Activation of renal angiotensin type 1 receptor contributes to the pathogenesis of progressive renal injury in a rat model of chronic cardiorenal syndrome

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Homma T, Sonoda H, Manabe K, Arai K, Mizuno M, Sada T, Ikeda M. Activation of renal angiotensin type 1 receptor contributes to the pathogenesis of progressive renal injury in a rat model of chronic cardiorenal syndrome. Am J Physiol Renal Physiol 302: F750–F761, 2012. First published December 7, 2011; doi:10.1152/ajprenal.00494.2011.—Although chronic cardiac dysfunction is known to progressively exacerbate renal injury, a condition known as type 2 cardiorenal syndrome (CRS), the mechanism responsible is largely unknown. The present study was undertaken to clarify the mechanism of renal injury in rats with both unilateral nephrectomy (NX) and surgically induced myocardial infarction (MI), corresponding to a model of type 2 CRS. Compared with a control group, rats with both MI and NX (MI+NX) exhibited progressive proteinuria during the experimental period (34 wk after MI surgery), whereas proteinuria was not observed in rats with MI alone and was moderate in rats with NX alone. The proteinuria in rats with MI+NX was associated with renal lesions such as glomerulosclerosis and infiltration of mononuclear cells and upregulation of the renin proinflammatory and -fibrotic cytokine and angiotensin II type 1a receptor (AT1aR) genes. In contrast, plasma renin activity was lowered in rats with MI+NX. Immunohistochemistry revealed that the increased AT1R protein was present mainly in renal interstitial mononuclear cells. Olmesartan medoxomil, an AT1R blocker, markedly reduced the proteinuria and infiltration of mononuclear cells. Olmesartan medoxomil, an AT1R blocker, markedly reduced the proteinuria and infiltration of mononuclear cells, whereas spironolactone, a mineralocorticoid receptor blocker, did not. The present findings demonstrate the pathogenic role of renal interstitial AT1R signaling in a model of type 2 CRS, providing evidence that AT1R blockade can be a useful therapeutic option for this syndrome.

IT HAS BEEN REPORTED THAT cardiac dysfunction induces renal injury and that, conversely, impaired renal function could be a risk factor for cardiovascular diseases (1, 3, 12, 19–22, 29, 41, 49). These findings have led to the recognition that there is an interaction between heart and kidney diseases, a condition referred to as cardiorenal syndrome (CRS) (3, 41, 42). Ronco et al. (41, 42) categorized CRS into five types according to the pathophysiology, time frame, and nature of the concomitant cardiac and renal dysfunction. Type 1 CRS involves a sudden deterioration of cardiac function, leading to acute kidney injury. Type 2 CRS includes chronic abnormalities of cardiac function, causing progressive chronic kidney disease. Type 3 CRS, also called acute renocardiac syndrome, is characterized by acute kidney injury that leads to acute cardiac dysfunction. Type 4 CRS is a state of chronic kidney disease contributing to decreased cardiac function. Type 5 CRS means a systemic condition causing both cardiac and renal dysfunction. For adequate diagnosis and treatment of CRS, it is important to clarify the mechanisms of each type in detail using animal models. However, established animal models for CRS are very limited, particularly so for chronic types of CRS such as type 2 and type 4 (41, 42).

The renin-angiotensin-aldosterone system (RAAS) is well known to play a role in the pathogenesis of both cardiac and renal injury (5, 7, 18). Blockade of the RAAS using angiotensin-converting enzyme (ACE) inhibitors and angiotensin II (ANG II) type 1 receptor (AT1R) blockers (ARB) effectively attenuates both forms of injury (23, 38, 51–53). Moreover, accumulating evidence indicates that aldosterone, an agonist for the mineralocorticoid receptor (MR), plays a pathogenetic role in tissue injuries (6, 44, 48) and that MR antagonists exert protective actions on both the heart and the kidney (13, 36, 37, 55).

The van Dokkum group (49) have shown that surgically induced myocardial infarction (MI) after unilateral nephrectomy (NX) results in progressive renal injury in rats, and to our knowledge, this is only the model that well reproduces the features of patients with type 2 CRS. Subsequently, based on results obtained using an ACE inhibitor, they suggested that the RAAS was involved in the pathogenesis of the progressive renal injury (54). In that study, however, the ACE inhibitor caused a dramatic decrease in blood pressure and an improvement in cardiac parameters. Therefore, at present, the pathogenetic role of RAAS in that model is still unclear. Furthermore, the contribution of aldosterone to the pathogenesis in that model has yet to be clarified.

To reveal some of the detail of the mechanism of renal injury in an animal model corresponding to human type 2 CRS, we studied several cardiac, renal, and neurohumoral parameters in rats with MI after NX. Also, the effects of olmesartan medoxomil, an ARB, and spironolactone, a MR antagonist, on the renal injury in this model were examined.

