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A reduction of unilateral ureteral obstruction-induced renal fibrosis by a therapy combining valsartan with aliskiren

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[1]*Graduate Institute of Clinical Medical Science, China Medical University;* [2]*Division of Nephrology, Chang-Bing Show Chwan Memorial Hospital;* [3]*Institute of Clinical Medicine, National Yang Ming University; Departments of* [4]*Research,* [5]*Pathology, and* [6]*Division of Nephrology, Department of Medicine, Taichung Veterans General Hospital;* [7]*Chung-Shan Medical University; and* [8]*Graduate Institute of Biomedical Science, National Chung Hsing University, Taichung, Taiwan*

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Wu WP, Chang CH, Chiu YT, Ku CL, Wen MC, Shu KH, Wu MJ. A reduction of unilateral ureteral obstruction-induced renal fibrosis by a therapy combining valsartan with aliskiren. *Am J Physiol Renal Physiol* 299: F929–F941, 2010. First published August 4, 2010; doi:10.1152/ajprenal.00192.2010.—The protective effect of combination therapy with valsartan and aliskiren against renal fibrosis remains to be defined. This study was undertaken to examine the protective effects of the combination of valsartan and aliskiren against renal fibrosis induced by unilateral ureteral obstruction (UUO). Combination therapy with valsartan (15 $mg \cdot kg^{-1} \cdot day^{-1}$) and aliskiren (10 $mg \cdot kg^{-1} \cdot day^{-1}$), valsartan monotherapy (30 $mg \cdot kg^{-1} \cdot day^{-1}$), and aliskiren monotherapy (20 $mg \cdot kg^{-1} \cdot day^{-1}$) all significantly ameliorated the increase in blood urea nitrogen and the degree of hydronephrosis determined by the increase in weight and length of the obstructed kidney. The dose titration study and blood pressure measurement confirmed that the combination therapy provided a greater benefit independent of the vasodilatory effect. There were no significant changes in serum levels of creatinine, sodium, and potassium in UUO rats and any treatment groups. Combination therapy also attenuated UUO-related increases in the scores of tubular dilatation, interstitial volume, interstitial collagen deposition, α-smooth muscle actin, the activation of ERK 1/2, the infiltration of monocytes/macrophages, the mRNA expression of snail-1, and transforming growth factor-β1 to a greater extent compared with aliskiren or valsartan used alone. The mRNA expression of renin and the (pro)renin receptor significantly increased after UUO. Combination therapy and monotherapy of valsartan and aliskiren had a comparable enhancing effect on the mRNA expression of renin, whereas all these treatments did not affect the expression of the (pro)renin receptor. In conclusion, a direct renin inhibitor in conjunction with an angiotensin II receptor blocker exerts increased renal protection against renal fibrosis and inflammation during obstruction over either agent alone.

direct renin inhibitor; angiotensin II receptor blocker

THE ACTIVATION OF the renin-angiotensin-aldosterone system (RAS) is an important contributing factor in the pathogenesis of many cardiovascular and renal diseases (4, 6, 11). The blockade of the RAS through an angiotensin-converting enzyme (ACE) inhibitor, angiotensin II receptor blocker (ARB), or direct renin inhibitor (DRI), is a proven effective treatment of hypertension, heart failure, diabetic nephropathy, and nondiabetic renal diseases (5, 29, 39, 54). A dual therapy of ACE inhibitor and ARB has been shown to significantly reduce proteinuria and retard the progression of renal disease (21, 32, 35, 41–43). However, the risk of hyperkalemia with a combination therapy which combines the ACE inhibitor with ARB has also shown to be higher than with the ACE inhibitor or ARB used alone. Meanwhile, no matter whether the ACE inhibitor or ARB is used alone or in combination therapy, they all result in the increase in plasma renin activity (34, 51).

Renin, which controls the first and rate-limiting step of the RAS, has been recognized since the late 1950s (16, 49) as a preferred means for blocking the RAS. Direct renin inhibitors block the activity of renin through the interaction with the active site of the enzyme (7, 12, 52). The plasma renin activity decreases with the use of DRIs (38). Aliskiren is the first oral DRI which has been approved for the treatment of hypertension as monotherapy or in combination with other antihypertensive medications by the US Food and Drug Administration in March 2007 (9, 56). Given the success of an ACE inhibitor and ARB in reducing morbidity and mortality among patients with hypertension, diabetes mellitus, cardiac failure, nephropathy, and atherosclerosis, DRI alone or a therapy combining DRI with ARB may have potential benefits in the same disease states (31, 45, 50). In an animal study, both perindopril and aliskiren have shown to reduce blood pressure, albuminuria, and structural injury in experimental diabetic nephropathy (25, 33). Aliskiren and perindopril both were equally effective in reducing albuminuria and glomerulosclerosis in diabetic animals. Nevertheless, aliskiren had a better effect than perindopril on reducing the magnitude of interstitial fibrosis. In an AVOID (for Aliskiren in the eValuation of prOteinuria In Diabetes) study, a combination therapy with aliskiren and lorsartan resulted in 20% more proteinuria reduction than with losartan monotherapy in adults with type 2 diabetes and nephropathy. This effect was shown to be independent of blood pressure control (4, 40).

Renal fibrosis is the final common pathological pathway of most progressive renal disease regardless of the underlying disease or the originating compartment (3, 13, 60). The model of unilateral ureteral obstruction (UUO) generates progressive renal fibrosis that is independent of hypertension or systemic immune disease (8, 26). The obstructed kidney after UUO

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results in marked renal hemodynamic and metabolic changes. It is followed by tubular injury and cell death by apoptosis or necrosis, with interstitial macrophage infiltration (8, 27, 48, 55). Previous studies showed that ARBs or ACE inhibitors could ameliorate renal tubulointerstitial fibrosis that was caused by UUO (15, 20, 22–24). In this study, we investigate the effect of a therapy combining aliskiren with valsartan, referred to as combination therapy, on UUO-induced renal fibrosis.

MATERIALS AND METHODS

Animals. The studies were conducted in male Sprague–Dawley rats (~200 g) obtained from the National Laboratory Animal Center (Taipei, Taiwan). The experimental protocol was approved by the Animal Care Committee of Taichung Veterans General Hospital. The rats had free access to tap water and a standard rat diet.

Experimental design. The UUO rats underwent surgery as described previously (58). The left kidney and ureter were exposed under intraperitoneal pentobarbital anesthesia via a flank incision. The left ureter was ligated with 4-0 silk at two points and cut between the ligatures to prevent retrograde urinary tract infection. Finally, the wound was closed in layers. Sham animals underwent identical surgical procedures, but the left ureter was simply manipulated.

The efficacy of aliskiren, valsartan, or combination therapy on UUO-induced renal fibrosis was examined on *days 7* and *14*. In both experimental sets, the rats were divided into the same seven groups: *1*) sham-vehicle, where animals underwent sham operations and were treated with vehicle ($n = 10$); *2*) UUO-vehicle, where the animals underwent UUO but were treated with vehicle (PBS; $n = 10$); *3*) UUO-valsartan, where the rats underwent UUO and were treated with valsartan (15 $mg \cdot kg^{-1} \cdot day^{-1}$, $n = 10$); *4*) UUO-valsartan, where the rats underwent UUO and were treated with valsartan (30 $mg \cdot kg^{-1} \cdot day^{-1}$, $n = 10$); *5*) UUO-aliskiren, where the rats underwent UUO and were treated with aliskiren (10 $mg \cdot kg^{-1} \cdot day^{-1}$, $n = 10$); *6*) UUO-aliskiren, where the rats underwent UUO and were treated with aliskiren (20 $mg \cdot kg^{-1} \cdot day^{-1}$, $n = 10$); and *7*) UUO-combination therapy, where the rats underwent UUO and were treated with valsartan (15 $mg \cdot kg^{-1} \cdot day^{-1}$) and aliskiren (10 $mg \cdot kg^{-1} \cdot day^{-1}$, $n = 10$).

The blood pressure of the tail artery of the rat was measured by tail-cuff plethysmography (BP-98A; Softron, Tokyo, Japan) before the start of drug treatment and before death. The day before death, a 24-h urine sample was collected from each rat in metabolic cages to measure urinary protein excretion. All rats were killed under pentobarbital sodium anesthesia on *day 7* or *day 14*. Whole-blood samples were collected from the tail vein of each rat for the measurement of blood urea nitrogen (BUN), serum creatinine (Cr), serum sodium, serum potassium, and plasma renin activity before harvesting.

