Augmentation of angiotensinogen expression in the proximal tubule by intracellular angiotensin II via AT_{1a}/MAPK/NF-κB signaling pathways

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Am J Physiol Renal Physiol 310: F1103–F1112, 2016. First published February 10, 2016; doi:10.1152/ajprenal.00350.2015—Long-term angiotensin II (ANG II) infusion significantly increases ANG II levels in the kidney through two major mechanisms: AT_{1} receptor-mediated augmentation of angiotensinogen (AGT) expression and uptake of circulating ANG II by the proximal tubules. However, it is not known whether intracellular ANG II stimulates AGT expression in the proximal tubule. In the present study, we overexpressed an intracellular cyan fluorescent ANG II fusion protein (Ad-sglt2-ECFP/ANG II) selectively in the proximal tubule of rats and mice using the sodium and glucose cotransporter 2 (sglt2) promoter. AGT mRNA and protein expression in the renal cortex and 24-h urinary AGT excretion were determined 4 wk following overexpression of ECFP/ANG II in the proximal tubule. Systolic blood pressure was significantly increased with a small antinatriuretic effect in rats and mice with proximal tubule-selective expression of ECFP/ANG II (P < 0.01). AGT mRNA and protein expression in the cortex were increased by >1.5-fold and 61 ± 16% (P < 0.05), whereas urinary AGT excretion was increased from 48.7 ± 5.7 (n = 13) to 102 ± 13.5 (n = 13) ng/24 h (P < 0.05). However, plasma AGT, renin activity, and AGT mRNA levels remained unaltered by ECFP/ANG II. The increased AGT mRNA and protein expressions in the cortex by ECFP/ANG II were blocked in AT_{1a}-knockout (KO) mice. Studies in cultured mouse proximal tubule cells demonstrated involvement of AT_{1a} receptor/MAP kinases/NF-κB signaling pathways. These results indicate that intracellular ANG II stimulates AGT expression in the proximal tubules, leading to increased AGT formation and secretion into the tubular fluid, which contributes to ANG II-dependent hypertension.

angiotensin II; angiotensinogen; kidney; NF-κB; proximal tubule

The important role of the renin-angiotensin system (RAS), especially its major effector peptide, angiotensin II (ANG II), in the development and maintenance of hypertension has been well established in all ANG II-dependent hypertension models, including two-kidney, one-clip (2K1C) (5, 30, 46), Ren-2 gene transgenic (40, 42, 59), and ANG II-infused animals (7, 56, 61, 63). One important and consistent feature of these ANG II-dependent hypertension models is that ANG II levels in the kidneys are increased to a greater extent than can be explained on the basis of elevated plasma ANG II which suppresses renin expression in the juxtaglomerular apparatus (JGA) of the kidney (12, 29, 44, 61). The mechanisms underlying this phenomenon remain incompletely understood. Currently, there are at least two potential mechanisms contributing to augmentation of ANG II levels in the kidney: AT_{1} receptor-mediated angiotensinogen (AGT) expression and uptake of circulating ANG II by the proximal tubules. We and others have shown that circulating ANG II or systemically infused ANG II is taken up and accumulated in the proximal tubule of the kidney, including endosomal, mitochondrial, and nuclear compartments (1, 15, 29, 47, 61). The uptake of circulating ANG II by the proximal tubule of the kidney appears to be mediated primarily by AT_{1} (AT_{1a}) receptors (29, 32, 55, 61, 64), but also partially by the multiligand endocytic receptor megalin (10, 37) and caveolin 1-dependent mechanisms (26). Our studies further suggest that internalized (or intracellular) ANG II may act on cytoplasmic and nuclear AT_{1} receptors to induce long-term transcriptional effects on the RAS (33, 34). Second, there is increasing evidence suggesting that ANG II may be generated intrarenally, especially in the proximal tubules and collecting ducts during ANG II-induced hypertension (11, 16, 22, 44). Indeed, we and others have shown that chronic infusion of ANG II is associated with marked increases in angiotensinogen (AGT) expression in the renal cortex and urinary AGT excretion, which is also AT_{1} receptor dependent (11, 16, 22, 44). The upregulation of AGT, along with angiotensin-converting enzyme (ACE) (14) and AT_{1} receptor expression in the proximal tubules by ANG II (14, 59), may therefore serve as a powerful feed-forward mechanism to promote local ANG II production and actions (44).