MATERIALS AND METHODS

Experimental materials. Olmesartan medoxomil was synthesized at Daiichi Sankyo (Tokyo, Japan). Spironolactone was purchased from Shanghai FWD Chemicals (Shanghai, China).

Experimental protocol and surgical procedures. All experiments were carried out in accordance with the Animal Experimentation Guidelines of Daiichi-Sankyo, the Law Concerning the Protection and Control of Animals (Japanese Law No. 105, October 1, 1973, revised on June 22, 1987), Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notification No. 88 of the Ministry of the Environment, Japan, April 28, 2006), and Guidelines for Animal Experimentation (the Japanese Association for Laboratory Animal Science, May 22, 1987).

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Male Slc:Wistar rats (6 wk of age) were purchased from Japan SLC (Shizuoka, Japan). The rats underwent surgical procedures for NX or sham at 7 wk of age and MI or sham at 8 wk of age. At 2 wk after the second surgery, rats were finally assigned to four groups: control, MI, NX, and MI+NX in study 1.

In the other series of experiments for evaluating the effects of drugs, MI+NX rats were divided into vehicle and olmesartan medoxomil (OLM) groups in study 2 or control and spironolactone (SPL) groups in study 3.

Under anesthesia with pentobarbital sodium (40 mg/kg ip), rats were subjected to unilateral right nephrectomy after ligation of the renal artery and vein and the urethra. The sham group underwent only laparotomy without nephrectomy.

MI was produced by ligation of the left ascending coronary artery. Briefly, rats under anesthesia were intubated, connected to a ventilator (SAR-830/AP Small Animal Ventilator, CWE, The Netherlands), and underwent a left-sided thoracotomy. The left ascending coronary artery was ligated ∼3 mm from its origin with 8-0 silk suture. The sham group underwent only a left-sided thoracotomy.

**Functional parameters.** Systolic blood pressure (SBP) was measured by the tail-cuff method (MK-2000; Muromachi Kikai, Tokyo, Japan) at the time points described in the text.

At the end of the study (week 37 in study 1 and week 16 in study 2), a pressure transducer (Micro-Tip 3Fr; Millar Instruments, Houston, TX) was inserted from the right carotid artery for measurement of cardiac function under pentobarbital sodium anesthesia. After stabilization, left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), heart rate (HR), and maximum rate of increase or decrease in left ventricular pressure (±dP/dt) were recorded using a polygraph system. To determine cardiac reserve, changes in these parameters after an intravenous injection of 6 µg/kg dobutamine were also measured. Thereafter, a blood sample was collected from the abdominal aorta. The heart and kidney were then removed and fixed for histopathological examination, and another piece was stored at −80°C for RNA analysis. In study 2, before fixation, the LV pressure-volume relationship was obtained using isolated hearts as described previously (24).

**Analyses of blood and urine samples.** Atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP, DRC Instruments) and plasma renin activity (PRA; Renin RIA beads, TFB, Tokyo, Japan) were measured with a radioimmunoassay kit. Twenty-four-hour urine was collected using a metabolic cage. Urinary total protein and creatinine were measured using a radioimmunoassay kit. Twenty-four-hour urine was collected from the abdominal aorta. The heart and kidney were then removed and fixed for histopathological examination, and another piece was stored at −80°C for RNA analysis. In study 2, before fixation, the LV pressure-volume relationship was obtained using isolated hearts as described previously (24).

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**Real-time PCR.** Total RNA was isolated from frozen kidneys using a RNeasy Plus Mini Kit (Qiagen, Tokyo, Japan). Five micrograms of total RNA from each preparation was reverse-transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen, Life Technologies, Carlsbad, CA). Real-time PCR analyses were performed using SYBR Premix Ex Taq II (TAKARA BIO, Shiga, Japan). The primers are shown in Table 3. GAPDH was used as an internal control. Real-time PCR and data analyses were performed using the 7900HT system (Applied Biosystems, Life Technologies).

**MI size and renal histological evaluation.** For measurement of MI size, hearts were cut into five transverse slices. The images of the sections were taken, and then the lengths of the infarcted wall and noninfarcted wall (epicardial surface) were measured using Photoshop 4 (Adobe Systems).

Histological and immunohistochemical analyses of the kidneys were performed as described previously (47). The kidneys were fixed in a 10% formaldehyde solution, and then the tissue was paraffin-embedded, cut into 2-µm-thick sections, deparaffinized, and rehydrated. Renal sections were stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS), or Masson’s trichrome. To determine the incidence of focal glomerulosclerosis (FGS), 50 glomeruli in each PAS-stained section were examined, and the severity of glomerulosclerosis was scored into four grades (0–3) in a blinded manner. The degree of mononuclear cell infiltration was quantified in areas across the kidney specimen, as described previously (11). For immunohistochemistry, antigens in the kidney sections were retrieved by incubating each specimen in distilled water at 121°C for 5 min. Each specimen was then incubated with anti-ATIR antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and immunoreactivity was visualized using Alexa Fluor 488 anti-rabbit IgG (Molecular Probes, Eugene, OR). Semiquantitative analysis was performed by measuring the intensity of fluorescence using an image-quantification system attached to the microscope (Olympus FV300, Tokyo, Japan), and the mean intensity was calculated per area unit. For each animal, >10 fields in each section were examined. When a conventional immunohistochemical analysis was performed, endogenous peroxidase was consumed using a 3% H2O2 solution, and then immunoreactivity was visualized using Envision System Labelled Polymer reagent (DAKO Japan, Tokyo, Japan), followed by treatment with 3,3′-diaminobenzidine tetrahydrochloride.

