Abstract—Hypertension induced by long-term infusion of angiotensin II (Ang II) is associated with augmented intrarenal Ang II levels to a greater extent than can be explained on the basis of the circulating Ang II levels. Although part of this augmentation is due to AT1 receptor-dependent internalization, the intracellular compartments involved in this Ang II accumulation remain unknown. In the present study, we sought to determine whether Ang II trafficking into renal cortical endosomes is increased during Ang II hypertension, and if so, whether the AT1 receptor antagonist, candesartan, prevents this accumulation. Compared with controls (n=12; 114±2 mm Hg), Ang II-infused rats (n=12; 80 ng/kg/min, SC, for 13 days) developed hypertension with systolic blood pressure rising to 185±4 mm Hg by Day 12. In Ang II hypertensive rats, plasma renin activity was suppressed, whereas plasma and kidney Ang II levels were increased by 3-fold (348±58 versus 119±16 fmol/mL) and 2-fold (399±39 versus 186±26 fmol/g). Intracellular endosomal Ang II levels were increased by more than 10-fold (1100±283 versus 71±12 fmol/mg protein), whereas intermicrovillar cleft Ang II levels were increased by more than 2-fold (88±22 versus 37±7 fmol/mg protein). Flow cytometric analysis detected significant increases in AT1A receptor antibody binding in endosomal and intermicrovillar clefts of Ang II–infused rats. The hypertension induced by Ang II was prevented in rats treated concurrently with candesartan (2 mg/kg/d, 119±3 mm Hg). Candesartan treatment (n=8) also prevented increases in kidney (215±19 fmol/g), endosomal (96±29 fmol/mg protein), and intermicrovillar cleft Ang II levels (11±2 fmol/mg protein). These results indicate that there is substantial intracellular accumulation of angiotensin peptides in renal cortical endosomes during Ang II–dependent hypertension via an AT1 receptor–mediated process. (Hypertension. 2002;39:116-121.)

Key Words: kidney ■ endosomes ■ angiotensin II ■ AT1 receptor ■ hypertension, experimental
ney,\textsuperscript{11} Renal endosomes and intermicrovillar clefts from rat renal cortex also contain angiotensin converting enzyme (ACE) and AT\textsubscript{1} receptors.\textsuperscript{11} In the present study, we first sought to determine whether intracellular accumulation of circulating or intrarenally formed Ang II into renal endosomes is increased during Ang II–dependent hypertension. Second, studies were performed to examine whether AT\textsubscript{1} receptor blockade with candesartan would prevent Ang II accumulation in whole kidney, renal endosomes, and intermicrovillar clefts. Third, flow cytometric analysis was used to measure AT\textsubscript{1} receptor antibody binding in renal intracellular endosomes isolated from normotensive and Ang II hypertensive rats. The present study demonstrates that during Ang II–induced hypertension, Ang II levels are markedly increased in parallel in the kidney, intermicrovillar clefts, and endosomes via an AT\textsubscript{1} receptor–mediated process.

Methods

Animal Preparation

Ang II–induced hypertensive rats were prepared as described previously.\textsuperscript{4,9,10} Adult male Sprague-Dawley rats (Charles River Laboratories, Montreal, Quebec, Canada) were divided into three groups: Group 1 rats served as controls and did not receive any treatment (n=12); Group 2 rats received Ang II infusions at 80 ng/min via osmotic minipumps (n=12) (Alza Corporation) for 13 days; and Group 3 rats received concurrently Ang II infusion and the AT\textsubscript{1} receptor antagonist, candesartan, at 2 mg/kg/d for 13 days (n=8). Systolic blood pressures were monitored by tail-cuff plethysmography one day before and on Day 6 and Day 12, respectively, following Ang II or candesartan treatment. All experiments were approved by the Tulane University Animal Care and Use Committee.