Histopathological and immunohistochemical analysis. Bilateral kidneys from each rat were fixed in 4% formalin in PBS and embedded in paraffin. Four-micrometer sections were stained with hematoxylin/eosin to assess the grade of tubulointerstitial damage. A standard point counting method, modified from previous reports, was used to determine the histological changes after UUO (58). Under high magnification (×400), 20 nonoverlapping fields from each section of the renal cortex were photographed. A grid containing 100 (10 × 10) sampling points was superimposed on each photograph. Points falling on glomerular structures or on large vessels were excluded from the total count. The tubular dilatation score was determined by the number of points overlying dilated tubular spaces and then converted to a percentage. The staining scores of interstitial volume, interstitial collagen deposition, and interstitial α-smooth muscle actin (SMA) expression were assessed accordingly. The interstitial collagen deposition score was determined by sirius staining, well established in our laboratory, as described previously (58). The method used was the selective binding of sirius red F3BA to all collagen proteins and Fast green FCF to noncollagen proteins when both were dissolved in aqueous saturated picric acid. All immunohistochemical studies were performed on paraffin-embedded sections as described previously (58). As a negative control, the primary antibody was replaced with normal rabbit IgG, without staining. The matrix score for α-SMA expression in the renal cortical interstitium was determined by procedures in accordance with previous reports (58). The monoclonal antibody against human α-SMA (1:200; EPOS System, Dako) was used.

To evaluate the infiltration of interstitial monocytes/macrophages, a rabbit polyclonal antibody against ED-1 (1:200; Serotec, Oxford, UK) was applied to the primary reaction, followed by a second reaction with biotin-labeled anti-rabbit IgG (Vector Laboratories, Burlingame, CA). Finally, a 3,3′-diaminobenzidine (DAB) reaction was performed on the section using a kit (DakoCytomation), and hematoxylin was used as the counterstain. The number of ED-1-positive cells was determined from 10 randomly chosen ×400 fields within the same section of the kidney from an individual animal (58). The average number of ED-1-positive cells from five separate rats was calculated.

Quantitative determination of collagen and total protein. The quantitative measurement of collagen and total protein content in formalin-fixed, paraffin-embedded tissue sections was preformed as described previously (58). The method used the selective binding of sirius red F3BA to collagen protein and Fast green FCF to noncollagen protein when both were dissolved in aqueous saturated picric acid. When the dye was eluted from the tissue sections with sodium hydroxide-methanol, the absorbances of 540 and 605 nm were determined for sirius red F3BA and Fast green FCF-binding proteins. The absorbances provided a relative measurement of collagen/total protein (μg/mg) quantity. Because the determination of collagen was relative to the concentration of protein per milligram in each tissue section, the thickness or the area of histological preparation was not a significant factor.

Western blot analysis. The expression of type IV collagen and α-SMA proteins in cell lysates and kidney tissue was analyzed by Western blotting as described previously (57, 58). The cell lysates (20 μg protein) were separated on 10% SDS-polyacrylamide gels. The proteins were electroblotted onto a nitrocellulose membrane (Amersham, Piscataway, NJ). Filters were incubated overnight at 4°C with antibodies directed against the type IV collagen (Dako), α-SMA (Dako), or actin (Santa Cruz Biotechnology) and then incubated with a secondary antibody.

RT-PCR. Total RNA isolation, reverse transcription of the RNA, and all PCR experiments were performed. Total RNA was prepared from a kidney tissue by using TRIzol reagent (Invitrogen, Carlsbad, CA) and was quantified by the determination of ultraviolet absorbance at 260 nm. The first strand of cDNA was synthesized using 2 μg RNA in 20 μl of reaction buffer by a reverse transcription using AMV-RT (Promega, Madison, WI) and random primers, at 42°C for 30 min. The PCR was performed using a standard PCR kit on 1-μl aliquots of cDNA and HotStarTaq polymerase (Qiagen, Valencia, CA) with gene-specific primer pairs. About 20–25 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for amplification in a linear range were used and followed by a final extension step at 72°C for 7 min. The products of PCR were size-fractionated on agarose gels and detected by ethidium bromide staining. After quantification of band intensities using densitometry, the relative steady-state level of mRNA was calculated after normalizing to β–actin. The sequences of primer sets were specified as follows: Snail, 5′-CAC TAT GCC GCG CTC TTT C-3′ (sense) and 5′-GGT CGT AGG GCT GCT GGA A-3′ (antisense); transforming growth factor-β1 (TGF-β1), 5′-CCT GAG TGG CTG TCT TTT GAC G-3′ (sense) and 5′-AGT GAG CGC TGA ATC GAA AGC-3′(antisense); renin, 5′-ATC TTT GAC ACG GGT TCA GC-3′ (sense) and 5′-CAC AGT GAT TCC ACC CAC AG-3′(antisense); prorenin receptor, 5′-TTC TGA ACT GCA AGT GCT GCA T-3′ (sense) and 5′-CTG CCA GCT CCA GTG AAT ACA AG-3′(antisense); and β–actin, 5′-CAG CTG AGA GGG AAA TCG TG-3′ (sense) and 5′-CGT TGC CAA TAG TGA TGA CC-3′ (antisense).

ELISA of TGF-β. Extracts were made from kidney tissue from control and 14 days UUO rats with or without valsartan and aliskiren treatment. Equal amounts of protein were analyzed for the presence of TGF-β using a TGF-β ELISA kit (R&D Systems, Minneapolis, MN). Each sample was assayed in duplicate.

Statistical analysis. All data were expressed as means ± SE. Statistical calculations were performed using SPSS software (SPSS, Chicago, IL). One-way analysis of variance and multiple comparison tests were used to determine the statistical significance. Statistical significance was defined as a P value <0.05.

RESULTS

Effect of each treatment on changes in kidney weight, kidney length, and cross-section morphological finding on UUO rats. As shown in Fig. 1, both the weight and length of the obstructed kidney increased significantly after UUO compared with the sham-operated kidney and contralateral nonobstructed kidney (Fig. 1, *A* and *B*) ($P < 0.001$). Compared with the vehicle group, the 30 mg·kg^{-1}·day^{-1} valsartan group significantly attenuated the increase of obstructed kidney length by 10.9% on *day 7* and 13.6% on *day 14* (both $P < 0.05$, Fig. 1*A*), and the reduction was similar to that in the 20 mg·kg^{-1}·day^{-1} aliskiren group (10.2% on *day 7*, $P < 0.05$ and 13.0% on *day14*, $P = 0.058$). In the combination therapy with 15 mg·kg^{-1}·day^{-1} valsartan and 10 mg·kg^{-1}·day^{-1} aliskiren, the reduction was better (19.5% on *day 7* and 28.6% on *day14*) than that obtained by either valsartan or aliskiren on *day 7* or *day 14* (all $P < 0.05$). Monotherapy with 15 mg·kg^{-1}·day^{-1} valsartan or 10 mg·kg^{-1}·day^{-1} aliskiren failed to reduce the increase in obstructed kidney length.

Being consistent with the data on obstructed kidney length, the increase in kidney weight was reduced in the valsartan group (18.5% on *day 7* and 19.9% on *day 14*, both $P < 0.005$, Fig. 1*B*), and the reduction was also significant in the aliskiren group (18.0% on *day 7* and 18.8% on *day 14*, both $P < 0.005$) and in the combination therapy group (34.4% on *day 7* and 36.1% on *day14*, both $P < 0.005$). In the meantime, the reduction of kidney weight in the combination therapy group was significantly better than in either the valsartan or the aliskiren group on *day 7* or *day 14* (all $P < 0.05$), whereas monotherapy with 15 mg·kg^{-1}·day^{-1} valsartan or mg·kg^{-1}·day^{-1} aliskiren failed to reduce the increase in obstructed kidney weight.

As shown in Fig. 2, monotherapy with 30 mg·kg^{-1}·day^{-1} valsartan and 20 mg·kg^{-1}·day^{-1} aliskiren significantly and similarly ameliorated the change in the cross-sectional morphological changes in the obstructed kidney on *days 7* and *14* after UUO. Combination therapy with 15 mg·kg^{-1}·day^{-1} valsartan and 10 mg·kg^{-1}·day^{-1} aliskiren ameliorated these morphological changes to a greater extent than monotherapy with 30 mg·kg^{-1}·day^{-1} valsartan and 20 mg·kg^{-1}·day^{-1}. aliskiren.