**Statistical analysis.** The differences from the control group were compared by two-way ANOVA followed by Dunnett’s tests for parameters determined in the multigroup study. For comparison between two groups, a t-test was used. In all tests, P < 0.05 was considered statistically significant.

**RESULTS**

**Basic characterization of model rats with MI after NX.** In the first series of experiments (study 1), rats were divided into four groups: double sham operation (control), MI, NX, and combined surgery (MI+NX). The time point at 2 wk after MI or sham operation was assigned as week 0. A: 24-h urinary protein excretion was measured at the indicated time points. B: systolic blood pressure (SBP) was measured by the tail-cuff method in awake, restrained animals. Values are means ± SE; n = 12–16/group. *P < 0.05 and ***P < 0.001 compared with the control group.

Fig. 1. Combined unilateral nephrectomy (NX) and myocardial infarction (MI) induces progressive proteinuria but no change in systemic blood pressure. Rats were assigned to 4 groups: double sham operation (control), MI, NX, and combined surgery (MI+NX). The time point at 2 wk after MI or sham operation was assigned as week 0. A: 24-h urinary protein excretion was measured at the indicated time points. B: systolic blood pressure (SBP) was measured by the tail-cuff method in awake, restrained animals. Values are means ± SE; n = 12–16/group. *P < 0.05 and ***P < 0.001 compared with the control group.
groups: a control group (subjected to a double sham operation), MI group (subjected to sham and MI surgery), NX group (subjected to NX and sham surgery), and MI+NX group (subjected to both MI and NX surgery). At 1 wk after NX or sham operation, MI or sham surgery (2nd surgery) was performed. The time point at 2 wk after the second surgery was assigned as week 0, and we continued to observe several parameters until week 37, when necropsy was performed.

When we measured body weights at week 37, no significant differences were evident among the four groups (control: 467 ± 10, MI: 404 ± 19, NX: 397 ± 13, MI+NX: 402 ± 19).

### Table 1. Cardiac function, myocardial infarction size, and plasma natriuretic peptide concentration in experimental groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>MI</th>
<th>NX</th>
<th>MI+NX</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, beats/min</td>
<td>392 ± 17</td>
<td>404 ± 19</td>
<td>397 ± 13</td>
<td>402 ± 19</td>
</tr>
<tr>
<td>LVSP, mmHg</td>
<td>138 ± 5</td>
<td>110 ± 7†</td>
<td>133 ± 5</td>
<td>124 ± 4</td>
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<td>−dP/dt, mmHg/s</td>
<td>6,120 ± 306</td>
<td>4,014 ± 391†</td>
<td>6,179 ± 393</td>
<td>4,413 ± 251†</td>
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<tr>
<td>Δ+dP/dt, mmHg/s</td>
<td>3.2 ± 0.7</td>
<td>13.8 ± 3.4*</td>
<td>4.7 ± 1.9</td>
<td>15.1 ± 3.4†</td>
</tr>
<tr>
<td>Δ−dP/dt, mmHg/s</td>
<td>1,691 ± 273</td>
<td>490 ± 127†</td>
<td>1,938 ± 572</td>
<td>369 ± 90*</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>−4,619 ± 272</td>
<td>−2,891 ± 274†</td>
<td>−4,695 ± 308</td>
<td>−3,237 ± 197†</td>
</tr>
<tr>
<td>Myocardial infarction size, %</td>
<td>35.6 ± 1.5</td>
<td>32.7 ± 2.5</td>
<td>32.7 ± 2.5</td>
<td>32.7 ± 2.5</td>
</tr>
<tr>
<td>ANP, pg/ml</td>
<td>248 ± 57</td>
<td>1,108 ± 126†</td>
<td>203 ± 58</td>
<td>1,239 ± 113†</td>
</tr>
<tr>
<td>BNP, pg/ml</td>
<td>106 ± 2</td>
<td>198 ± 28†</td>
<td>108 ± 3</td>
<td>213 ± 14†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 10–12 group. MI, myocardial infarction; NX, unilateral nephrectomy; HR, heart rate; LVSP, left ventricular systolic pressure; ±dP/dt, maximum rate of increase or decrease in left ventricle pressure; LVEDP, left ventricular end-diastolic pressure; Δ±dP/dt, change in values of ±dP/dt after 6 μg/kg dobutamine infusion; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide. All parameters were measured at week 37. *P < 0.05 and †P < 0.01 compared with the control group.
$n = 12$; MI: $451 \pm 11$, $n = 10$; NX: $479 \pm 8$, $n = 12$; MI+NX: $443 \pm 11$ g, $n = 14$), indicating that the procedures employed in this study did not produce any nonspecific toxicological effects. Figure 1A summarizes the time course of urinary protein excretion. Proteinuria gradually increased in the NX and MI+NX groups, the level being markedly higher in the latter. In contrast to both of these groups, the MI group did not exhibit significant proteinuria. To estimate renal function, we measured the plasma creatinine concentration at week 37. Plasma creatinine concentrations in the NX and MI+NX groups were slightly higher than that in the control group (control: $0.3 \pm 0.1$, $n = 12$; MI: $0.4 \pm 0.1$, $n = 10$; NX: $0.5 \pm 0.1$, $n = 12$, $P < 0.05$; MI+NX: $0.6 \pm 0.2$ mg/dl, $n = 13$, $P < 0.001$), but the values were within the normal range, indicating that renal function was little affected by the surgery.