While there is a general consensus on AT_{1} (AT_{1a}) receptor-mediated uptake of circulating and/or extracellular ANG II by the proximal tubule of the kidney, the major source of augmented AGT mRNA and protein expression in the kidneys by ANG II remains uncertain. Recently, Matsusaka et al. (39) reported that mice with kidney-specific knockout of AGT maintained similar levels of AGT and ANG II proteins in the kidneys, compared with wild-type control mice. These authors further showed that liver-specific AGT knockout (AGT-KO) in mice almost completely abolished plasma and kidney AGT and ANG II proteins (4). The findings of this study suggest that liver-derived AGT is the primary source of AGT and ANG II proteins in the kidneys under normal conditions. In contrast, previous studies have demonstrated that 1) AGT levels in the proximal tubule fluid are greater than in plasma (43); 2) proximal tubules secrete ANG II and/or AGT (3); and 3) there is augmented AGT mRNA and protein expression in ANG II-induced hypertension in rats and mice (11, 16, 17). Although it is possible that some liver-derived AGT may be filtered through the glomeruli to enter the proximal tubules, where AGT may be taken up by the multiligand endocytic receptor megalin (48), our studies also support an...
important role for augmented proximal tubule AGT expression, contributing to higher levels of intrarenal ANG II in ANG II-dependent hypertension.

In the present study, we tested the hypothesis that tissue-specific overexpression of a cyan fluorescent protein-tagged intracellular ANG II fusion protein, ECFP/ANG II, directly stimulates AGT mRNA and protein expression in the proximal tubule cells, independently of liver-derived AGT in rats and mice. We chose to overexpress the intracellular ANG II fusion protein selectively in the proximal tubule instead of systemic infusion of ANG II to exclude the direct stimulatory effect of circulating ANG II on AGT biosynthesis and release in the liver. Since overexpression of ECFP/ANG II selectively in the proximal tubules did not increase plasma AGT, renin activity (PRA), and ANG II levels, our approach provides evidence that the expression of AGT mRNA and protein in the proximal tubules is augmented by intracellular ANG II independently of liver-derived AGT and circulating ANG II and independently of cell surface AT₁ receptor activation.

METHODS

Animals. Forty-eight adult male Sprague-Dawley rats (SD; ≈250 g body wt), purchased from Charles River, 24 adult male C57BL/6J mice, purchased from Jackson Laboratories, and 24 adult male AT₁KO mice (≈25 g body wt), bred in our laboratory, were used in the present study. Upon arrival, all rats were maintained on a normal rodent chow and had free access to tap water. Furthermore, all animals were trained with the tail-cuff measurement of blood pressure daily for 1 wk before basal systolic blood pressure (SBP) and 24-h urine and urinary sodium excretion were determined. All animal surgical and experimental protocols were approved by the Institutional Animal Care and Use Committees of the University of Mississippi Medical Center and Tulane University School of Medicine.