Preparation of Plasma and Kidney Samples, Renal Endosomes, and Intermicrovillar Clefts

On Day 13 after initiation of the treatment, rats were decapitated and trunk blood samples collected for measurement of plasma renin activity (PRA) and Ang I and Ang II concentrations.\textsuperscript{4,9,10} Kidneys were immediately harvested, demedullated, and prepared separately for measurement of whole kidney Ang I and Ang II or for fractionation and purification of renal endosomes and intermicrovillar clefts as we described previously.\textsuperscript{11,12}

Measurement of PRA and Circulating, Kidney, and Endosomal Ang II

PRA was measured using a standard commercial kit (Incstar), and plasma and kidney Ang I and Ang II levels were determined by radioimmunoassay.\textsuperscript{4,9,10} Ang II levels in renal endosomes and intermicrovillar clefts were also determined by radioimmunoassay as we described previously\textsuperscript{13} and expressed as femtomole per milligram (fmol/mg) endosomal protein. The sensitivity of Ang II immunoassays was ~2 fmol, the specific binding was ~41% and the nonspecific binding was ~3%.

Flow Cytometric Analysis of Endosomal AT\textsubscript{1} Receptor Antibody Binding

AT\textsubscript{1} receptor antibody binding in renal endosomes and intermicrovillar clefts and its colocalization with entrapped marker fluorescein dextran were determined by flow cytometry as described previously.\textsuperscript{11} Briefly, aliquots (25 μL) of endosomal or intermicrovillar cleft vesicles were first preincubated in 50% goat serum for 2 hours at 22°C to reduce nonspecific binding and then incubated with serial dilutions of the AT\textsubscript{1} receptor antiserum at 4°C overnight.\textsuperscript{11} After washing, endosomal samples were further incubated with 1:40 of goat anti-rabbit phycocerythrin-conjugated secondary antiserum for 4 hours at 22°C. The AT\textsubscript{1A} receptor antibody binding tagged by phycocerythrin and fluorescein dextran were analyzed for each rat sample by a Becton-Dickinson FACSVantage flow cytometer using a dedicated Power Mac computer.\textsuperscript{11,12}

Data Analysis and Statistics

Data are presented as the mean±SEM. The differences between different groups of animals in systolic blood pressure, PRA, plasma and kidney Ang I and Ang II, and endosomal Ang II levels were analyzed using one-way ANOVA followed by Dunnett’s comparisons between groups’ means. A value of P<0.05 was considered statistically significant.

An expanded Methods section can be found in an online data supplement available at http://www.hypertensionaha.org.

Results

Effect of Long-Term Ang II Infusion and/or Candesartan on Systolic Blood Pressure, PRA, and Plasma Ang I and Ang II Levels

Rats receiving chronic Ang II infusions developed hypertension by Day 6, which rose further by Day 12 (Table). Concurrent administration of candesartan to Ang II–infused rats prevented the development of hypertension. Control rats remained normotensive throughout the duration of the experiment. PRA was markedly suppressed in Ang II–hypertensive rats, and conversely, it was elevated more than 6-fold in Ang II–infused rats treated concurrently with candesartan (Table). Long-term Ang II infusion also significantly decreased plasma Ang I levels compared with controls (38±9 versus 70±9 fmol/mL). In contrast, plasma Ang II concentrations increased by almost 3-fold in Ang II–infused rats compared with controls. Concurrent administration of candesartan elevated PRA markedly and increased plasma Ang II levels further above the levels seen in rats receiving Ang II infusion alone (Table). The increase in Ang II as a consequence of stimulation of PRA is presumably due to the blockade of the feedback inhibition of renin release by Ang II in the juxtaglomerular apparatus (JGA).\textsuperscript{9}
Effects of Long-Term Ang II and/or Candesartan on Kidney and Endosomal Ang II Levels
Kidney Ang II levels in control rats averaged 186 ± 26 fmol/g kidney weight and were increased to 399 ± 39 fmol/g in Ang II–infused rats (P < 0.05) (Figure 1). Unlike plasma Ang II levels, which increased further above the levels of Ang II–infused rats (Table), kidney Ang II levels in candesartan–treated Ang II–infused animals did not increase significantly from the levels observed in control rats (215 ± 19 fmol/g kidney weight). Endosomal Ang II levels increased from 71 ± 12 fmol/mg in control animals to 1100 ± 283 fmol/mg endosomal protein in the Ang II–infused rats, whereas intermicrovillar cleft Ang II levels increased from 37 ± 7 fmol/mg to 88 ± 22 fmol/mg endosomal protein. Candesartan prevented the Ang II–induced increases in both endosomal and intermicrovillar Ang II levels (Figure 1).

