Original Article

Augmentation of Intrarenal Angiotensin II Levels in Uninephrectomized Aldosterone/Salt-Treated Hypertensive Rats; Renoprotective Effects of an Ultrahigh Dose of Olmesartan

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Recent studies have suggested that aldosterone plays a role in the pathogenesis of renal injury. In this study, we investigated whether local angiotensin II (Ang II) activity contributes to the progression of renal injury in aldosterone/salt-induced hypertensive rats. Uninephrectomized rats were treated with 1% NaCl in a drinking solution and one of the following combinations for 6 weeks: vehicle (2% ethanol, s.c.; *n*=9), aldosterone (0.75 μg/h, s.c.; *n*=8), aldosterone+Ang II type 1 receptor blocker olmesartan (10 mg/kg/day, p.o.; *n*=8), or aldosterone+olmesartan (100 mg/kg/day, p.o.; *n*=9). Aldosterone/salt-treated hypertensive rats exhibited severe proteinuria and renal injury characterized by glomerular sclerosis and tubulointerstitial fibrosis. Aldosterone/salt-induced renal injury was associated with augmented expression of angiotensin converting enzyme and Ang II levels in the renal cortex and medullary tissues. Renal cortical and medullary mRNA expression of transforming growth factor-*β* (TGF-*β*) and connective tissue growth factor (CTGF) as well as the collagen contents were increased in aldosterone/salt-treated hypertensive rats. Treatment with olmesartan (10 or 100 mg/kg/day) had no effect on blood pressure but attenuated proteinuria in a dose-dependent manner. Olmesartan at 10 mg/kg/day tended to decrease renal cortical and medullary Ang II levels, TGF-*β* and CTGF expression, and collagen contents; however, these changes were not significant. On the other hand, an ultrahigh dose of olmesartan (100 mg/kg/day) significantly decreased these values and ameliorated renal injury. These data suggest that augmented local Ang II activity contributes, at least partially, to the progression of aldosterone/salt-dependent renal injury. (*Hypertens Res* 2006; 29: 169–178)

***Key Words*: angiotensin II (Ang II), aldosterone, kidney, olmesartan, rats**

Introduction

There is increasing evidence supporting potential roles of aldosterone in the pathophysiology of renal injury (*1–3*). Severe glomerular injury and tubulointerstitial fibrosis have been observed in rats treated with aldosterone/salt (*4*, *5*). Furthermore, mineralocorticoid receptor (MR) antagonists have

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been shown to have minimal effect on systemic blood pressure, but to markedly ameliorate glomerular injury and tubulointerstitial fibrosis in stroke-prone spontaneously hypertensive rats (SHRSP) (*6*) and rats treated with cyclosporine A (*7*) or radiation (*8*). In patients with chronic renal failure (*9*) and early diabetic nephropathy (*10*) as well as in rats with anti–Thy-1 nephritis (*11*), the addition of an MR antagonist to angiotensin-converting enzyme (ACE) inhibitors had no hemodynamic effects, but markedly reduced proteinuria. Other studies have shown that monotherapy with MR antagonists is more effective than ACE inhibitors in reducing proteinuria in hypertensive patients (*12*, *13*). These observations suggest that aldosterone/MR is involved in the pathogenesis of renal injury through mechanisms that cannot be simply explained by blood pressure and hemodynamic changes.

Aldosterone production and release in the adrenal gland are stimulated by angiotensin II (Ang II) (*1*, *3*). Several studies indicate that aldosterone participates in Ang II–dependent renal injury (*1*, *14–18*); for example, it has been shown that MR antagonists or an aldosterone synthase inhibitor attenuate renal injuries in rats treated with Ang II and a nitric oxide synthase inhibitor (*14*), in rats overexpressing human renin and angiotensinogen genes (*15*) or in two-kidney, one-clip renovascular hypertensive rats (*16*). Similarly, exogenous infusion of aldosterone reversed the renoprotective effects of Ang II blockade in remnant kidney hypertensive rats (*17*) and SHRSP (*18*). More recently, it has been suggested that aldosterone increases Ang II activity in cardiovascular tissues (*19–26*). Chronic treatment with aldosterone and salt increased aortic ACE binding (*19*) and myocardial Ang II type 1 (AT_1) receptor expression (*20*). *In vitro* studies have also shown that aldosterone treatment increased ACE expression in endothelial cells (*21*) and cardiac myocytes (*22*), and AT_1 receptors in vascular smooth muscle cells (*23*). Keidar *et al*. (*24*) showed that increased ACE gene expression in monocyte-derived macrophages of patients with congestive heart failure was decreased by treatment with an MR antagonist. Interestingly, it was also observed that an MR antagonist significantly increased the macrophage ACE2 gene, suggesting its effect on decreasing Ang II formation. Sun *et al*. (*25*) showed that the binding density of ACE and AT_1 receptors was significantly increased in kidneys of rats chronically treated with aldosterone and salt. Further *in vitro* studies showed that aldosterone increased renin gene expression and protein secretion in juxtaglomerular cells (*26*). These data suggest that aldosterone increases local Ang II activity in the kidney. However, to the best of our knowledge, there is no direct evidence showing that aldosterone actually increases intrarenal Ang II levels. In addition, the role of intrarenal Ang II in the pathogenesis of aldosterone-induced renal injury remains unclear.