Effect on blood pressure in UUO rats. As shown in Fig. 3*A*, the blood pressure of rats treated by UUO for 14 days was significantly higher than that of control normal rats. Combination therapy with valsartan (15 mg·kg^{-1}·day^{-1}) and aliskiren (10 mg·kg^{-1}·day^{-1}) and valsartan monotherapy (30 mg·kg^{-1}·day^{-1}) and aliskiren monotherapy (20 mg·kg^{-1}·day^{-1}) all slightly reduced the blood pressure of rats. At both 7- and 14-day UUO examination, the blood pressure of rats was comparable among the groups of combination therapy with valsartan (15 mg·kg^{-1}·day^{-1}) and aliskiren (10 mg·kg^{-1}·day^{-1}) and valsartan monotherapy (30 mg·kg^{-1}·day^{-1}) and aliskiren monotherapy (20 mg·kg^{-1}·day^{-1}).

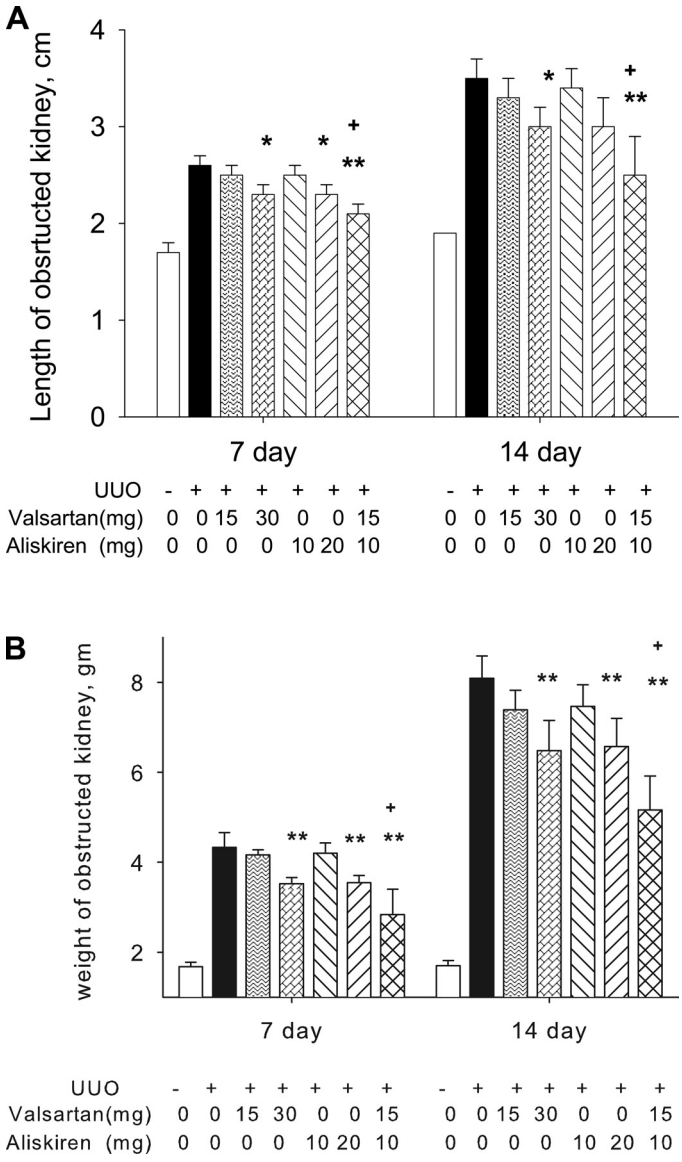

Fig. 1. Blockade of the renin-angiotensin-aldosterone system (RAS) significantly blunted the increase in obstructed kidney weight and length on *day 7* and *14* after unilateral ureteral obstruction (UUO). Values are means ± SE; $n = 5$/group. *$P <$ 0.05 vs. vehicle-treated rats after the same day of UUO. **$P < 0.005$ vs. vehicle-treated rats after the same day of UUO. +$P < 0.05$ vs. valsartan (30 mg·kg^{-1}·day^{-1})- or aliskiren (20 mg·kg^{-1}·day^{-1})-treated rats after the same day of UUO.

Effect on BUN, serum Cr, serum sodium, serum potassium, and 24-h proteinuria in UUO rats. As shown in Fig. 3*B*, BUN significantly increased in rats subjected to UUO for 7 and 14 days. Combination therapy with valsartan (15 mg·kg^{-1}·day^{-1}) and aliskiren (10 mg·kg^{-1}·day^{-1}) and valsartan monotherapy (30 mg·kg^{-1}·day^{-1}) and aliskiren monotherapy (20 mg·kg^{-1}·day^{-1}) all significantly ameliorated the increase in BUN of rats with UUO. Combination therapy with valsartan (15 mg·kg^{-1}·day^{-1}) and aliskiren (10 mg·kg^{-1}·day^{-1}) provided a greater reduction of BUN than monotherapy with valsartan (30 mg·kg^{-1}·day^{-1}) and aliskiren (20 mg·kg^{-1}·day^{-1}).

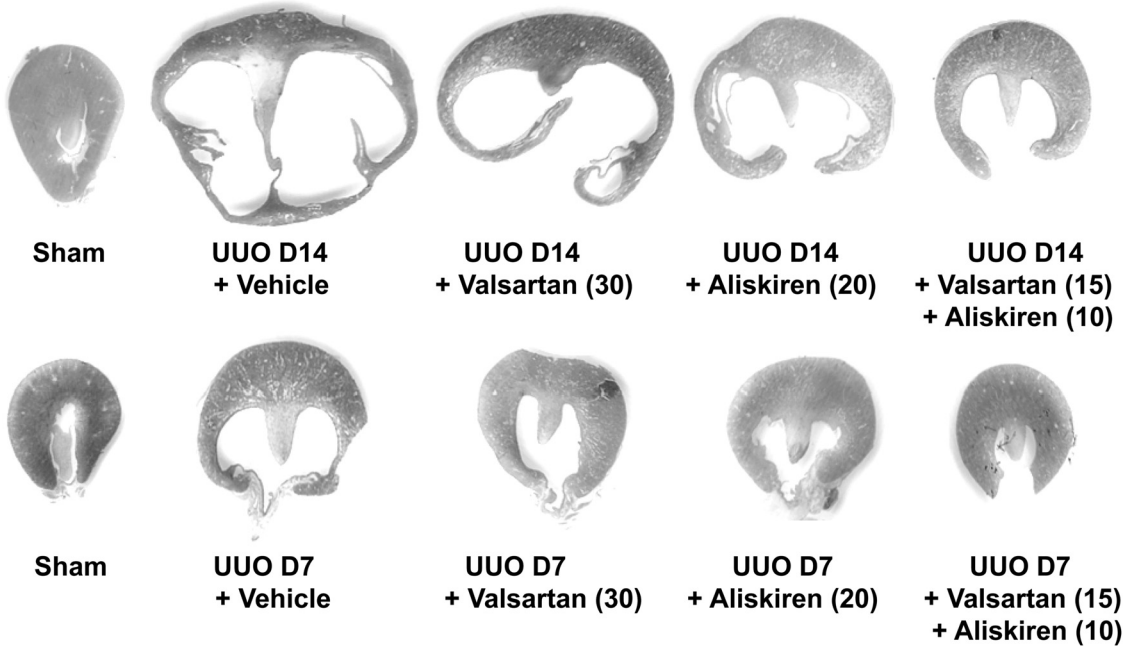

Fig. 2. Cross section of an obstructed kidney. (Effect of RAS system blockade on UUO-induced renal fibrosis in rats is shown).

Unlike BUN, the serum Cr, serum sodium, serum potassium, and 24-h proteinuria were not significantly affected by UUO and any drug treatment (Fig. 3, *C–F*).

Combination therapy with valsartan and aliskiren attenuated histological changes in the obstructed kidney induced by UUO. Figure 4 shows a representative histological finding by hematoxylin-eosin staining, α-SMA staining, and sirius staining on *day 14* of UUO in kidney tissue of UUO rats treated by vehicle, valsartan, aliskiren, and combination therapy. The obstructed kidney treated by the vehicle showed severe tubular dilatation, tubular atrophy, and widened interstitial space with a greater number of interstitial cells and infiltrating leukocytes. These changes were observed in the whole cortex, although the degree of severity was not homogeneously distributed. The administration of valsartan significantly attenuated the tubulointerstitial damage after UUO (Fig. 4*C*). The scores of tubular dilatation after UUO were significantly reduced by 30.1% on *day 7* and 20.6% on *day 14* (from 26.6 to 18.6 and from 36.0 to 28.6%, respectively, both $P < 0.05$, Fig. 5*A*). The effect resulting from the tubular dilation score reduction of aliskiren was similar (30.1% on *day 7* and 20.0% on *day 14*) with valsartan ($P = 1.0$, Fig. 4*D*). By contrast, there was a significantly better effect on the reduction of the tubular dilatation score by combination therapy (58.6% on *day 7* and 38.9% on *day 14*, $P < 0.05$, Fig. 4*E*).