Figure 1B shows the time course of SBP. Only in the MI group was the SBP significantly lower at week 23 or later compared with the control group.

Table 1 summarizes the cardiac parameters and MI size measured at the end of the observation period (week 37). A marked decrease in $\frac{dP}{dt}$ and an increase in LVEDP were observed in both the MI and MI+NX groups, accompanied by significant increases in the levels of ANP and BNP, hormonal markers of cardiac dysfunction. The response to dobutamine tended to be decreased in both the MI and MI+NX groups, indicating a reduction of myocardial contractile reserve.

**Fig. 3.** Combined NX and MI exacerbates indices of renal inflammation and fibrosis. Renal gene expressions of IL-1β (A) and transforming growth factor-β1 (TGF-β1; B) in the kidneys of the experimental groups were measured by real-time PCR. Values are means ± SE; $n = 10–14$ group. *$P < 0.05$ and **$P < 0.01$ compared with the control group.

**Fig. 4.** Combined NX and MI decreases plasma renin activity and increases renal angiotensin II type 1a receptor (AT1aR) mRNA. A: plasma renin activity at week 37 in the experimental groups was measured. Gene expression of AT1aR (B), angiotensin II type 2 receptor (AT2R) (C), and renin (D) in the kidney was measured by real-time PCR. Values are means ± SE; $n = 9–14$ group. **$P < 0.01$ compared with the control group.
parison between the MI and MI+NX groups revealed virtually no difference in cardiac parameters. Along with cardiac dysfunction, MI was macroscopically evident, and the infarct sizes in the two groups were quite similar. These data clearly indicated that our MI+NX model was characterized by progressive kidney injury, as evidenced by gradually increasing proteinuria without enhancement of cardiac injury.

Renal histology. At week 37, renal histology was evaluated, and the results are shown in Fig. 2. Compared with the control group, there were not significant changes in renal histology in the MI group. In contrast, lesions such as urinary cast formation, infiltration of mononuclear cells, an increase in the mesangial matrix, adhesion of Bowman’s capsule, interstitial fibrosis, and glomerulosclerosis were clearly evident in the MI+NX group. On the other hand, these changes in the NX group were moderate (data not shown).

Evaluation of renal inflammation and fibrosis. As renal inflammation and fibrosis were marked in the MI+NX group, we examined the renal expression of mRNAs for IL-1β, an inflammatory cytokine, and TGF-β1, a profibrogenic cytokine (56). As shown in Fig. 3, compared with the control group, the mRNAs of both renal IL-1β and TGF-β1 were significantly upregulated in the MI+NX group.

PRA and expression of RAS-related genes in the kidney. Although there is very limited understanding of the pathophysiology of type 2 CRS, it has been suggested that the RAS may be involved (54). Therefore we examined PRA and renal expression of mRNAs for RAS-related genes, such as AT1aR,
type 2 receptor (AT2R), and renin in the experimental groups. As shown in Fig. 4, PRA was significantly lower only in the MI+NX group relative to the controls (Fig. 4A). On the other hand, the gene expression level of AT1aR in the kidney was significantly higher only in the MI+NX group (Fig. 4B). Expression of mRNA for AT2R tended to be increased in the MI+NX group relative to the controls, but not to a significant degree (Fig. 4C). There was no difference between the groups in the level of renin mRNA (Fig. 4D).