In the present study, we hypothesized that intrarenal Ang II levels are augmented by chronic treatments of aldosterone and salt. To test this hypothesis, we measured Ang II levels in renal cortical and medullary tissues of uninephrectomized aldosterone/salt-treated rats. To assess the potential contribution of intrarenal Ang II to aldosterone/salt-dependent renal injury, we also examined the effects of an AT_1 receptor blocker (ARB), olmesartan. Since recent studies indicate that ultrahigh doses of ARBs have enhanced renoprotective effects (*27–30*), we administered olmesartan at a dose of 100 mg/kg/day in addition to the recommended dose of 10 mg/kg/day in rats (*31–33*).

Methods

Animal Preparation

All experimental procedures were performed under the guidelines for the care and use of animals as established by the Kagawa University Medical School. Male Sprague-Dawley rats (CLEA Japan, Inc., Tokyo, Japan) weighing 162 to 184 g at the beginning of the experiments were subjected to right uninephrectomy under anesthesia with sodium pentobarbital (50 mg/kg, i.p.). After 10 days, rats were randomly treated with one of the following combinations for 3 or 6 weeks: Group 1: 1% NaCl in the drinking solution+vehicle (2% ethanol, s.c.; $n=7$ and $n=9$, respectively); Group 2: 1% NaCl+aldosterone (0.75 μg/h, s.c.; $n=8$ each); Group 3: 1% NaCl+aldosterone+olmesartan (10 mg/kg/day, p.o.; $n=8$ each); and Group 4: 1% NaCl+aldosterone+olmesartan (100 mg/kg/day, p.o.; $n=8$ and $n=9$, respectively). Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and an osmotic minipump (model 2002; Alza Co., Palo Alto, USA) was implanted subcutaneously at the dorsum of the neck to infuse vehicle or aldosterone. Osmotic minipumps were replaced every 2 weeks. The doses of aldosterone (0.75 μg/h, s.c.) and olmesartan (10 mg/kg/day, p.o.) were determined on the basis of results from previous studies in rats (*4*, *5*, *20*, *31–33*).

In the 6 week treatment groups, systolic blood pressure (SBP) was measured in a conscious state by tail-cuff plethysmography (BP-98A; Softron Co., Tokyo, Japan) and 24-h urine samples were collected before uninephrectomy and at weeks 1, 3 and 6 during the treatment period. For all animals, blood and kidney samples were harvested at the end of week 3 or 6. After decapitation, trunk blood was collected into chilled tubes containing an inhibitor mixture and processed for measurements of plasma Ang II and creatinine levels (*34*, *35*). One third of the kidney was fixed in 10% buffered paraformaldehyde for histological examination. Renal cortical and medullary tissues were separated from the remaining kidney and used for measurements of kidney Ang II contents (*33*, *35*), protein or mRNA extraction and analysis of collagen contents (*4*, *33*, *35*).

Western Blotting

Protein levels of AT_1 receptor and ACE in renal cortical and medullary tissues were analyzed by Western blotting analy-

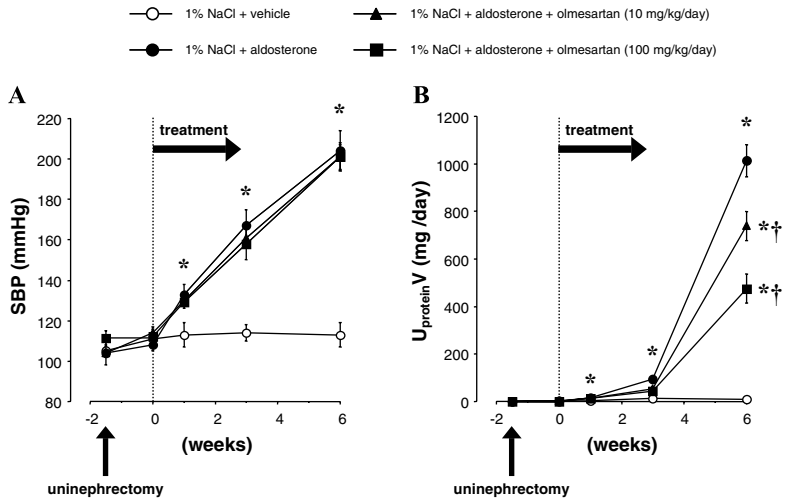

Fig. 1. *The profiles of systolic blood pressure (SBP) (A) and urinary protein excretion ($U_{protein}V$) (B) are shown. One % NaCl+aldosterone–treated rats showed severe hypertension and proteinuria. Concurrent administration of olmesartan at 10 or 100 mg/kg/day did not affect the development of hypertension. However, olmesartan significantly decreased $U_{protein}V$ in these animals.* *p < *0.05* vs. *1% NaCl+vehicle.* †p < *0.05: 1% NaCl+aldosterone* vs. *1% NaCl+aldosterone+olmesartan.*

ses, as previously described (*33–35*). Briefly, protein samples were separated by 8% or 10% polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. The membrane was reacted with a polyclonal anti-ACE antibody (1:400; Santa Cruz Biotechnology Inc., Santa Cruz, USA) or a polyclonal anti–AT_1 receptor antibody (1:1,000; Santa Cruz Biotechnology Inc.), followed by incubation with a horseradish peroxidase–conjugated secondary antibody (for ACE: donkey anti-goat IgG, 1:1,000, Santa Cruz Biotechnology Inc.; for AT_1 receptor: goat anti-rabbit IgG, 1:1,000, Cell Signaling Technology Inc., Beverly, USA). To check for equal loading, membranes were reprobed with an antibody against β-actin (Sigma Chemical Co., St. Louis, USA). All values were normalized by arbitrarily setting the integrated densitometric values of vehicle-infused rats to 1.0.