Overexpression of ECFP-tagged intracellular ANG II fusion protein, ECFP/ANG II, or its scrambled control, ECFP/ANG IIc, selectively in proximal tubules. Construction of adenovirus-mediated sglt2 promoter-directed ECFP/ANG II or ECFP/ANG IIc constructs and adenovirus-mediated ECFP/ANG II or ECFP/ANG IIc expression selectively in the proximal tubules of the kidney have been described previously (25, 28, 36). The constructs of ECFP/ANG II and ECFP/ANG IIc were provided by Dr. Julia Cook of the Ochsner Clinic Foundation (6), whereas the sglt2 promoter was provided by Dr. I. Rubera of the University of Nice-Sophia Antipolis (France), respectively (49). SD rats and C57BL/6J and AT₁KO mice were divided into three groups with 8 animals in each group: control, Ad-sglt2-ECFP/ANG II, or Ad-sglt2-ECFP/ANG IIc (a scrambled control of ECFP/ANG II). Briefly, the rats or mice were anesthetized with pentobarbital sodium (50 mg/kg ip), and both kidneys were exposed via left and right flank incisions, respectively (49). SD rats and C57BL/6J and AT₁KO mice were trained with the tail-cuff measurement of blood pressure daily (49). SD rats and C57BL/6J and AT₁-KO mice were divided selectively in the proximal tubules of the kidney have been described as previously described by Kobori et al. (17, 19). Briefly, total RNA was extracted from the renal cortex using a RNeasy mini kit (Qiagen, Chatsworth, CA), and the integrity and quality of the purified RNA were verified by the presence of the 28S and 18S rRNA bands after agarose gel electrophoresis. The extracted and purified RNA from each renal cortical sample was then reverse-transcribed using the SuperScript preamplification system for first-strand cDNA synthesis (Life Technologies BRL, Gaithersburg, MD) (17, 19). The AGT primers used in the present study included: sense, 5'-TTGTTGAAGCTTGCT- CCTCA-3' (exon 2, bases +638 to +661) and antisense, 5'- CACAGACTCACTGTTGTGCTCA-3' (exon 3, bases +901 to +911). For control, the GAPDH primers included sense, 5'- TCCCTCAAGATGTCAGCAA-3' (bases +421 to +440) and antisense, 5'-AGATCCACACGGCATACTT-3' (bases +728 to +709). Quantitative RT-PCR was then performed with each RT-PCR reaction containing 0.25–4 μl of the RT mixture, 2.5 μl of 10× buffer, 37.5 nmol of MgCl₂, 5 nmol of each dNTP, 5 pmol of each sense and antisense primer for AGT or GAPDH, and 0.625 U of Taq DNA polymerase (Life Technologies BRL), in a final volume of 25 μl, respectively (17, 19). PCR was carried out in a DNA thermal cycler 480 (PerkinElmer, Norwalk, CT) for 15–40 cycles, with a 45-s denaturation at 94°C, a 30-s annealing at 52°C, and a 90-s extension at 72°C, with a final 10-min extension at 72°C (17, 19). AGT mRNA expression was normalized as the AGT mRNA-to-GAPDH mRNA ratio for comparisons.

Measurement of AGT protein expression in the renal cortex by Western blot analysis. AGT protein expression in the renal cortex was determined by Western blot analysis as previously described (17, 19). Briefly, proteins samples from the cortex were extracted from each kidney in the buffer containing protease inhibitor cocktail (100 μg/ml PMSF, 1 μg/ml leupetin, 1 μg/ml aprotinin, and 2 mM EDTA, final concentrations in lysis buffer) and quantified accordingly. Protein samples were then electrophoretically separated on 3–8% Trit-glycine stacking gels at 125 V for 2.5 h and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) for 90 min at 25 V (XCell II Mini-Cell; Novex, San Diego, CA). The membranes were incubated with the primary AGT antibody for 3 h, washed, incubated with horseradish peroxidase-conjugated secondary antibody (donkey anti-sheep IgG, 1:30,000; Sigma) for 1 h, and washed again. This was followed by detection using enhanced chemiluminescence Western blotting (ECL system; Amersham Pharmacia Biotech) and exposed to X-ray film (Hyperfilm-ECL; Amersham Pharmacia Biotech). The integrated densitometric values (IDV) were normalized using the average values for the control group as previously described (17, 19).

Measurement of plasma AGT concentration and 24-h urinary AGT excretion. Plasma AGT levels and 24-h urinary AGT excretion were measured by a specific AGT ELISA as previously described (18, 20). Briefly, highly purified recombinant rodent AGT protein was used as the standard. One hundred microliters per well of rodent AGT (0.08–5.0 ng/ml for rat AGT diluted in ELISA buffer), plasma (1:2,500 diluted in ELISA buffer), and urine samples (1:25 diluted in ELISA buffer) were added into each well of the plates and incubated at 37°C for 1 h. After the incubation, the plates were washed with a washing buffer (PBS containing Tween 20, 0.05%, pH 7.5). This was followed by incubation with the 100 μl/well of the horseradish peroxidase-labeled C-terminal antibody (1:30 diluted in antibody solution) at
37°C for 30 min. Finally, the plates were washed with the washing buffer, and further incubated with the 100 μl/well of 3,3’,5,5’-tetramethylbenzidine solution under light-protected conditions at room temperature for 30 min. The reactions were stopped with the 100 μl/well of sulfuric acid (0.5 mol/l), and the absorbance were measured at 450 nm (18).