The administration of valsartan blunted the score increase of the interstitial volume by 20.6% on *day 7* and by 19.5% on *day 14* (from 33.0 to 26.2 and from 41.0 to 33.0%, respectively, both $P < 0.05$, Fig. 5*B*). The effect of aliskiren was also similar (19.4% on *day 7* and 19.5% on *day 14*) for valsartan. Surprisingly, an effect using the combination therapy (38.2% on *day 7* and 34.1% on *day 14*, $P < 0.05$) was found to be significantly better than that using either valsartan or aliskiren alone.

Combination therapy with valsartan and aliskiren decreased expression of α-SMA and collagen in the obstructed kidney induced by UUO. We examined the effect of blockage of the RAS on interstitial myofibroblasts characterized by the expression of α-SMA. This contrasted with a high number of cells with α-SMA expression surrounding the peritubular and periglomerular spaces that developed in Sprague-Dawley rats after UUO (Fig. 4*G*). Valsartan significantly reduced the score of α-SMA expression in the cortical interstitium of UUO rats by 15.3% on *day 7* and by 14.1% on *day 14* (from 27.4 to 23.2% and from 35.4 to 30.4%, respectively, both $P < 0.05$, Figs. 4*H* and 5*C*). The effect of aliskiren was found to be similar (16.8% on *day 7* and 13.0% on *day 14*) to valsartan (Fig. 4*I*). By contrast, the combination therapy (31.4% on *day 7* and 27.7% on *day 14*, $P < 0.05$) was shown to be significantly better than either valsartan or aliskiren alone (Fig. 4*J*).

The interstitial collagen deposition of the obstructed kidney was significantly increased on *day 7* after UUO compared with that of contralateral kidneys (Fig. 4*L*). The score of interstitial collagen deposition by sirius staining also showed improvement by the blockage of the RAS (Fig. 5*D*). The valsartan administration significantly blunted the increase in the score of interstitial collagen deposition of the obstructed kidney by 26.9% on *day 7* and by 15.4% on *day 14* (from 26.8 to 19.6 and from 37.6 to 31.8%, respectively, both $P < 0.05$, Fig. 4*M*). The effect of aliskiren was also similar (27.6% on *day 7* and 12.8% on *day 14*) to valsartan (Fig. 4*N*). Compared with aliskiren and valsartan, the combination therapy results in a much better effect (48.5% on *day 7* and 29.3% on *day 14*, $P < 0.05$) than either valsartan or aliskiren used alone (Fig. 4*O*).

Western blot analyses of the kidney tissue extracts also revealed that the expression of collagen IV and α-SMA in the obstructed kidney was significantly increased. Combination therapy and monotherapy with valsartan and aliskiren signifi-

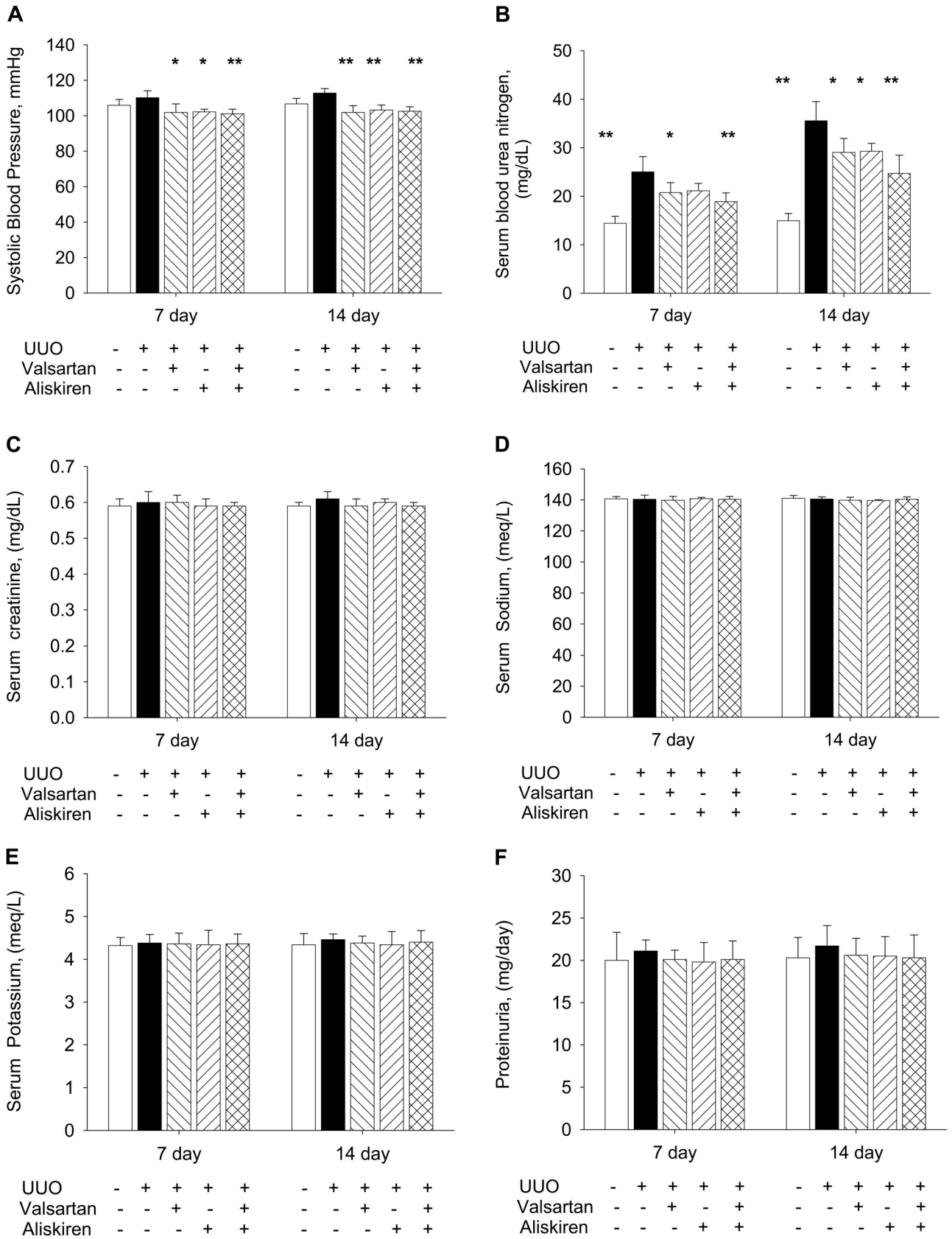

Fig. 3. Effect on blood pressure, blood urea nitrogen (BUN), serum Cr, serum sodium, serum potassium, and 24-h proteinuria of UUO rats treated by valsartan (30 mg·kg^{-1}·day^{-1}), aliskiren (20 mg·kg^{-1}·day^{-1}), and combination therapy with valsartan (15 mg·kg^{-1}·day^{-1}) and aliskiren (10 mg·kg^{-1}·day^{-1}). Values are means ± SE; $n = 5$/group. *$P < 0.05$ vs. vehicle-treated rats after the same day of UUO. **$P < 0.005$ vs. vehicle-treated rats after the same day of UUO.