Real-Time Reverse Transcription–Polymerase Chain Reaction (PCR)

The mRNA expression of glyceraldehydes-3-phosphate dehydrogenase (GAPDH), Ang II type 2 (AT_2) receptor, transforming growth factor-β (TGF-β) and connective tissue growth factor (CTGF) were analyzed by real-time PCR using a LightCycler FastStart DNA Master SYBR Green I kit or TaqMan Gene Expression Assay kits (Applied Biosystems, Foster City, USA). The oligonucleotide primer sequences and PCR conditions were as described previously (*33*). All data are expressed as the relative differences between vehicle-infused and treated rats after normalization to GAPDH expression.

Histological Examination

Kidneys were fixed with 10% formalin (pH 7.4), embedded in paraffin, sectioned into 4-μm slices, and stained with hematoxylin-eosin (HE) reagent. The severity of glomerular injury and tubular proteinaceous cast scores were evaluated using light microscopy according to previously described methods (*33*, *35*, *36*). Glomerular proliferative lesions were scored into 5 grades as follows: 0, no proliferation; 1, minor (segmental lesion<25%); 2, mild (25%<segmental lesion<50%); 3, moderate (diffuse proliferation without severe sclerotic change); and 4, severe (diffuse proliferation with nearly complete sclerosis). A minimum of 50 glomeruli were examined in each specimen. Tubular proteinaceous cast scores were scored into 5 grades as follows: 0, no damage; 1, mild (patchy isolated damage); 2, moderate (damage<25%); 3, severe (25%<damage<50%); and 4, very severe (50%<damage).

Analytical Procedures

The urinary excretion rate of protein ($U_{protein}V$) was determined using a protein assay kit (microTP-test; Wako Co., Tokyo, Japan). Renal cortical and medullary tissue collagen contents were determined on the basis of the hydroxyproline concentration (*4*, *33*, *35*).

Statistical Analysis

Values are presented as the means±SEM. Statistical comparisons of the differences were performed using one- or two-way analysis of variance for repeated measures combined with the Newman-Keuls post hoc test. Values of $p<0.05$ were

Table 1. Body Weight (BW), Left Kidney Weight (LKW), LKW/BW Ratio, Plasma Creatinine, Creatinine Clearance and Plasma Angiotensin II (Ang II) Levels in Uninephrectomized Rats Treated with 1% NaCl, Vehicle (2% Ethanol), Aldosterone (0.75 μg/h) and Olmesartan (10 or 100 mg/kg/day) for 6 Weeks

	1% NaCl+vehicle (n=9)	1% NaCl+aldosterone (n=8)	1% NaCl+aldosterone +olmesartan (10 mg/kg/day) (n=8)	1% NaCl+aldosterone +olmesartan (100 mg/kg/day) (n=9)
BW (g)	516±18	354±9*	347±9*	351±6*
LKW (g)	2.81±0.06	7.91±0.34*	6.57±0.59*	4.83±0.56*,†
LKW/BW (%)	0.54±0.03	1.97±0.39*	1.94±0.14*	1.48±0.16*,†
Plasma creatinine (mg/dl)	0.41±0.01	0.58±0.03*	0.53±0.02*	0.45±0.02†
Creatinine clearance (l/day)	0.92±0.04	0.46±0.05*	0.59±0.08*	0.81±0.08†
Plasma Ang II (fmol/ml)	17±6	23±4	64±12*,†	64±10*,†

Values are means±SEM. *$p<0.05$ *vs*. 1% NaCl+vehicle. †$p<0.05$: 1% NaCl+aldosterone *vs*. 1% NaCl+aldosterone+olmesartan.

Table 2. Left Kidney Weight (LKW)/Body Weight (BW) Ratio, Plasma Creatinine, Angiotensin II (Ang II) Levels in Plasma, Renal Cortical or Medullary Tissues, and Collagen Contents in Uninephrectomized Rats Treated with 1% NaCl, Vehicle (2% Ethanol), Aldosterone (0.75 μg/h) and Olmesartan (10 or 100 mg/kg/day) for 3 Weeks

	1% NaCl (n=7)	1% NaCl+aldosterone (n=8)	1% NaCl+aldosterone +olmesartan (10 mg/kg/day) (n=8)	1% NaCl+aldosterone +olmesartan (100 mg/kg/day) (n=8)
LKW/BW (%)	0.58±0.07	1.05±0.10*	0.83±0.07*,†	0.81±0.04*†
Plasma creatinine (mg/dl)	0.40±0.04	0.44±0.04	0.40±0.03	0.40±0.04
Creatinine clearance (l/day)	0.74±0.02	0.73±0.05	0.70±0.15	0.71±0.08
Plasma Ang II (fmol/ml)	25±2	30±6	76±18*,†	86±15*,†
Ang II in the cortex (fmol/g)	72±5	82±12	70±6	74±5
Ang II in the medulla (fmol/g)	126±17	120±13	109±13	110±12
Collagen in the cortex (μg/mg)	14±1	19±1*	14±1†	12±1†
Collagen in the medulla (μg/mg)	18±1	23±1*	18±1†	16±1†

Values are means±SEM. *$p<0.05$ *vs*. 1% NaCl +vehicle. †$p<0.05$: 1% NaCl+aldosterone *vs*. 1% NaCl+aldosterone+olmesartan.

considered statistically significant.