**Measurements of plasma and renal cortical ANG II levels.** Plasma and kidney cortical samples were collected at the end of the experiment to measure plasma and proximal tubule ANG II levels as described previously using a sensitive ELISA kit (S-1133; Bachem) (25, 36). ANG II was extracted from plasma and kidney samples using a phenyl-bonded solid-phase peptide extraction column (Elut-C18, Varian), vacuum dried overnight, and reconstituted in an ANG II assay buffer.

**Imaging of overexpression of ECFP/ANG II selectively in proximal tubules of rat and mouse kidneys.** To visualize whether ECFP/ANG II or its control ECFP/ANG IIc was similarly overexpressed selectively in the proximal tubules of the rat and mouse kidneys, fresh sections of the kidneys (6 μm thick) from control and experimental animals were cut on a cryostat and thaw-mounted on glass slides. Sections were briefly counterstained with the cell nuclear marker 4,6-diamidino-2-phenylindole (DAPI; 300 nM, Molecular Probes) for 5 min to stain the nuclei, or with the high-affinity F-actin probe Alexa Fluor 568 phalloidin to stain apical and basolateral membranes of the renal tubules, as well as the glomeruli with red-orange fluorescence (A12380, 1:40 from the stock solution, Molecular Probes). Sections were washed with phosphate-buffered saline and mounted on a microscopic stage. A Nikon-Eclipse TE2000-U inverted fluorescence microscope and a dual DAPI-CFP band-pass excitation filter set (excitation: 440 nm; emission: 495/50 nm) or a filter for Alexa Fluor 568 phalloidin (excitation: 578 nm; emission: 600 nm) were used for imaging as we described previously (25, 35, 36).

**Overexpression of intracellular ECFP/ANG II fusion proteins in mouse proximal tubule cells.** To determine the potential signaling mechanisms of intracellular ECFP/ANG II-induced augmentation of AGT expression in the kidneys, immortalized mouse proximal tubule cells (mPCTs) derived from the S1 segment of the proximal tubule of male C57BL/6J mice were used in vitro cell culture studies (57). mPCTs were a generous gift of Dr. Ulrich Hopfer of Case Western Reserve University. Briefly, mPCT cells were subcultured to 80% confluence in six-well plates, as appropriate, in the complete DMEM/F-12 growth medium at 37°C, supplied with 95% O2-5% CO2, 50 mM hydrocortisone, 5% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin as we described previously (27, 31, 35, 57). mPCTs were then transfected with the plasmid ECFP/ANG II (4 μg/well) for 48 h using the transfection protocol as we described previously (31, 35, 60, 62) and concurrently treated with or without the AT1 receptor blocker losartan (10 μM), the AT2 receptor blocker PD123319 (10 μM), the MEK1/MEK2 kinase inhibitor U0126 (1 μM), or the NF-κB inhibitor RO 106–9920 (1 μM), respectively (28). Protein and/or RNA samples were extracted from control and treated mPCTs, and the changes in NF-kB, p65, and AGT proteins were measured by Western blotting using a mouse monoclonal primary antibody recognizing an epitope overlapping the nuclear localization signal (NLS) of the p65 subunit of the NF-κB heterodimer (MAB3026, 1:500 dilution, Millipore) (24, 28) or a rabbit monoclonal primary anti-AGT antibody (MABC123, 1:1,000, Millipore), respectively.

**Statistical analysis.** All results are presented as means ± SE. One-way ANOVA was first to compare the differences in the same
parameters between groups of rats. If the $P$ value was $<0.05$, a post hoc Newman-Keuls multiple comparison test or Student’s unpaired $t$-test was performed to compare two different group means. The significance was set at $P < 0.05$.