Immunohistochemical localization of AT1aR in the kidneys of the MI+NX group. Next, we examined the renal expression level and distribution of AT1R protein using an immunohistochemical technique. As shown in Fig. 5, upregulation of AT1R was clearly observed in the renal cortex in the MI+NX group, relative to the control group (Fig. 5, A–C). In the renal cortex, when the expression level of AT1R protein was separately quantified in the glomerulus and interstitium, except for blood vessels, that in the interstitium was dramatically increased in the MI+NX group, while upregulation in the glomerulus was modest (Fig. 5, F and G). Higher magnification (Fig. 5E) revealed that most of the AT1R-positive cells in the interstitium were infiltrating mononuclear cells, and this staining pattern was quite consistent across the specimens. Furthermore, when we carefully observed serial sections from the MI+NX group stained with HE and anti-AT1R antibody (Fig. 6), most infiltrating mononuclear cells were positive for AT1R, while in the peritubular capillaries of the renal interstitium, AT1R-infiltrating mononuclear cells were positive for AT1R, while in the peritubular capillaries of the renal interstitium, AT1R-staining was hardly detected.

In the outer medulla, increased AT1R protein was also observed mainly in the interstitium in the MI+NX group (Fig. 5H). On the other hand, in the inner medulla, upregulation of AT1R protein was modest in the MI+NX group (data not shown).

Taken together, these results indicate that, in the MI+NX group, the expression level of AT1R protein was mainly increased in the infiltrating mononuclear cells in the interstitium of the cortex and outer medulla.

Effect of olmesartan medoxomil on renal injury in the MI+NX group. The above results suggested the involvement of renal AT1R signaling in the progressive renal injury seen in MI+NX rats. Therefore, we examined the effect of olmesartan, an ARB (31), at a dose that would not be expected to cause hypotension in normotensive rats (28), on proteinuria in the MI+NX group. For this series of experiments (study 2), rats were divided into two groups: a vehicle group (0.5% methylcellulose po) and an OLM group (5 mg·kg⁻¹·day⁻¹ po). At 2 wk after MI surgery, drug administration was started and continued for 16 wk, after which necropsy was performed. The start of drug administration was designated as week 0. As shown in Fig. 7A, proteinuria was significantly improved in the OLM group at weeks 8 and 12. Along with reduction of proteinuria, histological alterations resulting from the combined surgery were ameliorated (Fig. 8). On the other hand, when we measured SBP at week 12, there was no significant difference between the two groups (Fig. 7B). Figure 7C and Table 2 show the left ventricular pressure-volume relationship and cardiac parameters at week 16, respectively, and neither of the parameters differed significantly between the two groups. These data clearly indicate that ARB reduced proteinuria in MI rats after NX and that this effect was independent of blood pressure and cardiac parameters, suggesting that the reduction of proteinuria was mediated by direct inhibition of renal AT1R signaling.

Effect of spironolactone on the renal injury in MI+NX rats. Finally, to clarify the involvement of aldosterone, we investig-
gated the effect of spironolactone on the proteinuria in MI rats after NX (study 3). Rats were divided into two groups: a control group (normal diet) and SPL group (0.05% spironolactone-mixed diet). The treatment schedule was the same in study 2. As shown in Fig. 9, spironolactone did not decrease proteinuria in MI rats after NX. When we compared urinary volume between the two groups at week 11, the SPL group showed greater urinary volume excretion than the control group (control: 9.8 ± 0.6, n = 7; SPL: 13.3 ± 0.6 ml/day, n = 6, P < 0.01). As spironolactone is known to be a diuretic, the dose used in this study was sufficient to produce pharmacological effects.

DISCUSSION

In this study, MI surgery alone induced a reduction of cardiac function, but did not affect renal function or histology compared with control rats. NX alone caused a moderate degree of renal injury, as evidenced by a mild increase in urinary protein excretion, compared with rats subjected to sham or MI surgery. Combined MI and NX notably induced progressive renal injury compared with the other experimental groups, but did not induce further cardiac dysfunction compared with rats that underwent MI alone, at least during the experimental period we employed. Moreover, cardiac contraction was markedly inhibited at 2 wk after MI surgery (on the basis of M-mode echocardiography; data not shown), and proteinuria was evident at 10 wk or later after surgery, indicating that cardiac dysfunction clearly preceded the onset of renal injury in rats subjected to MI+NX. According to Ronco’s classification (41), among the five types of CRS in humans, there are two chronic forms: type 2 and type 4 CRS. Type 2 CRS involves chronic cardiac dysfunction causing progressive chronic kidney disease, whereas type 4 is a state of chronic kidney disease contributing to an increased risk of adverse cardiovascular events. Recently, it has been suggested that when cardiovascular disease precedes the onset of chronic kidney disease, patients should be classified as having type 2 CRS (3). Therefore, our rat model subjected to both MI and NX was considered to be a model for type 2 CRS, at least during the observation period we employed.

The model used in this study was first established by van Dokkum’s group (49). Subsequently, they observed amelioration of renal injury upon treatment with an ACE inhibitor and concluded that the RAS was involved in the pathogenesis of the progressive kidney injury in this model (54). However, in their study, the ACE inhibitor also caused a 30% reduction of SBP just after administration, relative to the pretreatment value, and this hypotension continued during the treatment period. They also observed that the ACE inhibitor improved cardiac parameters such as heart weight, LVEDP, left ventricular end-systolic pressure (LVESP), and +dP/dt max at the end of the experimental period compared with vehicle-treated rats.