Results

Blood Pressure, and Body and Kidney Weights

The temporal profile of SBP is depicted in Fig. 1A. SBP was identical among the groups at the beginning of the protocol and before treatments (10 days after uninephrectomy). SBP was also unaltered during the protocol in 1% NaCl/vehicle–treated rats (113±6 mmHg at week 6). On the other hand, 1% NaCl/aldosterone–treated rats developed hypertension (204±10 mmHg at week 6). Treatment with olmesartan did not result in significantly reduced SBP in aldosterone-infused rats (at week 6: 10 mg/kg/day, 201±7 mmHg; 100 mg/kg/day, 201±6 mmHg). The body and kidney weights are summarized in Tables 1 and 2. At week 6, aldosterone-infused rats showed lower body weight than vehicle-infused rats. Olmesartan treatment at a dose of 10 or 100 mg/kg/day did not affect the body weight in aldosterone-infused rats. On the other hand, aldosterone infusion significantly increased the kidney weight and the kidney weight/body weight ratio. Olmesartan at a dose of 10 mg/kg/day resulted in a slight but not significant change in kidney weight and kidney weight/body weight ratio. However, an ultrahigh dose of olmesartan (100 mg/kg/day) significantly decreased kidney weight and the kidney weight/body weight ratio in aldosterone-infused rats (Tables 1 and 2).

$U_{protein}V$, Plasma Creatinine and Creatinine Clearance

The temporal profile ratio of $U_{protein}V$ is depicted in Fig. 1B. Aldosterone-infused rats showed severe proteinuria (1,014±67 mg/day at week 6). Olmesartan at a dose of 10 mg/kg/day significantly decreased $U_{protein}V$ in aldosterone-infused rats (741±54 mg/day at week 6). A greater reduction in $U_{protein}V$ was observed in aldosterone-infused rats treated with olmesartan at 100 mg/kg/day (475±62 mg/day at week 6). Plasma creatinine levels and creatinine clearance are summarized in Tables 1 and 2. At week 3, none of the treatments altered plasma creatinine levels or creatinine clearance (Table