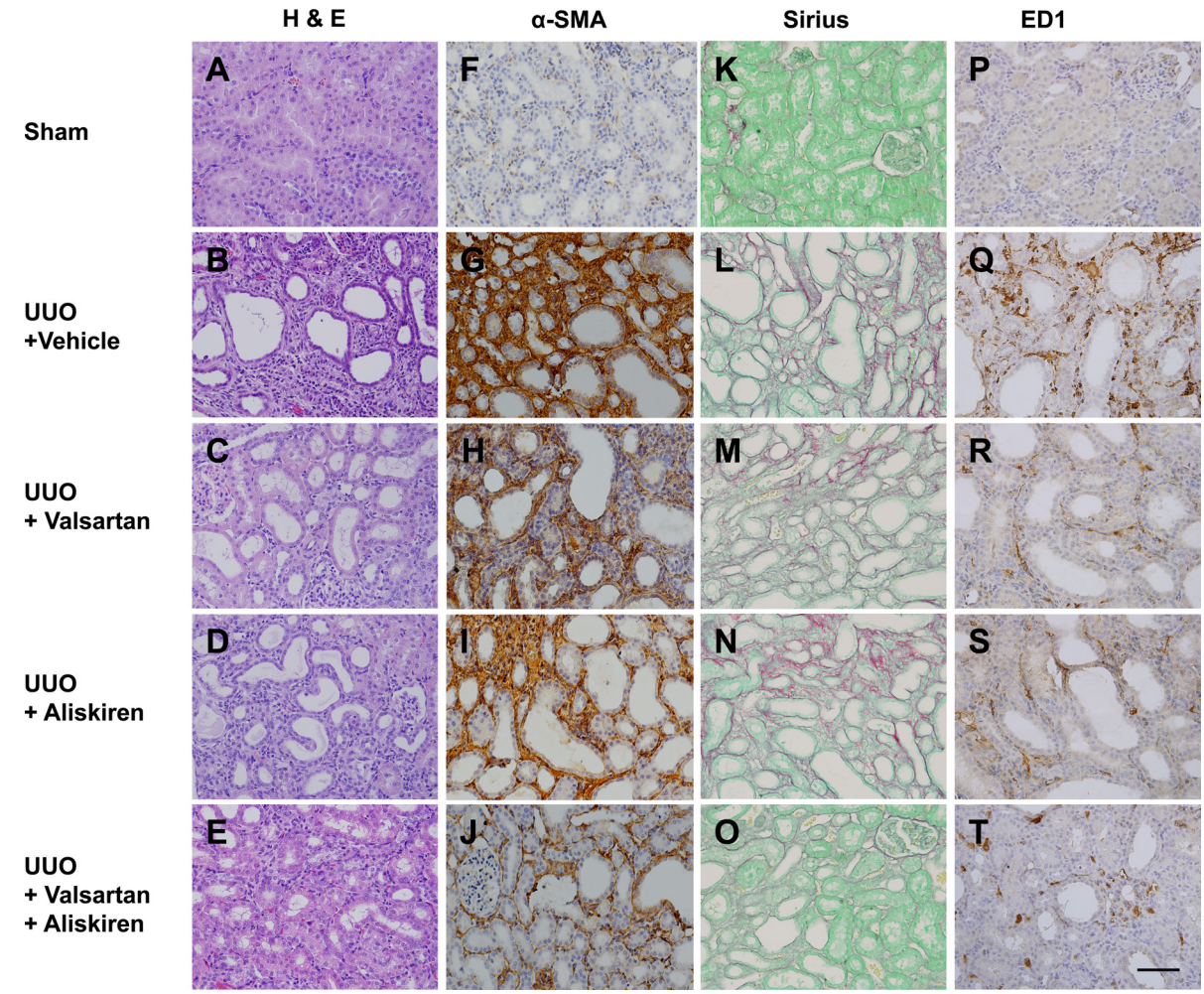

Fig. 4. Blockade of RAS attenuated the tubulointerstitial change of the obstructed kidney on *day 14* after UUO by different kinds of staining. Shown are representative micrographs of hematoxylin–eosin (H&E) staining (*A–E*), α-smooth muscle actin (α-SMA) staining (*F–J*), sirius staining (*K–O*), and ED-1 staining (*P–T*) in kidney tissue of normal rats that received sham operation (*A*, *F*, *K*, *P*) or rats that received UUO and were treated with vehicle (*B*, *G*, *L*, *Q*), valsartan (30 mg·kg^{-1}·day^{-1}; *C*, *H*, *M*, *R*), aliskiren (20 mg·kg^{-1}·day^{-1}; *D*, *I*, *N*, *S*), and combination therapy with valsartan (15 mg·kg^{-1}·day^{-1}) and aliskiren (10 mg·kg^{-1}·day^{-1}; *E*, *J*, *O*, *T*). Bar = 50 μm.

cantly attenuated the increase in collagen IV and α-SMA expression (Fig. 6).

We also determined the amounts of collagen and total protein content in the kidney tissue sections. Compared with the control kidney (12.5 ± 1.9 μg/mg) on *day 7*, the values were significantly higher ($P < 0.005$) in the obstructed kidney (26.2 ± 2.4 μg/mg on *day 7* and 34.0 ± 4.1 μg/mg on *day 14*). Valsartan effectively reduced the amounts of collagen by 14.7% on *day 7* and by 16.3% on *day 14* (from 26.2 ± 2.4 to 22.3 ± 1.7 and from 34.0 ± 4.1 to 28.5 ± 1.3 μg/mg, respectively, $P < 0.05$ on *day 14*, Fig. 5*E*). The effect of aliskiren was found to be similar (14.1% on *day 7* and 14.5% on *day 14*) to valsartan. Meanwhile, the combination therapy (27.1% on *day 7* and 21.9% on *day 14*) had a better effect than either valsartan or aliskiren used alone, but it was not significant.

Combination therapy with valsartan and aliskiren reduced infiltration of monocytes/macrophages and phosphorylation of ERK 1/2 in the obstructed kidney induced by UUO. The infiltration of ED-1-positive monocytes/macrophages was present as reported previously (Fig. 4*Q*) (58). Valsartan pretreatment resulted in a mild reduction in the number of infiltrating ED-1-positive cells by 9.4% on *day 7* and 6.5% on *day 14* (from 25.6 to 23.2 and from 33.8 to 31.6, respectively, Figs. 4*R* and 5*F*). The effect of aliskiren was better than that of valsartan (18.0% on *day 7* and 16.6% on *day 14*, both $P < 0.05$, Fig. 4*S*). Interestingly, the combination therapy (39.8% on *day 7* and 35.8% on *day 14*) showed the greatest inhibitory effect compared with either valsartan or aliskiren (both $P < 0.05$, Fig. 4*T*).

The Western blot analyses of the kidney tissue extracts showed that the ERK 1/2 pathway was activated after UUO as indicated in previous reports (Fig. 6). Both aliskiren and valsartan could reduce the activation of ERK 1/2. Once again, the combination of these two drugs inhibited the phosphorylation of ERK 1/2 to a greater extent than each drug used alone.

Effect of each treatment on mRNA expression of snail, TGF -β1, renin, and (pro)renin receptor in the obstructed kidney. As shown in Fig. 7, the mRNA expression of snail, TGF-β1, renin, and (pro)renin receptor were all increased in the obstructed kidney 14 days after UUO. Combination therapy and monotherapy with valsartan and aliskiren all exhibited a significantly ame-