Fig. 7. Blockade of AT1R signaling reduces progressive proteinuria in the present model of type 2 cardiorenal syndrome. Rats with MI+NX were assigned to 2 groups: a vehicle group (0.5% methylcellulose po) and OLM group (olmesartan medoxomil, 5 mg·kg\(^{-1}\)·day\(^{-1}\) po). Administration was started at 2 wk after MI surgery. Week 0 indicates the time when administration was started. A: 24-h urinary protein excretion was measured at the indicated time points. Values are means ± SE; n = 7–11/group. *P < 0.05 and **P < 0.01 compared with control group. B: SBP was measured by the tail-cuff method at 12 wk after the start of administration. Values are means ± SE; n = 5 for the vehicle group and n = 6 for the OLM group. N.S., not significant. C: left ventricular passive-volume curves were obtained at the end of the study using isolated hearts. The data from age-matched animals without surgery (the intact group) are also shown. The curves show a significant rightward shift in both the vehicle and OLM groups compared with the intact group (P < 0.01). No significant difference was evident between the vehicle and OLM groups. Values are means ± SE; n = 3 for the intact group, n = 6 for the vehicle group, and n = 6 for the OLM group.
Although these data suggested involvement of the RAS in renal injury, the possible contribution of hypotension and improvement of cardiac parameters could not be excluded. In the present study, we observed that combined MI and NX induced upregulation of the renal AT1R gene and protein, along with proteinuria and renal injury, and downregulation of PRA. Moreover, treatment with olmesartan medoxomil was dramatically effective in reducing both proteinuria and renal injury of Fig. 8. Blockade of AT1R signaling ameliorates histopathological alterations in the present model of type 2 cardiorenal syndrome. Kidney sections were stained with hematoxylin-eosin. Typical histopathological images in the vehicle (A and C) and OLM (B and D) groups are shown. Scale bars = 50 μm. Infiltration of mononuclear cells (A) and adhesion of the glomerulus to Bowman’s capsule (C) were evident in the vehicle group, and these changes were ameliorated in the OLM group. E: severity of infiltration of mononuclear cells was microscopically semiquantified, and the summarized data are shown. In each group, the specimens from 6 animals were evaluated.

Table 2. Effects of olmesartan medoxomil on cardiac function and MI size in rats that underwent both NX and MI surgery

<table>
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<th>Parameter</th>
<th>Vehicle</th>
<th>OLM</th>
<th>P Value</th>
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<tr>
<td>HR, beats/min</td>
<td>373 ± 6</td>
<td>377 ± 6</td>
<td>0.641</td>
</tr>
<tr>
<td>LVSP, mmHg</td>
<td>124 ± 5</td>
<td>110 ± 4</td>
<td>0.069</td>
</tr>
<tr>
<td>+dP/dt, mmHg/s</td>
<td>7,506 ± 446</td>
<td>6,402 ± 434</td>
<td>0.112</td>
</tr>
<tr>
<td>−dP/dt, mmHg/s</td>
<td>−5,715 ± 390</td>
<td>−5,060 ± 395</td>
<td>0.273</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>17.8 ± 4.4</td>
<td>19.8 ± 5.4</td>
<td>0.780</td>
</tr>
<tr>
<td>Δ+dP/dt, mmHg/s</td>
<td>1,614 ± 330</td>
<td>1,278 ± 377</td>
<td>0.528</td>
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<tr>
<td>Δ−dP/dt, mmHg/s</td>
<td>−1,277 ± 287</td>
<td>−1,153 ± 239</td>
<td>0.746</td>
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<tr>
<td>Myocardial infarction size</td>
<td>38.9 ± 2.9</td>
<td>38.4 ± 1.6</td>
<td>0.879</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5–6/group. OLM, olmesartan medoxomil. The infarct size was expressed as a ratio of the sum of infarct length relative to the entire left ventricular length. All parameters were measured at week 16.
rats after both MI and NX without either hypotension or improvement in cardiac parameters. In contrast to the effect of ARB, proteinuria was unaffected by spironolactone. Our data are the first to demonstrate that renal activation of AT1R is an important cause of renal injury in a model of type 2 CRS and that this seems to be independent of systemic RAS activation and aldosterone signaling.

Renin is the first, and rate-limiting, enzyme of the RAAS, and PRA has been reported to increase in heart failure after MI due to renal hypoperfusion (45). In this study, PRA showed a tendency to be high in rats after MI alone, but was rather reduced in rats with MI+NX. It has been shown that there is a feedback mechanism for renin release through the direct intrarenal action of ANG II (27). The present PCR and immunohistochemical studies suggested activation of the renal RAS in rats after MI+NX. Therefore, the reduced PRA level in rats with MI+NX may be due to a feedback mechanism. Furthermore, it has been reported that NX prevents the increase in PRA induced by renal ischemia through reduced renal cortical renin content (26), and therefore this mechanism may also play a role in the reduction of the PRA level in this model.