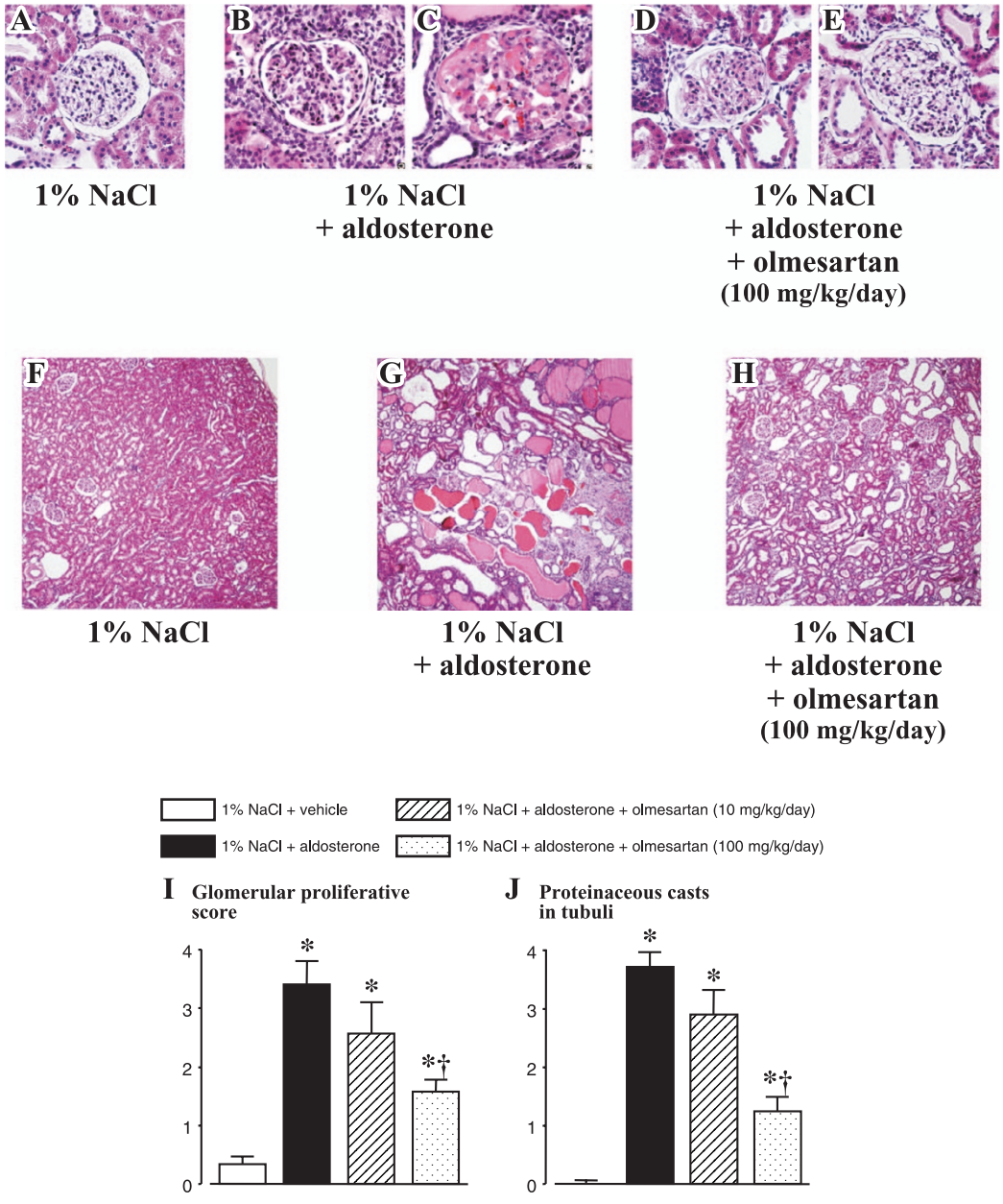

Fig. 2. *Photomicrographs of glomeruli (A–D, original magnification: ×400) and cortex (F–H, original magnification: ×100) are shown. The glomerular proliferative score (I) and proteinaceous casts in tubuli (C) are determined as described in Methods. One % NaCl+aldosterone–treated rats exhibited damaged glomeruli characterized by cell proliferation and sclerosis (B, C and I) and severe proteinaceous casts in tubuli (G and J). Treatment with olmesartan at 10 mg/kg/day did not significantly change aldosterone-induced glomerular injury or tubular proteinaceous casts (I and J). However, olmesartan at 100 mg/kg/day significantly attenuated these aldosterone-induced injuries (D, E, H, I and J).* *$p < 0.05$ vs. *1% NaCl+vehicle.* †$p < 0.05$: *1% NaCl+aldosterone* vs. *1% NaCl+aldosterone+olmesartan.*

2). At week 6, plasma creatinine levels were significantly higher in aldosterone-infused than in vehicle-infused rats. On the other hand, creatinine clearance was lower in aldosterone-infused than in vehicle-infused rats. Treatment with olmesartan at 10 mg/kg/day did not significantly change plasma creatinine levels or creatinine clearance in aldosterone-infused rats. However, olmesartan at 100 mg/kg/day significantly reversed aldosterone-induced changes in plasma creatinine levels and creatinine clearance (Table 1).

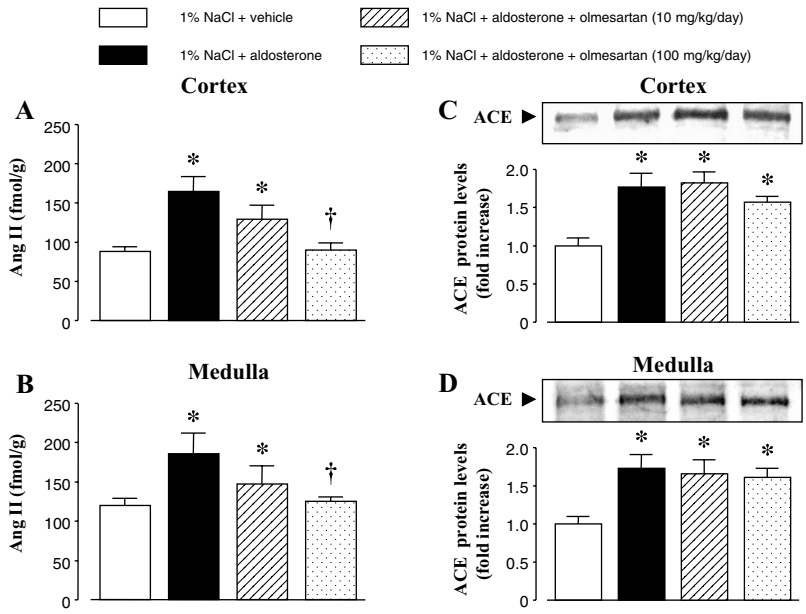

Fig. 3. *Ang II contents in renal cortical (A) and medullary (B) tissues are shown. One % NaCl+aldosterone–treated rats showed increased Ang II levels in both the cortex and medulla. Concurrent administration of olmesartan at 10 mg/kg/day to 1% NaCl+aldosterone–treated rats did not significantly change Ang II contents. However, olmesartan at 100 mg/kg/day significantly decreased both renal cortical and medullary Ang II levels. Protein expressions of ACE in renal cortical (C) and medullary tissues (D) are shown. Values were normalized by arbitrarily setting the densitometry of 1% NaCl+vehicle–treated rats to 1.0. One % NaCl+aldosterone–treated rats showed increased ACE protein levels in both the cortex and medulla. Concurrent administration of olmesartan at 10 or 100 mg/kg/day to 1% NaCl+aldosterone–treated rats did not change ACE protein levels. As a control study to check for equal loading, membranes were re-probed with an antibody against β-actin. The results showed that the densitometric values were unaltered in all groups (data not shown).* *$p < 0.05$ *vs. 1% NaCl+vehicle.* †$p < 0.05$*: 1% NaCl+aldosterone* vs. *1% NaCl+aldosterone+olmesartan.*

