–220 g) were divided into three groups: 1) sham operated ($n = 8$); 2) ANG II infused (80 ng/min, 13 days, $n = 8$); and 3) ANG II infused plus AT₁ receptor blocker (ARB), olmesartan (5 mg/days, $n = 8$). ANG II infusion increased systolic blood pressure (BP; 178 ± 4 vs. 122 ± 1 mmHg; $P < 0.001$) and suppressed plasma renin activity (PRA; 0.08 ± 0.1 vs. 5.3 ± 0.8 ng ANG I · ml⁻¹·h⁻¹). ARB treatment prevented the increase in BP (113 ± 6 mmHg) and led to increases in PRA (15.8 ± 1.5 ng ANG I·ml⁻¹·h⁻¹). Renin protein levels measured in the kidney medulla, to avoid contribution from juxtaglomerular apparatus cells, were higher in ANG II-infused rats [1.64 ± 0.3 vs. 1.00 ± 0.1 densitometric units (DU) compared with sham-operated rats; $P < 0.05$], and ARB treatment prevented this increase (1.01 ± 0.1). Similarly, renin immunoreactivity increased in medullary collecting ducts of ANG II-infused compared with sham-operated rats (2.5 ± 0.3 vs. 1.0 ± 0.2 DU; $P < 0.001$), which was also prevented by ARB (1.01 ± 0.06). Renin qRT-PCR in ANG II-infused rats showed higher mRNA levels in the kidney medulla compared with sham-operated rats (5.5 ± 2.3 vs. 0.04 ± 0.02 ratio to GAPDH mRNA levels; $P < 0.001$); however, renin transcript levels were normalized in the ARB-treated rats. These data demonstrate that the augmentation of distal nephron renin in ANG II-infused hypertensive rats is AT₁ receptor mediated. The augmented distal tubular renin may contribute to increased intratubular ANG II levels and distal nephron sodium reabsorption in ANG II-dependent hypertension.

Keywords

distal nephron; immunohistochemistry; Western blotting; qRT-PCR

ANGIOTENSIN II (ANG II) plays an important role in fluid, electrolyte, and blood pressure regulation. In ANG II-dependent hypertension, intrarenal ANG II levels increase to a greater extent than can be explained from the circulating levels (4,6,10,20,25,33,36,37). The augmented intrarenal ANG II levels are partially due to enhanced uptake of ANG II via an AT₁ receptor-mediated mechanism and augmentation of proximal tubule cell angiotensinogen (AGT) formation and secretion (11,13,14,35,37). Chronically elevated intrarenal ANG II levels exert a stimulatory action on proximal tubule AGT transcript and

protein which is mediated by the AT₁ receptor (17). The finding that urinary excretion of AGT also increases in a time- and dose-dependent manner indicates that proximally formed AGT is delivered to distal nephron segments, which could thus contribute to intratubular ANG I and ANG II formation in distal nephrons as long as adequate sources of renin and angiotensin-converting enzyme (ACE) are available. Substantial ACE activity in collecting duct fluid and urine have been demonstrated (1), in addition to an increment of intrarenal ACE binding in ANG II-dependent hypertension. In contrast, juxtaglomerular (JG) renin as well as plasma renin activity (PRA) are suppressed by chronic ANG II infusions (20,32).

Although renin is mainly produced by JG cells, renin transcript and protein are also present in renal tubular segments (2,5,9,19,27,29,30). Renin has been identified in the proximal tubules, and it has been suggested that it may be regulated differently than JG renin, because it can be activated by ACE inhibitors (21,30,31). Rohrwasser et al. (29) reported in human and mouse tissues the presence of apical renin immunostaining in connecting tubule (CNT) cells and renin secretion in isolated CNT cells in vitro, suggesting that these cells may secrete renin. It was also demonstrated that renin is present in principal cells of CNTs and of cortical and medullary collecting ducts of normal rat kidneys; furthermore, chronic infusion of ANG II led to an enhancement of renin protein in these distal nephron cells (27). Distal nephron renin could be an additional contributing factor to the development and maintenance of hypertension due to continued intratubular formation of ANG II, despite suppression of JGA renin. Because elevated ANG II levels were associated with increased distal tubular renin levels, further experiments were performed to determine whether the augmentation of distal nephron renin in chronic ANG II-infused hypertensive rats is dependent on an AT₁ receptor-mediated mechanism.