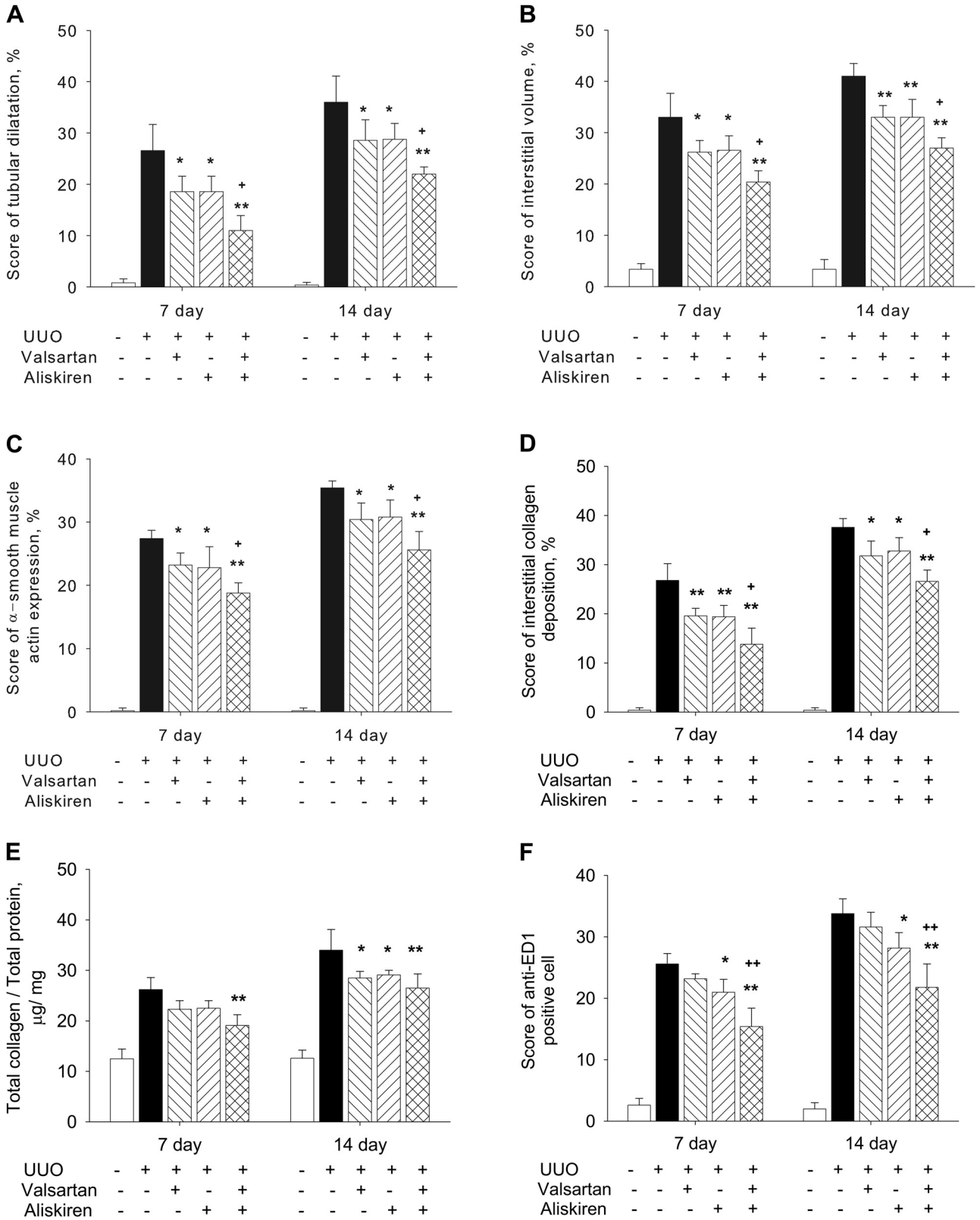

Fig. 5. Blockade of RAS attenuated the scores of tubulointerstitial damage of the obstructed kidney on *day 7* and *14* after UUO. The scores for tubular dilatation (*A*), interstitial volume (*B*), α-SMA expression (*C*), interstitial collagen deposition (*D*), collagen/total protein (μg/mg; *E*), and ED-1-positive cells of the obstructed kidney on *days 7* and *14* after UUO (*F*). Values are means ± SE; $n = 5$/group.*$P < 0.05$ vs. vehicle-treated rats after the same day of UUO. **$P < 0.005$ vs. vehicle-treated rats after the same day of UUO. +$P < 0.05$ vs. valsartan (30 $mg \cdot kg^{-1} \cdot day^{-1}$)- or aliskiren (20 $mg \cdot kg^{-1} \cdot day^{-1}$)-treated rats after the same day of UUO. ++$P < 0.005$ vs. valsartan- or aliskiren-treated rats after the same day of UUO.

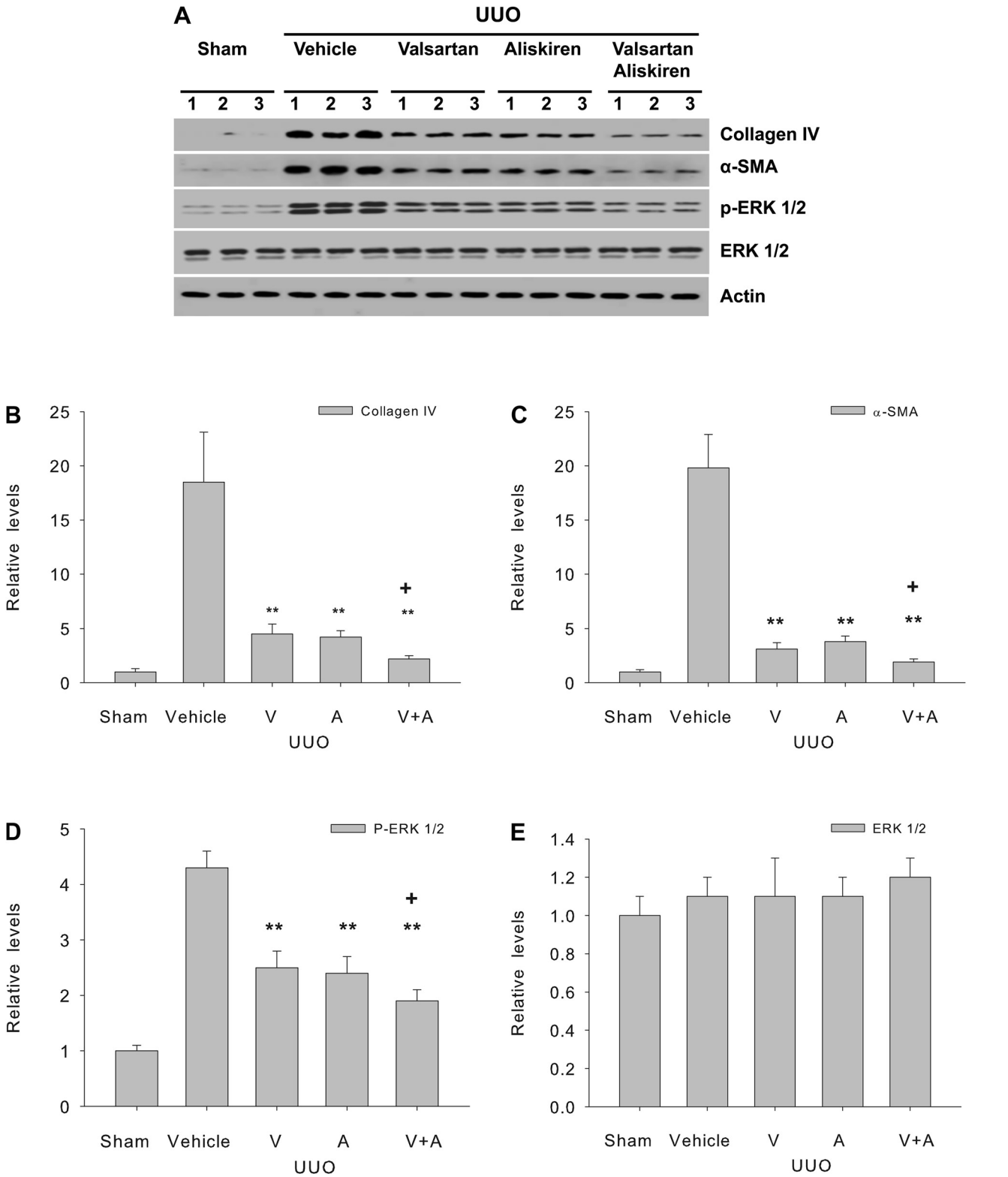

Fig. 6. Western blot showed that combination therapy has better efficacy than aliskiren or valsartan to block the increase of collagen IV and the de novo expression of α-SMA and phospho-ERK 1/2 on *day 14* after UUO. The same blot was stripped and reprobed with actin to confirm equal loading. Western blot results (*A*) and quantitative data (*B*~*-E*) are presented. Values are means ± SE; $n = 3$. V, valsartan (30 mg·kg^{-1}·day^{-1}); A, aliskiren (20 mg·kg^{-1}·day^{-1}); V+A, valsartan (15 mg·kg^{-1}·day^{-1}) and aliskiren (10 mg·kg^{-1}·day^{-1}). *$P < 0.05$ vs. vehicle-treated rats after the same day of UUO. **$P < 0.005$ vs. vehicle-treated rats after the same day of UUO. +$P < 0.05$ vs. valsartan (30 mg·kg^{-1}·day^{-1})- or aliskiren (20 mg·kg^{-1}·day^{-1})-treated rats after the same day of UUO.