Histological Findings

The glomerular and renal cortical histological findings at week 6 are illustrated in Fig. 2A–J. One % NaCl/vehicle–treated rats showed almost normal glomeruli (Fig. 2A, I) and tubulointerstitium (Fig. 2F, J). However, aldosterone-infused rats exhibited injured glomeruli characterized by cell proliferation and sclerosis (Fig. 2B, C, I). In aldosterone-infused rats, severe proteinaceous casts were also observed in tubuli (Fig. 2G, J). Treatment with olmesartan at 10 mg/kg/day did not significantly change glomerular injury or tubular proteinaceous casts (Fig. 2I, J). However, olmesartan at 100 mg/kg/day significantly attenuated these aldosterone-induced injuries (Fig. 2D, F, H–J).

Ang II, ACE, AT_1 and AT_2 Receptors

Aldosterone infusion for 3 or 6 weeks did not alter plasma Ang II levels (Tables 1 and 2). Similarly, aldosterone infusion for 3 weeks altered neither cortical nor medullary Ang II contents (Table 2). Cortical and medullary ACE expression were also unaltered in all groups (data not shown). On the other hand, aldosterone infusion for 6 weeks resulted in higher Ang II contents in the renal cortex (165±19 fmol/g) and medulla (186±26 fmol/g) as compared with those in vehicle-infused rats (88±6 and 120±9 fmol/g; Fig. 3A and B, respectively). Concurrent administration of olmesartan in aldosterone-infused rats significantly increased plasma Ang II levels (Tables 1 and 2). However, olmesartan at 100 mg/day significantly decreased Ang II contents in the renal cortex (90±9 fmol/g at week 6; Fig. 3A) and medulla (125±6 fmol/g at week 6; Fig. 3B). At week 6, increases in cortical and medullary tissue Ang II contents were associated with increases in ACE protein expression (cortex: 1.8±0.2-fold; medulla: 1.7±0.2-fold; Fig. 3C and D, respectively). Olmesartan did not alter ACE expression in the renal cortex or medulla. As a control study to check for equal loading, membranes were re-probed with an antibody against β-actin. The results showed that the densitometric values were not significantly altered in any of the groups (data not shown).

Neither cortical nor medullary AT_1 receptor protein levels were different among the groups at week 6 (data not shown). On the other hand, aldosterone infusion for 6 weeks markedly reduced mRNA levels of AT_2 receptor in the cortex (27±5% of vehicle-treated rats) and medulla (28±9% of vehicle-treated rats). Aldosterone-induced decreases in cortical and