METHODS

Animals and tissue preparation

All experimental protocols were approved by the Tulane Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (200 to 220 g, $n = 24$; Charles River Laboratories, Wilmington, MA) were cage housed and maintained in a temperature-controlled room with a 12:12-h light-dark cycle, with free access to tap water and standard rat chow (Ralston Purina, St. Louis, MO) for 2 wk. For minipump implantation (Alzet model 2002, Alza, Palo Alto, CA), rats were selected at random to be subjected to either sham operation ($n = 8$) or ANG II infusion ($n = 16$). For the ANG II infusions, an osmotic minipump containing ANG II (Human ANG II, Sigma, St. Louis, MO; 80 ng/min for 13 days) was implanted subcutaneously at the dorsum of the neck. One group of ANG II-infused rats ($n = 8$) also received an AT₁ receptor blocker (ARB), olmesartan (5 mg/day in the food, Sankyo, Japan) during the period of ANG II infusion. Systolic blood pressure (SBP) was monitored by tail-cuff plethysmography (Visitech, BP2000, Apex, NC) 1 day before and 3, 7, and 11 days following sham operation or minipump implantation as previously described (13-15).

Sample collection, preparation, and evaluation

Blood and kidney samples were harvested on *day 13*. Trunk blood was collected into two chilled tubes containing 5.0 mmol/l EDTA, which were centrifuged at 4,000 rpm for 30 min at 4°C. Plasma fractions were removed and assayed for PRA as previously described (33). PRA was expressed as nanograms per milliliter per hour of generated ANG I.

After decapsulation, the left kidney was cross sectioned and fixed in zinc-saturated formalin for immunohistochemical studies. Kidneys were embedded in paraffin blocks, sectioned (23 μ m), and mounted onto slides with Vectabond (Vector Laboratories, Burlingame, CA). Serial kidney sections were immunostained by the immunoperoxidase technique (with

modifications as described previously) (26). Briefly, after being dewaxed in xylene, the kidney sections were rehydrated in 100, 95, to 70% alcohols and treated with methanol and 3% H₂O₂ for 30 min to block peroxidase activity. Then, kidney sections were washed and sequentially incubated with 1) normal blocking rabbit serum for 20 min; 2) primary antibody, antipeptide renin polyclonal antibody diluted in PBS containing 1% bovine serum albumin at 1:4,000 or 1:8,000 concentrations (100 µl) for 90 min; 3) secondary antibody, biotinconjugated rabbit antimouse IgG for 30 min; and 4) avidin DH, biotinylated horseradish peroxidase H complex using an ABC Elite Vectastain kit (Vector Laboratories) for 45 min. Peroxidase activity was visualized with 0.1% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma). The slides were counterstained with hematoxylin, mounted using aqueous mounting media (Biomed, Fisher Scientific), and coverslipped. Renin immunolocalization was assessed using a polyclonal anti-rat specific renin antibody raised in rabbit (generously provided by Dr. T. Inagami) and an automatic robot system (DAKO, Carpinteria, CA), which allowed identical incubation time of all slides to reagents and antibody as previously described (27). Renin immunoreactivity was determined in 10 different microscopic fields per tissue section per animal, using an Olympus BX51TRF microscope, ×40 magnification, and an integrated Magnafire SP Digital "Firewire" Camera System for image processing. The intensity of distal nephron renin immunoreactivity in ANG II-infused and sham rat kidney sections was analyzed using ImagePro Plus Software (version 4.5.1 for Windows 2000 XP; Media Cybernetics), which allowed a computerized determination of the area of positive staining (µm) and the intensity of immunoreactivity (sum of density of positive tubules in an analyzed area). For this analysis, JGA renin immunostaining was analyzed separately. The results were expressed in arbitrary units of the relative intensity normalized to the renin immunostaining average of the sham group.