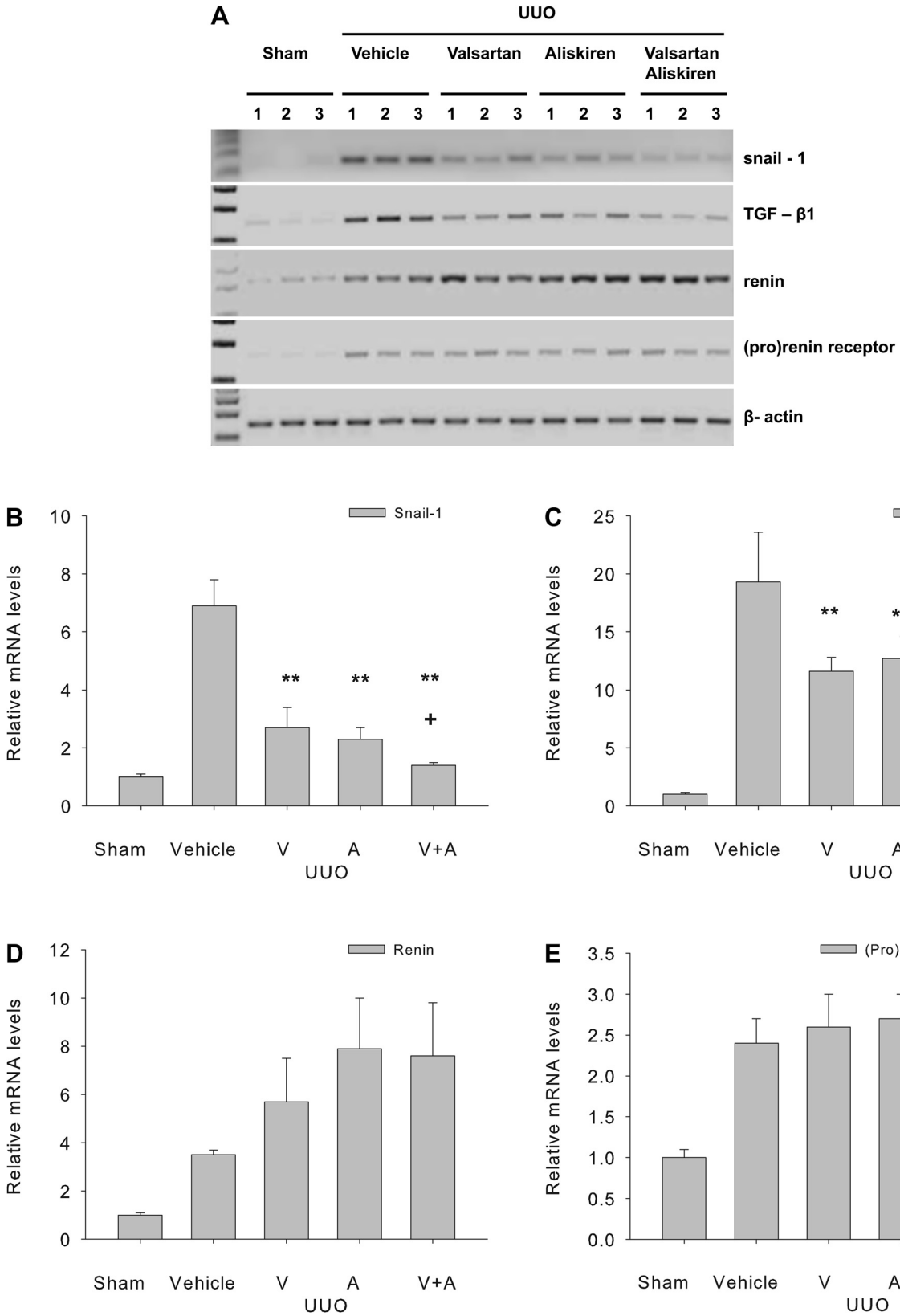

Fig. 7. Expression of snail 1 and TGF-β1, renin, and (pro)renin receptor in sham-operated kidneys, UUO kidneys treated with vehicle, valsartan, aliskiren, and combination therapy on *day 14*. Representative RT-PCR (*A*) and quantitative determination of mRNA (*B–E*) showed the expression in the obstructed kidney after various treatments. Values are means ± SE; $n = 3$. Abbreviations for treatments is as in Fig. 6. *$P < 0.05$ vs. vehicle-treated rats after the same day of UUO. **$P < 0.005$ vs. vehicle-treated rats after the same day of UUO. +$P < 0.05$ vs. valsartan (30 mg·kg^{-1}·day^{-1}) or aliskiren (20 mg·kg^{-1}·day^{-1})-treated rats after the same day of UUO.

liorating effect on the mRNA expression of snail and TGF-β1 in the obstructed kidney induced by UUO. Again, combination therapy showed a greater beneficial effect on the mRNA expression of snail and TGF-β1 than monotherapy with valsartan and aliskiren. Combination therapy and monotherapy with aliskiren had a comparable greater increase in renin mRNA expression than valsartan monotherapy, whereas all these treatments did not affect the expression of the (pro)renin receptor.

ELISA of TGF-β in the obstructed kidney induced by UUO. Being consistent with the data on mRNA expression, TGF-β ELISA showed that TGF-β was increased 6.1-fold in the kidney tissue of 14-day UUO rats compared with control normal rats (2.26 ± 0.41 vs. 0.37 ± 0.04, $P < 0.05$). Combination therapy with valsartan (15 $mg \cdot kg^{-1} \cdot day^{-1}$) and aliskiren (10 $mg \cdot kg^{-1} \cdot day^{-1}$) and valsartan monotherapy (30 $mg \cdot kg^{-1} \cdot day^{-1}$) and aliskiren monotherapy (20 $mg \cdot kg^{-1} \cdot day^{-1}$) all significantly ameliorated the increase in TGF-β in obstructed kidney tissues. Combination therapy with valsartan (15 $mg \cdot kg^{-1} \cdot day^{-1}$) and aliskiren (10 $mg \cdot kg^{-1} \cdot day^{-1}$) provided a greater reduction of TGF-β than monotherapy with valsartan (30 $mg \cdot kg^{-1} \cdot day^{-1}$) and aliskiren (20 $mg \cdot kg^{-1} \cdot day^{-1}$) (0.76 ± 0.04 vs. 1.12 ± 0.10 and 1.18 ± 0.07, $P < 0.05$).

Combination therapy with valsartan and aliskiren changes plasma renin activity. Plasma renin activity was significantly higher ($P < 0.05$) in the UUO kidney (10.7 ± 3.0 $ng \cdot ml^{-1} \cdot h^{-1}$ on *day 7* and 12.7 ± 1.6 $ng \cdot ml^{-1} \cdot h^{-1}$ on *day 14*) compared with the control normal kidney (5.7 ± 1.5 $ng \cdot ml^{-1} \cdot h^{-1}$ on *day 7* and 5.5 ± 1.6 $ng \cdot ml^{-1} \cdot h^{-1}$ on *day 14*, Fig. 8). The valsartan group had a higher level of plasma renin activity than the UUO group by 34.0% on *day 7* and 35.4% on *day 14* (from 10.7 ± 3.0 to 14.3 ± 4.3 $ng \cdot ml^{-1} \cdot h^{-1}$ on *day 7* and from 12.7 ± 1.6 to 17.1 ± 3.0 $ng \cdot ml^{-1} \cdot h^{-1}$ on *day 14*, $P < 0.05$ on *day 14*). The effect of aliskiren was the reverse; the plasma renin activity level was lower than in UUO rats treated with vehicle by 83.7% on *day 7* and 85.8% on *day 14* (from 10.7 ± 3.0 to 1.7 ± 1.1 $ng \cdot ml^{-1} \cdot h^{-1}$ on *day 7* and from to 1.8 ± 0.5 $ng \cdot ml^{-1} \cdot h^{-1}$ on *day 14*, both $P < 0.005$). The combination therapy group also had a lower plasma renin activity level than the UUO group by 50.8% on *day 7* and 50.6% on *day 14* (from 10.7 ± 3.0 to 5.3 ± 1.8 $ng \cdot ml^{-1} \cdot h^{-1}$ on *day 7* and from 12.7 ± 1.6 to 6.3 ±1.6 $ng \cdot ml^{-1} \cdot h^{-1}$ on *day 14*, both $P < 0.05$).