Effects of Long-Term Ang II Infusion on AT1 Receptor Antibody Binding in Renal Endosomes and Intermicrovillar Clefts
To determine whether the increases in kidney, endosomal, and intermicrovillar Ang II levels in Ang II–infused rats are associated with alterations of AT1 receptor expression, flow cytometry was employed to measure AT1 receptor antibody binding in renal cortical endosomes and intermicrovillar clefts (Figure 1). As shown in Figure 2, compared with control animals, AT1 receptor antibody binding was significantly higher in both intracellular endosomes (control 115 ± 5.5 fluorescence intensity units versus Ang II–infused 161 ± 17.6 fluorescence intensity units, P < 0.05) and intermicrovillar clefts (control 26.4 ± 4.8 fluorescence intensity units versus Ang II–infused 44.7 ± 8.6 fluorescence intensity units, P < 0.05) in Ang II–infused rats at 1:1000 antibody dilution.

Colocalization of Entrapped Endosomal Marker Fluorescein Dextran and AT1 Receptor Antibody Binding
Colocalization of entrapped fluorescein dextran as an endosomal marker and AT1 receptor antibody binding in renal intracellular endosomes by flow cytometry is shown in Figure 3. Panel A shows the background level of autofluorescence without entrapped fluorescein and minimal level of AT1 receptor antibody binding. Colocalization of AT1 receptor antibody binding with entrapped fluorescein dextran in a representative control endosomal sample is shown in Panel B. A marked shift to the right of the 3D frequency histogram (colocalization) was observed in renal endosomes of Ang II–infused rats, indicating an increase in intracellular endosomal AT1 receptor antibody binding (Panel C). Concurrent administration of candesartan prevented the shift to the right of the 3D frequency histogram in renal endosomes of Ang II–infused rats (Panel D). Only a single population of renal endosomes was observed in all 3 groups of rats.

Discussion
Internalization of the Ang II–AT1 receptor complex following binding of the agonist to its membrane-bound receptors has been suggested to play an important role in mediating biological actions of Ang II in kidney cells. Although fluorescence-labeled Ang II and AT1 receptors have been localized in endosomes and/or lysosomes, the actual presence of internalized Ang II and AT1 receptors in intracellular compartments has been reported only recently. We demonstrated that rat renal cortical endosomes and intermicrovillar...
clefts contain detectable ACE, Ang I, Ang II, and AT1 receptor, therefore suggesting that the potential site(s) of intrarenal accumulation of circulating and intrarenally formed Ang II in Ang II–hypertensive rats are the intracellular endosomal compartments. In the present study, we observed marked increases in renal endosomal Ang II levels in rats receiving long-term Ang II infusion. This enhanced endosomal Ang II accumulation was associated with an increase in AT1 receptor antibody binding in renal endosomes and intermicrovillar clefts and was prevented by the treatment of the Ang II–infused rats with the AT1 receptor blocker, candesartan. Thus, our results confirm our hypothesis that Ang II levels are markedly elevated in renal intracellular endosomes and intermicrovillar clefts freshly isolated and purified from normotensive and Ang II–infused rats, and in rats treated concurrently with Ang II infusion and candesartan, serve as a direct in vivo corollary to the in vitro studies. The present findings provide direct evidence for increased Ang II accumulation in renal endosomes during Ang II–induced hypertension and for the important role of AT1 receptors in mediating this process.