To avoid the contribution of JGA renin to distal nephron renin expression, protein and total RNA were extracted from renal medulla dissected from cortex of the right kidney. Renin Western blot analysis using 10 µg of protein against rat renin (1:4,000) was performed with a standard protocol as described previously (14,27).

For quantitative real-time PCR (qRT-PCR), 50 ng/well of total RNA was used as previously described (14,27). Although we previously were not able to show upregulation due to inadequate sensitivity, we optimized the conditions and procedures since the previous report (14,27). Amplifications of the rat *renin 1c* gene were performed using 1) a specific probe (5'-TTCAAAGTCATCTTTGACACGGGTTTCAG-3') labeled with 5'-FAM and 3'-black hole quencher 1 and 2) a set of primers (sense 5'-AGTACTATGGTGAGATCGGCATT-3'; antisense 5'-AGATTCACAACCTCTATGACTCCTC-3'). In addition, coamplifications of the GAPDH gene were performed using the following primers (sense, 5'-CAGAACATC ATCCCTGCATC-3'; antisense, 5'-CTGCTTACCACCTTCTTGA-3'; and the probe, 5'-CCT GGA GAA ACC TGC CAA GTA TGA TGA-3') labeled with 5'-HEX and 3'-black hole quencher-2.

Statistical analysis

Results are expressed as means ± SE. The data were analyzed using a one-way factorial or a two-way repeated ANOVA with post hoc Newman-Keuls and Bonferroni tests, respectively. Statistical significance is defined at a value of $P < 0.05$.

RESULTS

SBP and PRA

SBP values were similar among the groups before implantation of the osmotic minipumps. By day 7 of ANG II infusion, SBP was significantly elevated in ANG II-infused rats ($157 \pm$

8 vs. 120 ± 3 mmHg) and was increased further by *day 11* (178 ± 4 vs. 122 ± 1 mmHg; $P < 0.001$). ARB treatment with olmesartan prevented the development of hypertension (110 ± 6 and 113 ± 6 mmHg at *days 7* and *11*, respectively). PRA was suppressed in ANG II-infused rats (0.1 ± 0.1 vs. 5.3 ± 0.8 ng ANG I·ml⁻¹·h⁻¹) compared with sham-operated rats. ARB treatment with olmesartan led to large increases in PRA (15.8 ± 1.5 ng ANG I·ml⁻¹·h⁻¹).

Renin immunohistochemistry in JGA and in cortical and medullary distal nephron segments

Figures 1 and 2 show renin immunostaining of rat kidney sections stained in the same run using an automatic immunostainer system and a polyclonal rat anti-renin antibody raised in rabbit at 1:8,000 concentration. Figure 1 shows augmentation of renin immunoreactivity in principal cells of cortical CNTs and cortical (cortex, *top*) and medullary (medulla, *bottom*) collecting ducts of ANG II-infused rats (Fig. 1, *B* and *E*) compared with sham-operated rats [Fig. 1, *A* and *D*; 5.0 ± 0.6 vs. 1.0 ± 0.1 densitometric units (DU) cortex; 2.5 ± 0.3 vs. 1.0 ± 0.2 DU medulla; $P < 0.001$]. Treatment with olmesartan, a specific AT₁ receptor blocker, prevented the increases in renin immunoreactivity induced by chronic ANG II infusion (0.1 ± 0.0 DU cortex; 0.1 ± 0.0 DU medulla; Fig. 1, *C* and *F*). In contrast, positive renin immunoreactivity observed in JGA cells (Fig. 2) of sham-operated (*A*), ANG II-infused (*B*), and ANG II-infused plus ARB treatment (*C*) rats was significantly suppressed in ANG II-infused rats (*B*) compared with sham-operated (*A*) rats (0.1 ± 0.0 vs. 1.0 ± 0.1 DU relative to sham; $P < 0.001$), but it markedly increased in ARB-treated rats (*C*; 2.1 ± 0.3 DU relative to sham). In addition, renin-specific immunostaining was observed in medullary interstitial cells of rats infused with ANG II and treated with olmesartan. Densitometric analysis of the intensity of renin immunoreactivity in JGA (Fig. 3A), cortical (Fig. 3B), and medullary (Fig. 3C) collecting duct cells expressed in arbitrary units is presented in the Fig. 3.