RESULTS

Overexpression of ECFP/ANG II selectively in the proximal tubule of the kidney. Adenovirus-mediated sglt2 promoter-driven overexpression of intracellular ECFP/ANG II or its scrambled control, ECFP/ANG IIc, selectively in the proximal tubule of the rat kidney is shown in Fig. 1. We previously reported in detail that the overexpression of ECFP/ANG II peaked at 7–14 days but persisted 4 wk after intrarenal gene transfer (25). The present study focused on the relatively long-term expression and its effect on AGT expression in the kidney and blood pressure 4 wk after the overexpression was initiated. In control rats, there were minimal levels of CFP visualized in the cortex (Fig. 1A) and medulla (Fig. 1D), respectively. However, high levels of ECFP/ANG II expression were observed in nearly all proximal tubules in the superficial cortex of the kidney, with blue showing the nuclei (Fig. 1B). Both apical and basolateral membranes of the proximal tubules, as well as the glomeruli, were imaged in red-orange fluorescence using the high-affinity F-actin probe Alexa Fluor 568 phalloidin. Very low levels of ECFP/ANG II expression were observed in the glomeruli and cortical collecting ducts of the cortex (Fig. 1B). Furthermore, no ECFP/ANG II expression was detected in distal tubules and collecting ducts of the renal medulla (Fig. 1E). In the rat kidneys transferred with the scrambled Ad-sglt2-ECFP/ANG IIc, similar levels of CFP expression were visualized in the proximal tubules and glomeruli in the cortex (Fig. 1C), but not in the medulla (Fig. 1F).

Effects of proximal tubule-selective overexpression of ECFP/ANG II on blood pressure and urinary sodium excretion. The time course of blood pressure and urinary electrolyte excretory responses to the proximal tubule-selective overexpression of ECFP/ANG II in the rat kidney was reported previously (25). At 4 wk after ECFP/ANG II overexpression in the proximal tubules (Fig. 2A), a moderate and significant increase in systolic blood pressure persisted in rats receiving the transfer of ECFP/ANG II (140 ± 5 mmHg), but not the scrambled control ECFP/ANG IIc (122 ± 6 mmHg, $P < 0.01$). No difference in basal blood pressure was found among the three groups (Fig. 2). The blood pressure response was associated with a moderate, but significant, decrease in 24-h urinary sodium excretion in rats receiving the transfer of ECFP/ANG II.

![Fig. 2. Effects of expression of intracellular ECFP/ANG II or its scrambled ECFP/ANG IIc selectively in the proximal tubules of the rat kidneys for 4 wk on systolic blood pressure (A) and 24-h urinary sodium excretion (UNaV; B). **$P < 0.01$ vs. basal in the same group of animals. +++$P < 0.01$ vs. the time-control group at week 4.]

![Fig. 3. Effects of expression of intracellular ECFP/ANG II selectively in the proximal tubules of the rat kidneys for 4 wk on plasma angiotensinogen (AGT; A), renin activity (PRA; B), and ANG II levels (C). No significant difference (n.s.) was detected between the time control and ECFP/ANG II-overexpressing groups.]

AJP-Renal Physiol • doi:10.1152/ajprenal.00350.2015 • www.ajprenal.org
II (1.82 ± 0.1 mmol/24 h vs. basal: 2.33 ± 0.13 mmol/24 h, P < 0.01), but not the scrambled control ECFP/ANG IIc [2.16 ± 0.06 mmol/24 h vs. basal: 2.10 ± 0.12 mmol/24 h, not significant (n.s.)] (Fig. 2B).

Effect of proximal tubule-selective overexpression of ECFP/ANG II on plasma AGT, PRA, and ANG II levels. Unlike systemic infusion of ANG II which markedly increases circulating and tissue ANG II levels and directly stimulates AGT biosynthesis and release from the liver (45, 48), adenovirus-mediated overexpression of ECFP/ANG II selectively in the proximal tubules of the rat kidneys had no significant effects on plasma AGT (Fig. 3A), PRA, or ANG II levels (Fig. 3B).

Effect of proximal tubule-selective overexpression of ECFP/ANG II on AGT mRNA and protein expression and ANG II levels in the renal cortex. Figure 4 shows that the expression of AGT mRNA in the renal cortex was significantly increased by 10.2 ± 0.3 fold in response to overexpression of ECFP/ANG II selectively in the proximal tubules (Fig. 4, left, P < 0.05). Similarly, there were corresponding increases in AGT protein expression, as estimated by Western blot analysis (Fig. 4, P < 0.01) and ANG II levels in the renal cortex (Fig. 5, P < 0.01), respectively. By comparison, overexpression of the control ECFP/ANG IIc selectively in the proximal tubules of the mouse kidney had no effects on AGT mRNA and protein expression (see Fig. 8), as well as on ANG II levels in the renal cortex (Fig. 5).