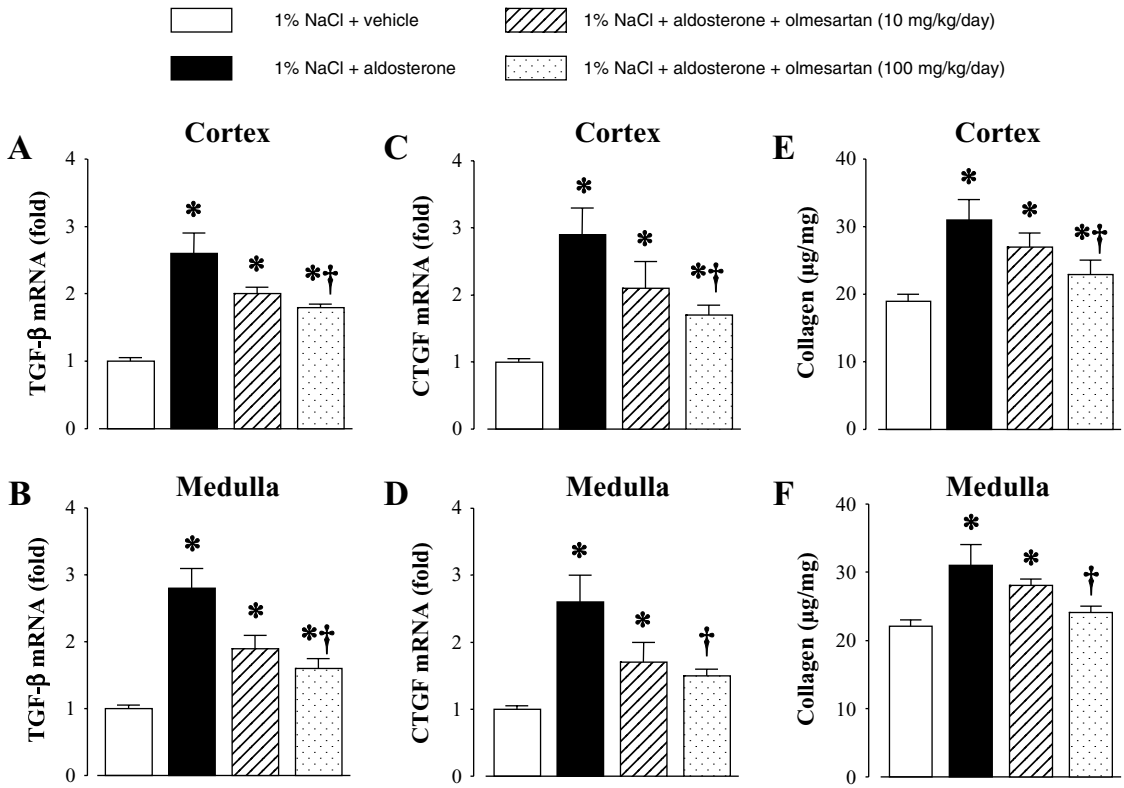

Fig. 4. *Gene expression of TGF-β (A and B) and CTGF (C and D), and collagen contents (E and F) are shown. Data of mRNA levels are expressed as the relative differences in 1% NaCl+aldosterone– or 1% NaCl+aldosterone+olmesartan (10 or 100 mg/kg/day)–treated rats compared to 1% NaCl+vehicle–treated rats after normalization to the expression of GAPDH. One % NaCl+aldosterone–treated rats showed increased mRNA levels of TGF-β and CTGF, and increased collagen contents in both the cortex and medulla. Concurrent administration of olmesartan at 10 mg/kg/day to 1% NaCl+aldosterone–treated rats did not significantly change these parameters. However, olmesartan at 100 mg/kg/day significantly decreased renal cortical and medullary TGF-β and CTGF mRNA levels, and collagen contents.* *$p < 0.05$ vs. *1% NaCl+vehicle.* †$p < 0.05$: *1% NaCl+aldosterone* vs. *1% NaCl+aldosterone+olmesartan.*

medullary AT_2 receptor expression were partially attenuated by treatment with an ultrahigh dose of olmesartan (63±16 and 66±14% of vehicle-treated rats, respectively).

TGF-β, CTGF and Collagen Content

At week 6, aldosterone-infused rats showed significantly higher renal cortical and medullary mRNA expression of TGF-β than did vehicle-infused rats (2.6±0.4 and 2.8±0.3-fold; Fig. 4A and B, respectively). Similarly, the renal cortical and medullary mRNA expression of CTGF was significantly increased in aldosterone-infused rats (2.9±0.3 and 2.6±0.4-fold; Fig. 4C and D, respectively). Treatment with olmesartan at 10 mg/day did not significantly alter these levels; however, olmesartan at an ultrahigh dose (100 mg/day) significantly decreased TGF-β and CTGF mRNA levels in both renal cortical and medullary tissues (Fig. 4A–D).

At week 6, collagen contents in the cortical or medullary tissues of aldosterone-infused rats were both 31±3 μg/mg, and these values were significantly higher than those of vehicle-treated rats (19±1 and 22±1 μg/mg; Fig. 4E and F, respectively). Treatment with olmesartan at 10 mg/day did not significantly alter these levels (27±1 and 28±1 μg/mg, respectively); however, olmesartan at an ultrahigh dose (100 mg/day) significantly decreased collagen contents in the renal cortical and medullary tissues (23±2 and 24±1 μg/mg; Fig. 4E and F, respectively).

Discussion

Recent studies suggest that aldosterone stimulates Ang II activity in cardiovascular tissues, including the kidney. In agreement with previous studies (*5*, *37*), uninephrectomized aldosterone/salt-treated hypertensive rats in the present study showed severe proteinuria and renal injury, characterized by glomerular sclerosis and tubular proteinaceous casts. The present study also provides the first direct evidence that Ang II levels are actually increased in renal cortical and medullary

tissues of aldosterone/salt-treated hypertensive rats. In addition, the data showed that strict AT_1 receptor blockade with an ultrahigh dose of olmesartan ameliorated aldosterone/salt-induced renal injury without changing blood pressure. These data support our hypothesis, which was based on previous studies (*25*, *26*, *38*), that augmented intrarenal Ang II activity contributes in part to aldosterone/salt-dependent renal injury.

It has been reported that plasma renin activity (PRA) and circulating Ang II levels are low in patients with primary aldosteronism (*39*, *40*). Animal studies have also shown that PRA is reduced in rats chronically treated with salt and aldosterone (*25*). In the present study, uninephrectomized aldosterone/salt-treated rats showed similar plasma Ang II levels compared with vehicle/salt-treated ones. Furthermore, AT_1 receptor blockade with olmesartan failed to prevent the development of hypertension even at an ultrahigh dose (100 mg/kg/day). These data are consistent with previous studies (*37*, *38*) showing that ARB did not affect blood pressure in aldosterone/salt-treated rats, and are consistent with the concept that the systemic renin-angiotensin system is not a predominant mediator of aldosterone/salt-induced hypertension. However, we found that intrarenal Ang II levels were significantly augmented in aldosterone/salt-induced hypertensive rats. Thus, these results indicate that in aldosterone/salt-induced hypertensive rats, the intrarenal renin-angiotensin system is regulated in a manner distinct from the circulating renin-angiotensin system. Since increases in intrarenal Ang II contents are associated with increases in ACE protein levels, augmentation of intrarenal Ang II levels may, at least partially, depend on local overexpression of ACE.