Renin protein expression of renin in rat kidney cortex and medulla

Analysis of integrated densitometric values (IDV) showed that the ratios for renin protein were significantly suppressed in cortical renal tissues from ANG II-infused hypertensive rats (Fig. 4A; 0.5 ± 0.2 vs. 1.0 ± 0.1 densitometric ratio compared with the average of sham-operated rats; $P < 0.05$); whereas the renin levels in ANG II-infused hypertensive rats treated with olmesartan increased (1.2 ± 0.3 densitometric ratio compared with the average of sham-operated rats). In contrast, renin protein levels were higher in the kidney medulla samples of ANG II-infused than sham-operated rats (Fig. 4B, 1.6 ± 0.3 vs. 1.0 ± 0.1 densitometric ratio compared with the average of sham-operated rats; $P < 0.05$). Treatment of ANG II-infused rats with olmesartan prevented the increases (1.0 ± 0.1 densitometric ratio compared with average of sham-operated rats). As a control study to assess for equal loading, membranes were reprobbed with an antibody against β -actin. The results showed that IDV levels were unaltered among the groups.

qRT-PCR for renin in rat kidney medulla

Expression of renin mRNA in renal medullary tissues is shown in Fig. 4. ANG II-treated rats showed significantly higher renin mRNA levels compared with sham-operated rats (5.5 ± 2.3 vs. 0.04 ± 0.02 DU; $P < 0.001$). Treatment with olmesartan prevented ANG II-induced increases in renin mRNA expression (0.1 ± 0.0 DU; Fig. 5). These results indicate that chronic ANG II infusion exerts a stimulatory effect on medullary renin mRNA which reflects the renin mRNA level in the tubular cells as the medulla does not contain JGA cells.

DISCUSSION

This study extends previous findings that chronic ANG II infusions lead to progressive increases in SBP along with suppression of PRA and JGA renin but augmentation of

medullary collecting duct renin protein and mRNA levels. These findings are consistent with previous results demonstrating that ANG II-infused hypertensive rats have increases of renin immunoreactivity in principal cells of cortical CNT cells and cortical and medullary collecting duct cells (27). The novel issue addressed in the present study is whether the augmentation of distal tubular renin in ANG II-dependent hypertension is mediated by an AT₁ receptor mechanism. To test this hypothesis, ANG II-infused rats were treated with olmesartan, a specific AT₁ receptor blocker. The data demonstrate that the increases in distal nephron renin mRNA and protein expression were prevented by treatment with ARB. Therefore, AT₁ receptor activation is involved in the mechanism by which increases in circulating and intrarenal ANG II levels stimulate renin in distal nephron segments. In contrast to the increases in PRA and JGA renin caused by blockade of ANG II-induced inhibition of JGA renin release, ARB treatment markedly reduced the expression of renin in distal nephron segments. Despite the fact that ARB-treated rats exhibited substantial increases in PRA and renin immunoreactivity by recruited cells from the afferent arteriole, there was suppression of renin in medullary collecting tubules. These findings provide further evidence that renin in principal cells of distal nephron segments is differentially regulated and is not the consequence of uptake of renin produced by JGA cells. Renin mRNA levels measured in the kidney medulla samples may reflect renin mRNA not only in tubular epithelial cells but also in endothelial and interstitial cells. Renin immunoreactivity was detected in medullary interstitial cells particularly under conditions of olmesartan treatment; however, no consistent trends were observed in the present study. Nevertheless, it is possible that interstitial cell renin expression could be upregulated in certain conditions such as ANG II inhibition with ACE inhibitors or under ANG II receptor blockade as has been reported in renin expressing cells in the preglomerular vasculature (7,8,23). In addition, these data firmly establish that medullary renin mRNA is also upregulated by the chronic ANG II infusions and that this increase is prevented by ARB treatment. Although it has also been shown that aldosterone exerts positive effects on renin gene expression in JGA cells probably by stabilizing renin mRNA via a mineralocorticoid receptor-mediated mechanism (12), the present study did not rule out the aldosterone effects on distal nephron renin. Whether aldosterone exerts a positive action on distal tubular renin is an issue that remains to be investigated.