Effect of proximal tubule-selective overexpression of ECFP/ANG II on urinary protein and AGT excretion. To determine whether AGT was secreted into the proximal tubule luminal fluid and then excreted in urine, 24-h urinary protein and AGT excretion were compared in rats receiving proximal tubule-specific overexpression of ECFP/ANG II or vehicle control (Fig. 6A). While there was no significant difference in 24-h urinary protein excretion between groups, 24-h urinary AGT excretion was significantly increased in rats overexpressing ECFP/ANG II in the proximal tubules 4 wk after the gene transfer (basal: 58.4 ± 6.3 ng/day vs. ECFP/ANG II: 123.6 ± 20.9 ng/day, P < 0.01) (Fig. 6B). Interestingly, 24-h urinary AGT excretion was also increased moderately in control rats (P < 0.05). However, the increase in 24-h urinary AGT excretion in control rats was significantly smaller than in rats overexpressing the intracellular ECFP/ANG II selectively in the proximal tubules.

Role of AT1a receptors in mediating the effects of proximal tubule-selective overexpression of ECFP/ANG II on blood pressure and AGT expression. In a previous study, we reported that the AT1 receptor blocker losartan largely blocked the effects of proximal tubule-selective overexpression of ECFP/ANG II on blood pressure responses in rats and mice (25). To determine whether AT1a receptors are involved in mediating the effect of ECFP/ANG II overexpression on AGT mRNA and protein expression, additional experiments were performed in AT1a-receptor-KO mice. Figure 7 shows that compared with a representative control mouse (Fig. 7A), ECFP/ANG II was
expressed similarly in the proximal tubules, but not the glomeruli, of the renal cortex in a male representative C57BL/6J mouse (Fig. 7C). Compared with control C57BL/6J mice, overexpression of ECFP/ANG II selectively in the proximal tubules was associated with a significant increase in systolic blood pressure (Fig. 7D) and a significant decrease in 24-h urinary sodium excretion (Fig. 7E) in C57BL/6J, but not in AT1a-KO mice. Twenty-four-hour urinary potassium excretion was not significantly altered by proximal tubule-selective overexpression of ECFP/ANG II. Overexpression of ECFP/ANG II in the proximal tubules had no significant effects on AGT mRNA expression and AGT proteins in the renal cortex of AT1a-KO mice compared with control C57BL/6J mice (Fig. 8).

Roles of MAP kinases and NF-κB signaling mechanisms. To elucidate the signaling mechanism involved, mPCTs were transfected with ECFP/ANG II and treated with ANG receptor blockers, MAP kinase inhibitor U0126, or NF-κB inhibitor RO 106–9920. Overexpression of ECFP/ANG II in mPCTs increased NF-κB-p65 proteins by >3-fold (P < 0.01), and the response was significantly attenuated by the AT1 receptor blocker losartan (10 μM) and the MAP kinase MER1 and MEK2 inhibitor U0126 (1 μM, P < 0.01), but not by the AT2 receptor blocker PD123319 (10 μM, n.s.) (Fig. 9A). The overexpression of ECFP/ANG II also significantly increased AGT protein (P < 0.01), which was significantly attenuated by the MAP kinase MER1 and MEK2 inhibitor U0126 and the NF-κB inhibitor RO 106–9920 (P < 0.01), respectively (Fig. 9B).

**DISCUSSION**

The role of circulating, endocrine, or extracellular ANG II in the regulation of AGT expression in the kidney has been extensively investigated previously in ANG II-induced hypertensive rats and mice (11, 16, 17, 21, 22). In those studies, chronic infusion of pressor doses of exogenous ANG II for weeks was found to induce severe hypertension and kidney injury, associated with significant augmentation of renal AGT expression and urinary AGT excretion (11, 16, 17, 21, 22). These studies indicate that unlike renin expression in the JGAs of the cortex, AGT mRNA expression and AGT protein production in the kidney are directly stimulated by ANG II, which provides a powerful and sustained feed-forward mechanism to further increase intrarenal ANG II formation and therefore lead to persistent hypertension. Indeed, intrarenal ANG II levels are significantly higher than can be explained by circulating AGT and ANG II under physiological conditions as well as during ANG II-induced hypertension (44, 53, 56, 61, 63). However, the source of intrarenal AGT in physiology and renal diseases such as in ANG II-dependent hypertension remains an issue of current debate. Recently, the study of Matsusaka et al. (39) has suggested that liver-derived AGT is the primary source of intrarenal AGT and ANG II and taken up via the megalin-dependent mechanism in normal mice, but they did not address the responses in hypertensive mice. Similarly, Pohl et al. (48) reported that immunoreactive AGT that was detected in the early proximal tubule was primarily derived from the uptake of filtered liver AGT, whereas AGT mRNA expression was detected in the proximal straight tubule. Nevertheless, the latter

![Image](image_url)
two studies may not be adequate to determine whether liver-derived or kidney-derived AGT contributes to augmented kidney or intratubular ANG II levels in ANG II-dependent hypertension or kidney diseases (45).