To ascertain whether enhanced intracellular trafficking of Ang II into renal endosomes is associated with increased expression of AT1 receptors, we employed flow cytometry to quantify endosomal AT1 receptors in normotensive and Ang II–infused rats. Using a rabbit anti-AT1 receptor antibody that was raised against the cytosolic tail of the AT1 receptor, we detected significant increases in AT1 receptor antibody binding in both endosomal and intermicrovillar clefts of Ang II–infused rats compared with their normotensive counterparts. These data demonstrate that increased endosomal Ang II levels in Ang II hypertensive rats are associated with increased AT1 receptors in endosomes and intermicrovillar clefts. Uregulation of Ang II receptors or AT1 receptors by Ang II has been documented previously in cultured explant–derived aortic vascular smooth muscle cells, in the primary culture of bovine adrenal medullary chromaffin cells in vitro, and in Chinese hamster ovary cells transfected with an AT1 receptor linked to green fluorescent protein. The above cited studies examined AT1 receptor–Ang II complex internalization in isolated or cultured cells in vitro, but the content of internalized Ang II and AT1 receptors in intracellular compartments was not determined. Our measurements of Ang II levels in renal intracellular endosomes and intermicrovillar clefts freshly isolated and purified from normotensive and Ang II–infused rats, and in rats treated concurrently with Ang II infusion and candesartan, serve as a direct in vivo corollary to the in vitro studies. The present findings provide direct evidence for increased Ang II accumulation in renal endosomes during Ang II–induced hypertension and for the important role of AT1 receptors in mediating this process.

To determine whether increased renal endosomal and intermicrovillar cleft Ang II levels were dependent on AT1 receptor activation, an additional group of Ang II–infused rats was treated with the AT1 receptor blocker, candesartan. We previously reported that losartan prevented intrarenal accumulation of infused exogenous Val5–Ang II in the kidney, indicating that intrarenal uptake of circulating Ang II is mediated by AT1 receptors. The results of the present study with candesartan demonstrate that AT1 receptor blockade prevents the accumulation of Ang II in intracellular endosomal compartments. Indeed, blockade of AT1 receptors with candesartan in Ang II–infused rats prevented not only the increase in whole kidney Ang II levels, but also the increases in endosomal and intermicrovillar cleft Ang II levels. These results support the hypothesis that increased intracellular accumulation of Ang II in renal endosomes during Ang II–induced hypertension is due to AT1 receptor–mediated endocytosis and/or internalization. Internalization of Ang II via AT1 receptors and blockade of Ang II internalization by losartan have been reported previously in cultured explant–derived aortic vascular smooth muscle cells, in the primary culture of bovine adrenal medullary chromaffin cells in vitro, and in Chinese hamster ovary cells transfected with an AT1 receptor linked to green fluorescent protein. The above cited studies examined AT1 receptor–Ang II complex internalization in isolated or cultured cells in vitro, but the content of internalized Ang II and AT1 receptors in intracellular compartments was not determined. Our measurements of Ang II levels in renal intracellular endosomes and intermicrovillar clefts freshly isolated and purified from normotensive and Ang II–infused rats, and in rats treated concurrently with Ang II infusion and candesartan, serve as a direct in vivo corollary to the in vitro studies. The present findings provide direct evidence for increased Ang II accumulation in renal endosomes during Ang II–induced hypertension and for the important role of AT1 receptors in mediating this process.

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increased expression of AT\textsubscript{1} receptors is responsible, at least in part, for enhanced Ang II accumulation in intracellular endosomal compartments during Ang II–dependent hypertension.