Previous studies demonstrated that the augmented intrarenal ANG II levels after chronic ANG II infusions are due to an enhanced internalization of ANG II (11,35,37) coupled with stimulation of AGT expression (13,14,17), both involving activation of AT₁ receptors. Additionally, it has also been reported that chronic ARB treatment limits the increases in AGT protein levels in renal cortex as well as its urinary excretion rate (15,16). Our data extend these findings, demonstrating that chronic treatment with olmesartan blocks the increases in renin protein expression in principal cells of CNTs and collecting ducts and renin mRNA in the renal medulla as well. Therefore, the chronic effect of ARB treatment to block the internalization of ANG II into kidney cells (11,35,37), the stimulation of intrarenal AGT expression (13,14), as well as the increases of renin in distal nephron segments may all be acting in a synchronized manner to ameliorate the enhanced intrarenal ANG II content seen during ANG II-dependent hypertension.

Renin in distal nephron segments may provide a possible pathway for intratubular ANG I generation in distal nephron segments. Intrarenal AGT mRNA and protein have been localized to proximal tubule cells, indicating that the intratubular ANG II could be derived from locally formed and secreted AGT (3,13,14,22). Furthermore, intrarenal AGT is regulated by an amplification mechanism such that proximal tubule AGT mRNA and protein are enhanced during chronic ANG II infusions associated with increased passage of AGT in the urine (16). This effect helps to maintain or even increase further the production of intrarenal ANG II in ANG II-dependent hypertension (13,14). The present findings

suggest that ANG II mediates enhancement of tubular renin through an AT₁ receptor-dependent mechanism. Once ANG I is formed, intratubular ANG II formation can occur because ACE is present in distal nephron segments as well as on the brush border of proximal tubule cells (1). Indeed, ACE production has been demonstrated in isolated inner medullary collecting duct cells, suggesting a new site of ACE secretion in collecting duct in the nephron (28). Therefore, intratubular ANG II formation may occur not only in the proximal tubule but also beyond the CNT. The increased intratubular ANG II formation may partially lead to augmented ANG II renal content and to enhancement of fractional sodium reabsorption rate characteristic of the ANG II-infused rat model.

Recent studies showed that ANG II directly stimulates epithelial sodium channel activity in cortical collecting duct cells (24) and that there is intraluminal conversion of ANG I to ANG II in cortical collecting ducts (18). Thus renin in distal nephron segments may synergistically contribute to the ANG II stimulatory effect on distal tubular renin and could help explain the marked stimulation of sodium reabsorption and suppression of the pressure-natriuresis relationship observed in ANG II-infused hypertensive rats (34). Therefore, distal nephron renin may play a crucial role in the sustained high intrarenal ANG II levels and hence contribute to the progressive high blood pressure observed in ANG II-dependent hypertension.