The present study may provide some new insights into the potential role of kidney derived AGT and intracellular ANG II to the current debate. One important difference in the experimental approach of the present study was that pressor doses of exogenous ANG II were not infused to induced severe hypertension, as we previously reported (11, 17, 21). Systemic administration of ANG II is well recognized to stimulate AGT biosynthesis and release from the liver (45, 48). Increased liver-derived AGT by circulating ANG II is expected to be delivered into the kidney, making it difficult to separate the role of kidney AGT from that of liver-derived AGT. Instead, we used a proximal tubule-specific sglt2 promoter to overexpress an intracellular ECFP-tagged ANG II fusion protein selectively in the proximal tubules of the kidney in rats and mice (Figs. 1 and Fig. 7) (25, 28, 36). With this unique approach, the expression of ECFP/ANG II is confined within the infected cells and increases intracellular ANG II levels in the proximal tubules, but does not release or secrete ANG II into the peritubular interstitium or microcirculation to increase plasma ANG II, or into the proximal tubular lumen to act on AT1 receptors or tubular cells (25). Indeed, plasma AGT, PRA, and ANG II were not significantly elevated in rats with overexpression of intracellular ECFP/ANG II selectively in the proximal tubules in the present study (Fig. 3); therefore, the contribution of systemically ANG II-stimulated, liver-derived AGT biosynthesis and secretion may most likely be excluded. The present study demonstrates that overexpression of intracellular ECFP/ANG II selectively in the proximal tubules of rats and mice significantly increased AGT mRNA expression and AGT proteins in the renal cortex (Fig. 4), ANG II levels in the cortex (Fig. 5), as well as augmented urinary AGT excretion (Fig. 6). Our results may be best interpreted as a direct effect of intracellular ANG II, independently of the circulating RAS, to enhance proximal tubule-specific AGT formation. As reported previously (25, 28, 36), we again found that ECFP/ANG II was expressed selectively in the proximal tubules of the renal cortex, with very low levels of ECFP/ANG II expressed in the glomeruli, the cortical collecting tubules, or the entire medulla (Figs. 1 and Fig. 7). Furthermore, we expressed the Ad-sglt2-ECFP/ANG IIc, a scrambled ECFP fusion protein, in different groups of rats or mice as a vehicle control to determine whether the effect of ECFP/ANG II is specific. Indeed, overexpression of the Ad-sglt2-ECFP lacking ANG II fusion protein in the proximal tubules had no effects on blood pressure, urinary sodium excretion in rats (Fig. 2), or AGT mRNA and AGT protein in the renal cortex of mice (Fig. 8). Thus our approaches effectively exclude the potential nonspecific, or off-target, effects of intracellular ECFP on blood pressure and intrarenal AGT expression in the present study.