Previous studies have examined the effects of Ang II blockade on aldosterone-induced renal injury (*37*, *38*); however, the data obtained have not been consistent. Iglarz *et al*. (*38*) showed that AT_1 receptor blockade with losartan (10 mg/kg/day) attenuated renal collagen accumulation in non-nephrectomized rats treated with aldosterone and normal salt intake for 6 weeks. However, Sorooshian *et al*. (*37*) reported that losartan (180 mg/l in the drinking solution) did not attenuate renal injury in uninephrectomized rats treated with aldosterone and salt for 8 weeks. The reasons for the discrepancies between these observations (*37*, *38*) are not clear; however, they may be due to differences in the experimental settings, including the doses and/or durations of aldosterone and salt. Alternatively, the discrepancies may be due to the differences in the doses of losartan. In the present study, we examined the effects of olmesartan at two different doses; *i.e.*, at a recommended dose in rats (10 mg/kg/day) (*31–33*) and an ultrahigh dose (100 mg/kg/day). The results showed that olmesartan at 10 mg/kg/day significantly attenuated proteinuria in uninephrectomized aldosterone/salt-treated rats. However, the scores of renal injury and collagen accumulation, as well as mRNA expression of TGF-β and CTGF, were not altered by olmesartan at 10 mg/kg/day. On the other hand, the ultrahigh dose of olmesartan significantly attenuated aldosterone/salt-induced increases in TGF-β and CTGF mRNA levels and collagen contents, and ameliorated renal injury. TGF-β has been shown to play a predominant role in mediating Ang II–induced extracellular matrix production (*41*, *42*) through activation of its downstream effector, CTGF (*42*). These data suggest that strict AT_1 receptor blockade is necessary to prevent aldosterone-induced activation of Ang II in the kidneys, and support the suggestion of recent studies (*27–30*) that ultrahigh doses of ARB are useful for improving renal injury in some pathophysiological conditions. In the present study, an ultrahigh dose of olmesartan markedly decreased kidney Ang II contents; therefore, in aldosterone/salt-induced hypertensive rats, some of the renoprotective effects of AT_1 receptor blockade are accompanied by reductions in intrarenal Ang II contents, as has also been demonstrated in other animal models (*33*, *35*, *43*). Two different AT_1 receptor–dependent mechanisms are involved in the maintenance of high levels of intrarenal Ang II: 1) intracellular trafficking/accumulation of circulating and/or intrarenally formed Ang II into cortical tubular endosomes and 2) Ang II–induced angiotensinogen formation in proximal tubules (*44*). Although we did not investigate these mechanisms in the present experimental models, it is possible that treatment with an ultrahigh dose of olmesartan would prevent the cascade involving not only ligand-receptor activation but also internalization of Ang II and angiotensinogen formation, and thereby reduce Ang II levels in the kidney.

Chronic treatment with aldosterone and salt increase cardiac AT_1 receptor expression (*20*). *In vitro* studies also show that AT_1 receptor expression in vascular smooth muscle cells is increased by aldosterone (*23*). The present study showed that chronic aldosterone/salt treatment had no effect on AT_1 receptor protein levels in renal tissues. Our data are inconsistent with those of previous studies showing that the binding density of AT_1 receptors in the kidney is significantly increased in rats chronically treated with aldosterone and salt (*25*). Further studies will be required to clarify regional and tubular segment–specific regulation of intrarenal AT_1 receptor expression and binding during the development of aldosterone/salt-induced hypertension. In the present study, we found that renal AT_2 receptor mRNA levels were significantly decreased by chronic treatment with aldosterone/salt. Morrissey *et al*. (*45*) examined the effects of AT_2 receptor blockade with PD123319 on renal fibrosis induced by ureteral obstruction in rats, and found that PD123319 exacerbated fibrosis of the tubulointerstitium in obstructive nephropathy. These data suggest that AT_2 receptor has potential antifibrotic effects in the kidney. Although the functional roles of AT_2 receptors in the kidneys remain to be elucidated (*46*), it can be speculated that excessive upregulation of AT_2 receptor expression in the kidneys might be involved in aldosterone-induced renal injury. Since olmesartan significantly attenuated the aldosterone-induced reduction in AT_2 receptor expression, it can also be speculated that some of the renoprotective effects of olmesartan may be mediated through maintenance of AT_2 receptor expression. However, the precise

mechanisms responsible for aldosterone-, Ang II– or ARB-dependent regulation of intrarenal AT_2 receptor expression were not clarified by the present experiments.

In conclusion, the present study provides the first direct evidence that Ang II levels are increased in renal cortical and medullary tissues of uninephrectomized aldosterone/salt-treated hypertensive rats. Further, strict AT_1 receptor blockade with an ultrahigh dose of olmesartan ameliorated aldosterone/salt-induced renal injury without changing blood pressure. These data support our hypothesis, which was based on previous studies (*25*, *26*, *38*), that augmented intrarenal Ang II activity contributes to aldosterone/salt-dependent renal injury. It is also possible that strict AT_1 receptor blockade with an ultrahigh dose of ARB will be useful for improving aldosterone-dependent renal injury. Since increased intrarenal Ang II levels were associated with increases in ACE expression in the present study, further studies will be needed to examine the effects of ACE inhibitors on aldosterone/salt-dependent renal injury.

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