In summary, these data demonstrate that chronic ANG II infusions to normal rats significantly increase renin protein and mRNA levels in principal cells of connecting ducts and collecting tubules. This augmentation is dependent on activation of AT₁ receptors. Although PRA and JGA renin are markedly suppressed in ANG II-induced hypertension, increased distal nephron renin associated with an increased proximal tubular AGT production and spillover into the distal nephron segments may collectively contribute to elevated and sustained intratubular ANG I and ANG II formation in this hypertensive model. ANG II actions on the distal nephron could be acting synergistically with aldosterone to increase distal nephron sodium reabsorption and contribute to overall enhanced sodium reabsorption. These results provide an additional mechanism by which ARB treatment may exert a protective effect on the kidney.

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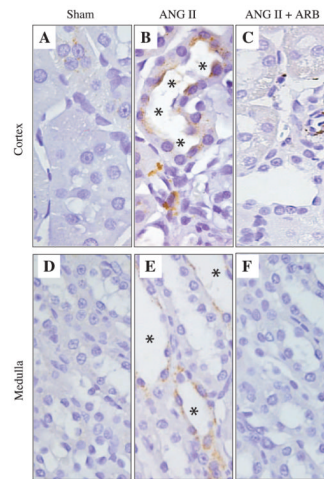


Fig. 1.

Renin immunoreactivity in paraffin-embedded rat kidney sections. Positive renin immunostaining is observed in cortical collecting tubule cells (cortex, *A-C*) and medullary collecting duct cells (medulla, *D-F*) of sham ($n = 8$; *A* and *D*), ANG II-infused ($n = 8$; *B* and *E*), and ANG II-infused rats treated with AT₁ receptor blocker ($n = 8$; ANG II + ARB, *C* and *F*). *Renin immunostaining present in collecting duct cells. Renin antibody concentration 1:4,000. ARB, AT₁ receptor blocker; olmesartan, 5 mg/day orally.