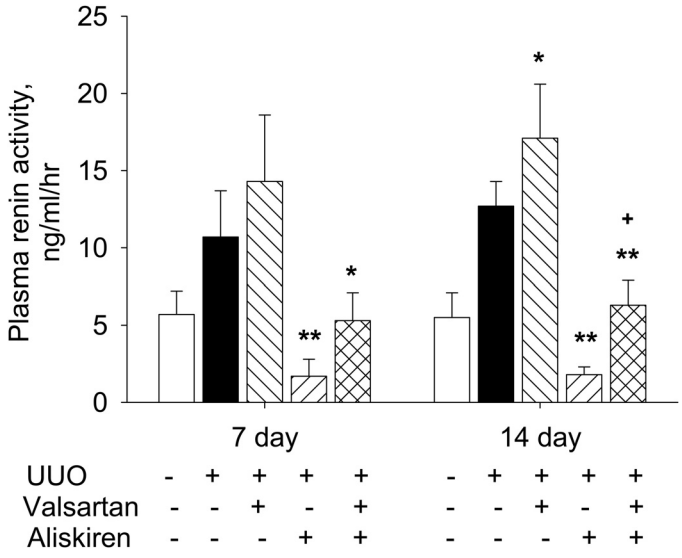

Fig. 8. Plasma renin activity of the obstructed kidney of UUO rats treated by valsartan (30 $mg \cdot kg^{-1} \cdot day^{-1}$), aliskiren (20 $mg \cdot kg^{-1} \cdot day^{-1}$), and combination therapy with valsartan (15 $mg \cdot kg^{-1} \cdot day^{-1}$) and aliskiren (10 $mg \cdot kg^{-1} \cdot day^{-1}$). Values are means ± SE; $n = 5$/group. *$P < 0.05$ vs. vehicle-treated rats after the same day of UUO. **$P < 0.005$ vs. vehicle-treated rats after the same day of UUO. +$P < 0.05$ vs. valsartan or aliskiren-treated rats after the same day of UUO.

DISCUSSION

The results presented in this study are believed to be the first to demonstrate a protective effect of the combination therapy using valsartan and aliskiren, a DRI, on renal fibrosis induced by UUO. The combination of ARB and DRI has also shown superior efficacy in protecting against renal fibrosis in models of diabetic nephropathy and hypertensive cardiac and renal injury in mice (10, 59). The rationale for the combination therapy of valsartan and aliskiren is based on the different mechanisms of actions of these two drug classes. The mechanisms of renal fibrosis involve several contributory steps. Each RAS inhibitor has significantly, but not completely, renoprotective effects on renal fibrosis. Thus appropriate combinations of the RAS inhibitors may have a better effect on renal fibrosis theoretically. The renoprotective effect of the combination therapy with an ACE inhibitor and ARB has been reported in animal and clinical studies (21, 28). The ACE inhibitor has been well known to decrease the formation of angiotensin II and thus attenuates renal fibrosis in obstructive uropathy (23). There were also several combination studies with ACE inhibitors which showed very good renoprotective effects in a rat model of renal fibrosis induced by UUO (28). One study showed that mycophenolate mofetil (MMF) and the ACE inhibitor lisinopril attenuated the progression of the fibrogenic process of UUO in an equivalent manner (14). The combination of both drugs did not show further improvement in the collagen content. Another study of UUO showed that paricalcitol blocked renin induction in the absence or presence of trandolapril (53). The combination therapy had additional efficacy in retarding renal scar formation during obstructive nephropathy. However, the risk of hyperkalemia, cough, and an insufficient response of the ACE inhibitor limits its clinical use (2). The insufficient response of the ACE inhibitor is caused by an incomplete blockade of the angiotensin converting enzyme after chronic ACEI inhibitor treatment or the generation of angiotensin II by ACE-independent pathways such as chymase (17, 37). On the other hand, a number of studies have shown that treatment with ARB is effective in reducing the rate of renal disease progression (5, 20, 30). ARB showed better tolerability than the ACE inhibitor, with less cough, angioedema, and hyperkalemia (2, 18). Besides, ARB may result in an incomplete blockage of the actions of angiotensin II mediated through the angiotensin II type 1 receptor (1). In the AVOID trial, the overall rate of hyperkalemia was similar between the aliskiren and the placebo groups (39). However, the rate of hyperkalemia was slightly higher when aliskiren was administered concomitantly with valsartan (4%) compared with aliskiren monotherapy (2%) or valsartan monotherapy (2%) (40). In fact, the combination therapy of DRI and

ARB, similar to the monotherapy of DRI and ARB, did not exert any effect on serum potassium in this study.

Hypertension and albuminuria are common in diabetic patients and are important risk factors for chronic kidney disease and renal fibrosis. Aliskiren reduced albuminuria and glomerulosclerosis in the rat model of advanced diabetic nephropathy by the blood pressure-independent pathway and also attenuated tubulointerstitial fibrosis to a greater extent than did perindopril (25). Aliskiren did not reduce the systemic blood pressure as much as did perindopril, but both compounds were equally effective in reducing albuminuria and glomerulosclerosis in diabetic animals. Indeed, UUO and any treatment group did not affect the 24-h proteinuria in this study. Since both valsartan and aliskiren are antihypertensive drugs, the information of blood pressure is very critical to correctly interpret the results. Comparable reduction of blood pressure in all treatment groups in this study confirmed that the greater beneficial effect of combination therapy is independent of the vasodilatory effect. Besides, we also performed the titration study to provide justification for the medication dosage.

DRI has been proven to be beneficial in diabetic and nondiabetic renal disease.(31, 45, 50). Theoretically, the DRI could decrease both angiotensin II formation and plasma renin activity despite that DRI may also incompletely block the activity of renin through interaction with an active site of the enzyme (52). In this study, the degree of hydronephrosis determined by kidney weights and lengths, and the scores for tubular dilatation, interstitial volume, interstitial collagen deposition, and α-SMA expression were all decreased under the blockage of valsartan, aliskiren, or the combination therapy. Western blot analysis also showed that the blockage of the RAS system could reduce the activation of ERK, as well as the expression of type IV collagen and α-SMA. In addition, the RT-PCR data also proved that the treatment with valsartan and aliskiren could inhibit the expression of snail-1 and TGF-β1. Especially, aliskiren has similar effects on inhibition of renal fibrosis compared with valsartan, whereas the therapy of combining these two classes of drugs showed a significantly better effect than with monotherapy of either valsartan or aliskiren on renal fibrosis induced by UUO. Similar to previous reports, our study also found that plasma renin activity was elevated in the UUO group and even higher in the valsartan group (17). By contrast, the aliskiren decreased the plasma renin activity level, which was reduced to the level near the sham-operated group, when aliskiren was added with valsartan for dual inhibition.

The binding of renin and prorenin to the (pro)renin receptor induces an intracellular signal with phosphorylation of serine and tyrosine residues which are associated with an angiotensin II that is an independent activation of the mitogen-activated protein kinases, In fact, angiotensin II can also activate the ERK 1/2 pathway which is known to be involved in cell hypertrophy and proliferation (36). In this study, we found that the activation of ERK 1/2 was ameliorated by the combination therapy and monotherapy with valsartan and aliskiren in a similar pattern to the histological index of renal fibrosis. As predicted, our results showed that the mRNA expression of renin and the (pro)renin receptor significantly increased after UUO. Aliskiren monotherapy and combination therapy of aliskiren and valsartan had a comparable greater increase in renin mRNA expression than valsartan monotherapy. However, we found that all these treatments did not affect the expression of the (pro)renin receptor in the obstructed kidney tissues. (Pro)renin receptor activation also has been shown to stimulate profibrotic pathways in the kidney that are independent of angiotensin II and therefore unaffected by ARBs and ACE inhibitors and presumably unaffected by DRI (19). Although the reactive increase in pro(renin) secretion with the DRI which potentially activates the fibrotic pathways, the feedback-suppressive mechanism of the (pro)renin receptor expression by a high concentration of pro(renin) provides one possible explanation for our findings (44). DRI may also provide additional protection over other RAS inhibitors by interfering with the enhanced catalytic activity of (pro)renin after the binding of these molecules to the (pro)renin receptor (47).

Ureteral obstruction causes the infiltration of the kidney by monocytes/macrophages. The tubulointerstitial influx of inflammatory cells has been observed in many forms of chronic kidney disease, and persistent inflammation in the kidney is thought to contribute to the development of tubulointerstitial fibrosis with functional impairment (46). The role of macrophages in inducing tissue injuries by releasing reactive oxygen species, nitric oxide, complement factors, and proinflammatory cytokines, or even an opposite role in resolution of inflammation or assisting regeneration has also been reported (46). Our experiments indicate that the infiltration of monocytes/macrophages in the obstructed kidney was significantly reduced by the administration of the DRI, but not by the ARB.

One the whole, our results demonstrate compelling evidence that a DRI in conjunction with an ARB provides increased renal protection against renal fibrosis and inflammation during obstruction over either agent alone.

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GRANTS

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DISCLOSURES

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

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