The results of the present study clearly indicated that renal activation of the RAS is involved in renal injury in rats after combined MI and NX surgery. The mechanism responsible for activation of the renal RAS under these experimental conditions is an important issue. Although the present study was unable to address this question, the findings of several previous investigations may be informative. Using the same MI+NX animal model, van Dokkum et al. (49) observed that the degree of proteinuria increased in parallel with MI size. MI is known to cause heart failure, leading in turn to hypoperfusion of the kidney, and thereby systemic activation of the RAS (45, 49). It has also been shown that ANG II infusion causes renal mononuclear cell infiltration and that the mononuclear cells contain ACE, renin, renin receptors, angiotensinogen, and AT1R (10, 25, 35, 39, 40, 46, 50). On the basis of these observations, it is thought that in our model the RAS is systemically activated immediately after MI surgery and that this in turn triggers renal mononuclear cell infiltration. Subsequently, the systemically activated RAS returns to its original level or drops even lower because of the aforementioned feedback mechanism and also nephrectomy. Thereafter, the mononuclear cells in the kidney self-activate the RAS and/or activate the renal RAS components. The degree of this activation is sufficient to injure the kidney in NX rats, but not in rats retaining both kidneys. Studies to investigate the time courses of systemic and renal activation of the RAS after MI alone, NX alone, or their combination would be helpful for clarifying this putative mechanism.

It has been reported that olmesartan medoxomil is effective in rats with MI (43). However, in this study we observed no beneficial effect of olmesartan medoxomil on cardiac parameters in rats with MI+NX. The reason for this discrepancy is currently unclear. However, the administration regimen we employed in this study was quite different from that of the other study. Here, drug administration was started 2 wk after MI surgery, whereas in the other study it was started 1 wk before surgery. In general, pretreatment with drugs is known to be more effective than posttreatment, and this may have been one reason for the discrepancy. Other factors, including differences in the number of kidneys, handling of rats, feeding regimes, and intestinal microbiota, may also have contributed to the difference.

In the present study, combined MI and NX led to proteinuria and an increased level of renal AT1R mRNA. Our immunohistochemical study clearly showed that AT1R protein was increased mainly in the renal interstitium, especially in infiltrating mononuclear cells. Previously, it has been reported that in animal models of kidney injury accompanied by proteinuria, such as % nephrectomy (15) and chronic inhibition of nitric oxide synthesis (16), AT1R is overexpressed in the renal interstitium, particularly in areas of inflammation. Furthermore, losartan, an ARB, is effective against renal injury in these animal models. Although these animals exhibited progressive hypertension, unlike the present model, the overall data suggest that activation of AT1R in the renal interstitium

Table 3. Primer sequence for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1aR</td>
<td>CCGACTCAAGCTGTCTAACGA</td>
<td>GTGCTTTTGACCTGTCACTCC</td>
</tr>
<tr>
<td>AT2R</td>
<td>CTGGAATGGTTGACCTGGAGT</td>
<td>AGGCTTTTACAACGGAGAATAC</td>
</tr>
<tr>
<td>Renin</td>
<td>AGGCAGGACCTCACTGGGCA</td>
<td>ATGGTGGTGAAGACGCCAGTA</td>
</tr>
<tr>
<td>IL-1β</td>
<td>GCTGTGAGGGATACATCGCGTG</td>
<td>AGGTGCTGCAATCCGACAGAG</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>TGCGCTGCAAGATGGCAAAG</td>
<td>AGGTAAAGCGAAGATGTGCTA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GGGCGAGTCAAGGCTGAGAATG</td>
<td>ATGGTGTTGAAAGGCCAAGTA</td>
</tr>
</tbody>
</table>

AT1aR, angiotensin II type 1a receptor; AT2R, angiotensin II type 2 receptor; ACE, angiotensin-converting enzyme; TGF-β1, transforming growth factor-β1.
may be an important cause of proteinuria. Interestingly, a recent human study has shown that renal interstitial expression of AT1R is increased in patients with proteinuria due to progressive glomerulopathies (4), thus supporting this notion.

In addition to mononuclear cell infiltration, disturbances of blood flow in the peritubular capillaries of the renal interstitium and resulting tissue oxygenation have been reported to be involved in proteinuria. Furthermore, RAS inhibition has been reported to restore blood flow and improve renal oxygenation (34). Given that AT1R is intensely expressed in the renal vasculatures (30), inhibition of RAS in the efferent glomerular arteriolar by treatment with ARB could exert renoprotective effects in rats with MI+NX through an increase in blood supply to the downstream interstitium. In future studies, blood flow in the peritubular capillaries of the renal interstitium should be determined in rats with NX+MI.