We and others have previously shown that ANG II stimulates AGT mRNA and AGT protein expression in the kidney or cultured rat or human proximal tubule cells via AT1 receptors (22, 51). However, these effects were induced by extracellular ANG II acting via cell surface AT1 receptors. No study has been reported for a role of intracellular ANG II on AGT expression in cultured proximal tubule cells or the proximal tubules of the rat and mouse kidney. One exception is a recent study in which overexpression of this intracellular ECFP/ANG II did not alter AGT accumulation in major tissues, such as kidney, liver, and brain (54). However, this study was different from the current study in a critical way. The overexpression of ECFP/ANG II in that study was driven by the mouse metallothionein promoter in all tissues, whereas in the present study the proximal tubule-specific sglt2 promoter was used to drive the expression of this intracellular protein selectively in the proximal tubules of the kidneys. The results of the present study suggest that intracellular ANG II significantly augments AGT mRNA expression and AGT protein production in the proximal tubules of the cortex, and the effects are also mediated by AT1a receptors (Figs. 7–9). This conclusion is supported by experiments using mutant mice with a deficiency of AT1a receptors, in which the effects of ECFP/ANG II on AGT mRNA and AGT protein expression in the renal cortex, blood pressure, and 24-h urinary sodium excretion were largely blocked (Figs. 8 and 9). This AT1a receptor-dependent mechanism is consistent with previous studies showing that the effects of exogenous ANG II to augment AGT mRNA and protein expression in rats or mice or in cultured proximal tubule cells were blocked by AT1 receptor blockers (22, 51). Whether AT1b receptors, which are also expressed in rodents (8), play a role in ECFP/ANG II-induced AGT mRNA or AGT protein expression in the kidney was not addressed in this study. The present study was not designed to determine the role of the AT1b receptor due to the lack of AT1b receptor-specific
Although the present study clearly shows that overexpression of an intracellular ANG II fusion protein selectively in the proximal tubules of the rat and mouse kidney significantly augmented AGT mRNA and AGT protein expression, a number of limitations should be recognized. First, the present study did not systematically determine the time- and dose-dependent effects of ECFP/ANG II on AGT expression in cultured mPCTs (Fig. 9). This suggests that intracellular ANG II may further support the reports that AT1 (AT1a) receptors are localized in the endosomal (15, 33, 38, 47). However, it should be pointed out that, not confined to AGT expression, the AT1a/MAP kinase signaling pathways also mediate the effects of ECFP/ANG II on AGT expression in cultured mPCTs (Fig. 9). This suggests that intracellular ANG II may stimulate cytoplasmic or nuclear AT1 (AT1a) receptors to activate the MAP kinase pathways, which alone and/or in turn activate NF-κB signaling to augment AGT expression in the proximal tubules (Figs. 9 and Fig. 10). This explanation is further supported by the reports that AT1 (AT1a) receptors are localized in the endosomal (15, 61), mitochondrial (1), and nuclear compartments (13, 33, 38, 47). However, it should be pointed out that, not confined to AGT expression, the AT1a/MAP kinase signaling pathways also mediate the effects of ECFP/ANG II on transcriptional responses of transforming growth factor-β, MCP-1, and NHE3 expression (28, 33).

The present study clearly shows that overexpression of an intracellular ANG II fusion protein selectively in the proximal tubules of the rat and mouse kidney significantly augmented AGT mRNA and AGT protein expression, a number of limitations should be recognized. First, the present study did not systematically determine the time- and dose-dependent effects of ECFP/ANG II on AGT expression in the kidney.

**Fig. 9. Effects of overexpression of intracellular ECFP/ANG II in cultured mouse proximal tubule cells (mPCTs) on NF-κB (A) or AGT protein expression (B).**

**Fig. 10. Schematic summary showing that expression of an intracellular ANG II fusion protein selectively in the proximal tubules of the kidney stimulates AT1 (AT1a) receptors in the cytoplasm and nuclei, leading activation of MAP kinases and NF-κB signaling and subsequent AGT mRNA and protein expression. Some of increased AGT proteins may be secreted from the proximal tubules into the tubular lumen and appear in urine.**
various sodium transporters, solute transporters, and cytokines or proinflammatory factors ultimately play important roles. Consistent with this interpretation, overexpression of human AGT and renin genes selectively in the proximal tubules of mice driven by the kidney androgen-regulated protein promoter (KAP) resulted in significantly elevated blood pressure and renal injury (9, 23, 50, 58).

In summary, the present study demonstrates that overexpression of an intracellular ANG II fusion protein, but not its scrambled control, selectively in the proximal tubules of the rat and mouse kidney significantly augments AGT mRNA and AGT protein expression, as well as increases urinary AGT excretion. This effect is associated with a moderately antinatriuretic response and elevated blood pressure and appears to involve the AT1 (AT1a) receptors/ MAP kinase/NF-κB signaling pathways. Our results suggest that the effect of ECFP/ANG II on AGT expression may at least in part contribute to hypertension. Our results suggest that the effect of ECFP/ANG II on AGT expression may at least in part contribute to hypertension. This work was supported by grants from National Institute of Diabetes and Digestive and Kidney Diseases to J. Zhuo (2RO1DK067299-06A2, 1R01DK102429-01) and the National Institute of General Medical Sciences IDeA Program to L. G. Navar (COBRE, P30GM103337).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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