The physiological consequence of increased endosomal AT\textsubscript{1} receptor expression and enhanced intracellular accumulation of Ang II in renal endosomes during Ang II–dependent hypertension is yet to be determined. Accumulating evidence suggests that internalization of AT\textsubscript{1} receptors following binding of the agonist is not solely for the purpose of Ang II trafficking to the lysosomes for degradation and recycling of the receptors back to cell membranes. Rather, this process may be important for full expression of biological actions in different cells.\textsuperscript{14,15,18,21} A recent study showed that microinjection of Ang II directly into vascular smooth muscle cells induces an increase in intracellular calcium via an AT\textsubscript{1} receptor–mediated process.\textsuperscript{22} In the kidney, endocytosis of the Ang II–AT\textsubscript{1} receptor complex increases phospholipase C and sodium flux and decreases cAMP in cultured proximal tubule epithelial cells following exposure to Ang II in vitro.\textsuperscript{21,23} Likewise, AT\textsubscript{1} receptor–mediated endocytosis of Ang II and increased recycling of AT\textsubscript{1} receptors have been linked to increased phospholipase A\textsubscript{2} activity and increased sodium flux in a tubule cell line expressing rabbit AT\textsubscript{1} receptors (LLC-PK).\textsuperscript{24} Thus, increased internalization and/or accumulation of Ang II in renal endosomes may enhance proximal tubular sodium reabsorption and, therefore, contribute to the blunted pressure natriuresis relationship in Ang II–dependent hypertension. Chen et al\textsuperscript{18} demonstrated Ang II–driven internalization of an AT\textsubscript{1} receptor–green fluorescent protein (AT\textsubscript{1}R-GFP) receptor complex and increased colocalization of the GFP fluorescence in nuclear regions. Long-term Ang II infusions have been shown to lead to increased angiotensinogen mRNA levels causing enhanced intrarenal production of angiotensinogen.\textsuperscript{25,26} Because of the evidence suggesting that the Ang II–AT\textsubscript{1} receptor complex may migrate to the nucleus,\textsuperscript{18} it is possible that Ang II–dependent stimulation of angiotensinogen mRNA requires Ang II internalization and direct genomic action as proposed by Re.\textsuperscript{27} Furthermore, increased accumulation of endosomal Ang II levels and AT\textsubscript{1} receptors may also play important roles in causing structural injury or derangements during Ang II hypertension. Intracellular Ang II may induce transforming growth factor-\textbeta or nuclear transcription factor-kB expression to promote Ang II–induced tubulo-interstitial inflammation.\textsuperscript{28} Glomerulosclerosis, and renal microvascular hypertrophy.\textsuperscript{3,9} Overall, internalization of Ang II and AT\textsubscript{1} receptors into renal endosomes and intermicrovillar clefts may play important paracrine and intracrine roles in the hypertensinogenic and pathologic actions of Ang II on the kidney.\textsuperscript{27}

In summary, the present study demonstrates that during Ang II hypertension, Ang II levels are markedly increased in the kidney and in renal cortical endosomes and intermicrovillar clefts. These increases in endosomal and intermicrovillar cleft Ang II levels are associated with enhanced expression of AT\textsubscript{1} receptors in these intracellular compartments and are prevented by concurrent administration of AT\textsubscript{1} receptor blockers. These findings support the hypothesis that intrarenal trafficking/accumulation of angiotensin peptides into renal cortical tubular endosomes is enhanced during Ang II hypertension, and this process is mediated by AT\textsubscript{1} receptors. Thus, increased AT\textsubscript{1} receptor expression and internalization of Ang II–AT\textsubscript{1} receptor complex may be important for full expression of hypertensinogenic, paracrine, and intracrine actions of Ang II in the kidney during Ang II–dependent hypertension.

Acknowledgments

This work was supported by National Institutes of Health Grants HL-26371 (to L.G. Navar), HL-59699 (to J.D. Imig), and DK-46117 (to T.G. Hammond); National Aeronautics and Space Administration Grant 9-811 Basic (to T.G. Hammond); and a Veterans Affairs Research Associate Career Development Award (to T.G. Hammond). Flow cytometry equipment was provided by the Louisiana Board of Regents Support Fund. J. Zhuo was supported by an Institute Block Grant to Howard Florey Institute from the National Health and Medical Research Council of Australia (No. 983001). Candesartan was provided by AstraZeneca. This work was completed while Dr. Zhuo undertook his sabbatical leave in Dr. Navar’s laboratory.

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Ang II Accumulation in Rat Renal Endosomes During Ang II-Induced Hypertension: Role of AT1 Receptor
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Hypertension. 2002;39:116-121
doi: 10.1161/01.HYP.0000021387.47481.AB

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