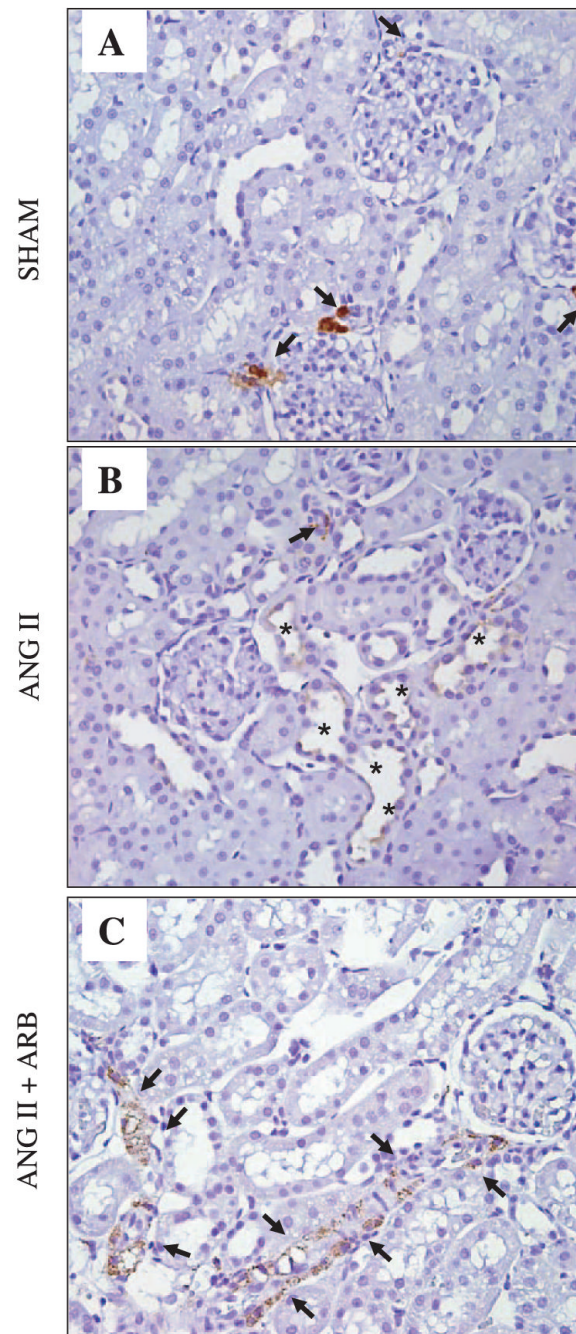


Fig. 2. Renin immunoreactivity in paraffin-embedded rat kidney sections. Positive renin immunostaining is observed in juxtaglomerular cells (arrows) of sham ($n = 8$; A), ANG II-infused ($n = 8$; B), and ANG II-infused rats treated with AT₁ receptor blocker ($n = 8$; ANG II + ARB; C). Recruitment of arterial smooth muscle cells beyond the afferent arterioles positive for renin immunostaining can be seen in the ANG II+ARB rat kidney. Renin immunostaining is also present in cortical collecting duct cells (*) of ANG II-infused rat kidney. Ten microscopic fields per kidney section were analyzed from 5 different kidneys sections/animal in each group. Renin antibody concentration 1:4,000. Olmesartan, 5 mg/day orally.

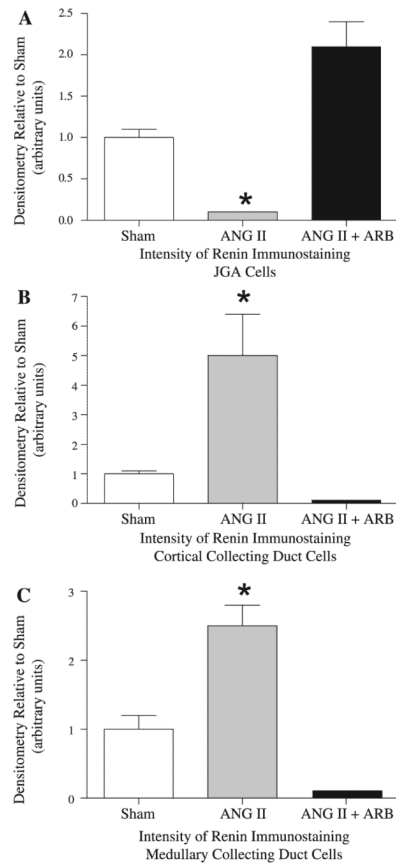
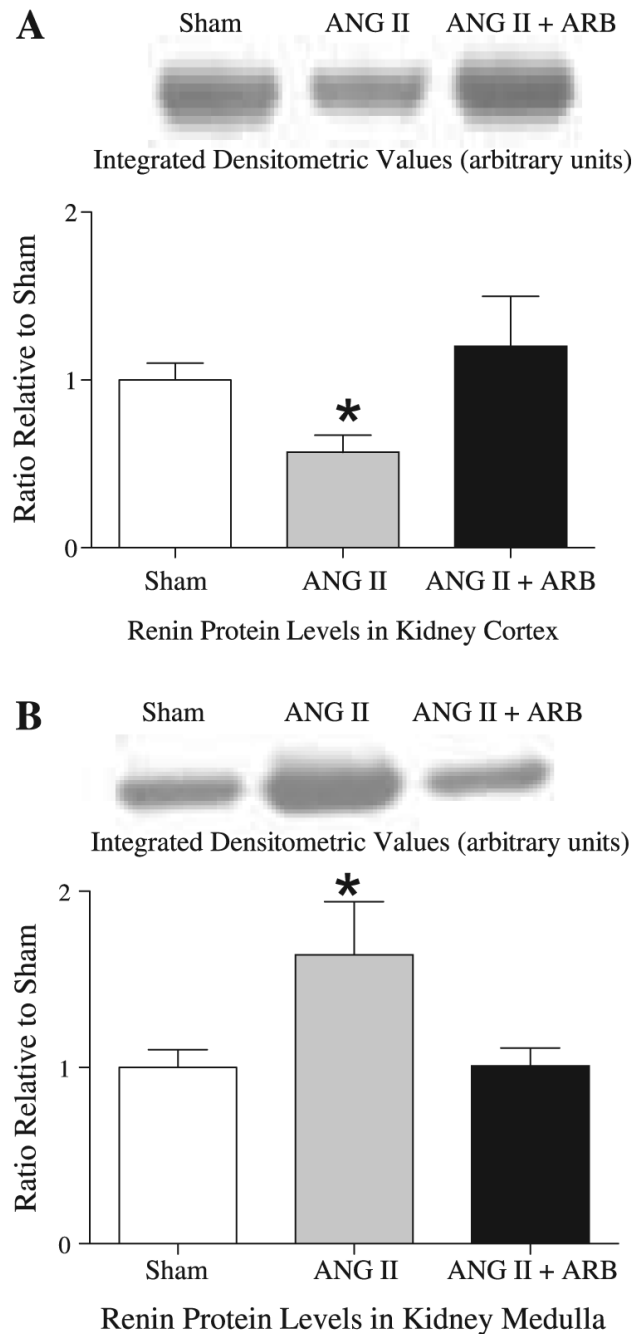