The present study did not address the types of cells involved in the increase in AT1R in the renal interstitium. Several types of inflammatory cells expressing ANG II and/or AT1R, such as lymphocytes and macrophages, have been suggested to infiltrate the renal interstitium in renal injury models (10, 25, 32, 35, 39, 40, 46, 50). Among these inflammatory cells, studies with mice lacking lymphocytes have suggested a pathogenetic role of T lymphocytes in ANG II–induced tissue injury, including the kidney (9, 17), while the results obtained with AT1R-deficient mice have suggested a protective role of macrophages against kidney injury resulting from unilateral ureteral obstruction (35). On the other hand, in recent years, evidence has emerged to suggest that specific macrophage phenotypes are involved in the pathobiology of renal injury. For example, it has been thought that M1 macrophages exacerbate renal cell damage, M2c macrophages promote epithelial and vascular repair, and M2a macrophages accelerate fibrogenesis (2). To further examine the mechanisms underlying renal injury in rats after MI+NX, future studies will need to assess the types of infiltrating AT1R-positive mononuclear cells.

In our study, although it did not reach significance, the level of AT2R mRNA tended to be increased in the NX+MI group compared with the control group. Furthermore, we also showed that ARB was effective in ameliorating the progression of renal injury in MI+NX rats. It has been thought that the beneficial effect of ARB is largely linked to blockade of AT1R action. Hypothetically, however, an increase in the level of unbound ANG II after AT1R blockade, being diverted to and activating the AT2R, could also be contributory. In fact, Naito et al. (33) have shown that the beneficial effect of ARB on renal injury induced by % nephrectomy is attributable to not only blockade of the AT1R but also an increase in the effect of ANG II mediated via the AT2R. In this study, therefore, olmesartan medoxomil might have exerted its beneficial effect on renal injury in rats with MI+NX partly through activation of AT2R. In contrast, however, Cao et al. (8) using the same % nephrectomy animal model have reported that blockade of the AT2R alone confers a degree of renal protection. In future studies, it will be necessary to clarify the extent to which AT2R activation by treatment with ARB contributes to renal protection in rats with MI+NX.

In the present study, we administered olmesartan medoxomil to rats at a dose of 5 mg/kg, which is higher than the daily therapeutic dose in humans (40 mg·body wt $^{-1}$·day$^{-1}$). In rats, however, this dose is not extremely high, because the serum concentration becomes equivalent to that in humans (maximum concentration 850 ng/ml in rats administered 5 mg/kg or in humans administered 40 mg/body wt; respective areas under the curve during 24 h: 4,900 and 5,200 ng·h$^{-1}$·ml$^{-1}$). In addition, we did not observe any serious adverse effects after administration of this dose of olmesartan medoxomil to rats. Therefore, it is unlikely that clinically irrelevant blood concentration of olmesartan medoxomil causes renoprotection observed in the present study.

The findings in our animal model suggest that living kidney donors and patients with one kidney could have a greater risk of renal damage after cardiovascular events than those with two kidneys. So far, to our knowledge, no published study has investigated this issue. On the other hand, one reported study has examined whether cardiovascular events are increased after kidney donation (14). The results of that study suggested that the risk of cardiovascular disease was unchanged in the first decade after kidney donation and that living kidney donors were more frequently diagnosed as having hypertension, probably due to nephrectomy. Since hypertension itself is a well-known risk factor for kidney injury, the available data indicate that it may be difficult to interpret the findings of any study examining the risk of progressive kidney disease after cardiovascular events in humans with one kidney.

Although there is plenty of clinical evidence for the interaction between kidney and cardiovascular diseases, clinical studies of type 2 CRS have been very limited (1, 3, 12, 19–22, 29, 41, 42, 49). However, among the studies available, the CATS randomized trial has examined the effect of RAS inhibition by captopril, an ACE inhibitor, in patients with type 2 CRS. The trial population comprised 298 patients with a first anterior wall MI (22). Renal function, as judged by glomerular filtration rate, declined by 5.5 ml·min$^{-1}$·yr$^{-1}$ in the placebo group but by only 0.5 ml·min$^{-1}$·yr$^{-1}$ in the ACE inhibitor group. This suggests the pathogenetic role of the RAS in patients with type 2 CRS, thus supporting our data obtained with this animal model and, furthermore, indicating that blockade of the ANG II signal would be a therapeutic or preventive strategy against type 2 CRS. To verify this possibility, further clinical evaluations in humans will be required.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: T.H., T.S., and M.I. provided conception and design of research; T.H., H.S., M.M., and M.I. analyzed data; T.H., H.S., M.M., and M.I. interpreted results of experiments; T.H., H.S., and M.I. prepared figures; T.H., M.M., and M.I. performed experiments; M.I. edited and revised manuscript; M.I. approved final version of manuscript.

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