Fig. 3.

Quantification of intensity of renin immunostaining in juxtaglomerular (JGA; *A*), cortical collecting duct (*B*), and medullary collecting duct (*C*) cells of rat kidneys. *A*: densitometric analysis of the renin immunostaining in JGA cells. JGA renin immunoreactivity was suppressed in ANG II-infused compared with sham-operated rat kidneys; however, it substantially increased after ARB. *B* and *C*: densitometric analysis of renin immunoreactivity in cortex and medulla, respectively. Notice that in contrast to the JGA renin immunoreactivity suppression observed in ANG II-infused rats, an enhancement of renin immunoreactivity is observed in collecting duct cells of the renal cortex (*B*) as well as of the renal medulla (*C*). Relative to sham-operated rats ($n = 8$), densitometric analysis of the renin immunoreactivity in JGA cells, cortical and medullary CCDs of ANG II-infused rats treated ($n = 8$) or not with ARB ($n = 8$) was determined in 5 kidney sections/animal; 10 analyzed microscopic fields/kidney section. Values are means \pm SE. * $P < 0.0001$ vs. sham rats.

**Fig. 4.**

Renin Western blot analyses in kidney cortex and medulla from sham-operated, ANG II-infused, and ANG II-infused rats treated with AT₁ receptor blocker (ANG II + ARB, olmesartan). Representative autoradiographies of renin kidney cortex (Fig. 3A) and kidney medulla (Fig. 3B) from sham-operated ($n = 8$), ANG II-infused ($n = 8$), and ANG II + ARB ($n = 8$) rats are shown. Densitometric analysis showed that chronic ANG II infusion significantly decreased renin levels in the cortex (Fig. 3A), but in contrast increased renin levels in the medulla (Fig. 3B). ARB treatment prevented these effects. Relative expression of renin protein was corrected by β -actin in all groups in 3 independent experiments. Renin antibody concentration 1:4,000. * $P < 0.05$ vs. sham rats.

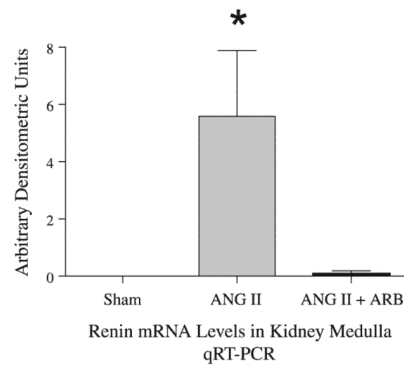


Fig. 5. Quantitative renin mRNA levels in rat kidney medulla measured by qRT-PCR. Renin qRT-PCR in ANG II-infused rats showed higher mRNA levels in the kidney medulla compared with sham-operated rats; however, renin transcript levels were normalized in the ARB (olmesartan)-treated rat group. Renin mRNA concentration was determined in 3 independent experiments and expressed relative to GAPDH. ARB, olmesartan (5 mg/day \times 5 days). * $P < 0.001$ vs. Sham and ANGII+ARB.