博士論文

The study of the mechanism of progressive renal injury in chronic cardiorenal syndrome, and the effects of mineralocorticoid receptor blockers on glucose tolerance

慢性心腎症候群における進行性腎傷害メカニズムに関する研究、及び、ミネラロコルチコイド受容体拮抗薬の耐糖能に対する作用

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Contents

General introduction ·····1

Chapter I:

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

(1) Introduction ·····8

(2) Materials and Methods

(i) Experimental materials ·····11

(ii) Experimental protocol and surgical procedures ·····11

(iii) Functional parameters ·····12

(iv) Analyses of blood and urine samples ·····13

(v) Real-time PCR ·····13

(vi) Myocardial infarct size and renal histology evaluation ·····14

(vii) Statistical analysis ·····15

(3) Results

(i) Basic characterization of model rats with myocardial infarction after unilateral nephrectomy ·····16

(ii) Renal histology ····· 18

(iii) Evaluation of renal inflammation and fibrosis ····· 18

(iv) Plasma renin activity and expression of rennin-angiotensin system-related genes in the kidney ····· 18

(v) Immunohistochemical localization of AT1aR in the kidney of MI+NX ····· 19

(vi) Effect of olmesartan medoxomil on the renal injury in MI+NX ·· 20

(vii) Effects of spironolactone on the renal injury in MI+NX ····· 21

(4) Discussion ····· 23

Tables and Figures ····· 32

Chapter II:

Spironolactone, but not eplerenone, impairs glucose tolerance in a rat model of metabolic syndrome

(1) Introduction ····· 55

(2) Materials and Methods

(i) Experimental materials ····· 58

(ii) Experimental protocol ····· 58

(iii) Measurement of urinary parameters and blood chemical parameters ····59
(iv) Oral glucose tolerance test ····60
(v) Statistical analysis ····60

(3) Results
(i) Increase of uNa^+/K^+ ratio in ND-SPL100 is similar in extent to that in ND-EPL ····61
(ii) SPL, but not EPL, increases the blood glucose level and improves lipid profiles in ND rats ····61
(iii) SPL, but not EPL, induces impairment of glucose tolerance in ND rats ····62
(iv) SPL does not affect serum insulin or adiponectin concentration in ND rats ····63
(v) SPL increases the serum aldosterone concentration and reduces the serum corticosterone concentration in ND rats ····63

(4) Discussion ····65

Tables and Figures ····70

Further discussion ····82

Conclusion 86

References 87

Summary 100

Acknowledgements 105

General introduction:

Cardiovascular disease is represented as a group of disorders of the heart and blood vessels, such as coronary heart disease, cerebrovascular disease, and peripheral arterial disease. Although a number of drug classes and individual agents have been developed and are available as treatment to improve various risk factors of cardiovascular disease, the prevalence of these risk factors, including hypertension, diabetes mellitus, hyperlipidemia, and obesity, has remained largely unchanged and in some cases has even been increasing. For the development of appropriate therapeutic strategies for each patient (e.g. Which agent or which combination is the best for the treatment of each disease? When should the medication be initiated?), it is important to know in detail about both the pathophysiology of each disease and the features of each drug.

For a long period, heart and kidney diseases have been recognized as independent disease states, and therefore, each disease has been treated separately. However, a number of reports have demonstrated a closed linkage between cardiac and renal dysfunction, and we have realized that the therapeutic strategies for heart or kidney diseases must be developed in consideration of each other. This interaction between heart and kidney termed the cardio-renal syndrome (CRS) is recently a well-recognized and -accepted definition in clinical (1, 2). As shown in Figure 1, Ronco et al. 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 known as 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. To clarify the pathophysiological mechanisms underlying each subtype will help to understand the clinical derangement and provide the rationale for management strategies for the patients with cardiac or renal dysfunction.

The renin-angiotensin-aldosterone system (RAAS) has been well-known as a risk factor of both cardiovascular diseases and renal injuries, and in fact, a number of reports have demonstrated that the abnormal activation of RAAS could be a cause of hypertension (3), atherosclerosis (4), and various forms of cardiovascular and renal injury (5). As shown in Figure 2, renin, which is the first rate-regulating enzyme of RAAS, cleaves angiotensinogen to generate the inactive peptide angiotensin I (Ang I). The angiotensin converting enzyme (ACE) converts Ang I to the physiologically active peptide angiotensin II (Ang II). Ang II can interact with at least 2 receptors, Ang II type 1 receptor and type 2 receptor (AT1R and AT2R, respectively). Most of the pathological actions of Ang II are considered to be mediated by AT1R. Ang II binding to AT1R, induces vasoconstriction,

oxidative stress, inflammation, and sodium and fluid retention, causing blood pressure elevation and organ damages (heart, vessel, kidney, etc.). Although many of the AT2R functions remain unknown, activation of AT2R may elicit counterregulatory effects to those mediated by the AT1R on cardiovascular tissue (6, 7).

Ang II is also known as a strong inducer of aldosterone production, a physiological ligand of the mineralocorticoid receptor (MR). Aldosterone is classically recognized as a major regulator of electrolyte balance, enhancing sodium reabsorption and potassium excretion, through the activation of MR in renal tubular epithelial cells. In addition to its role in tubular epithelia, a growing body of evidence suggests that aldosterone is directly involved in organ damage in the vasculature (heart, vessel, kidney, etc.) via enhancement of oxidative stress (8), inflammation (9), and fibrosis (10).

The impairment of glucose metabolism is known to be a major risk of cardiovascular diseases. RAAS has also been reported to be associated with the impairment of glucose metabolism (11). Although the mechanisms remain to be fully elucidated, both AT1R and MR activation contribute to downstream signaling pathways that attenuate the signaling mechanisms of insulin, which is a main regulator of blood glucose, and induces insulin resistance (12, 13) in the heart, vasculature, liver, and skeletal muscle, associated with the increased production of reactive oxygen species and oxidative stress (14).

Therefore, the blockade of RAAS at various steps is an attractive

therapeutic strategy for patients with cardiovascular disease risks, and 4 groups of RAAS blockers, including direct renin inhibitor (DRI), ACE inhibitor, AT1R blocker (ARB), and MR blocker, which are currently available in the clinical setting as antihypertensive/cardiorenal protective agents (Figure 1). In fact, a number of clinical studies using these RAAS blockers have demonstrated some beneficial effects on hypertension, acute myocardial infarction, chronic systolic heart failure, stroke and diabetic renal disease (15). To clarify the pathophysiology of diseases and the features of drug, I focused ARB and MR blocker in my research, and the effects of drugs in these groups on various parameters were evaluated,

Olmesartan medoxomil (OLM) is a best-in-class of ARB, which has been developed by Daiichi Sankyo Co., Ltd. (16), and a number of reports have demonstrated its notable antihypertensive (16), cardioprotective (17, 18), renoprotective (19) and antiatherosclerotic effects (20). Spironolactone (SPL) and eplerenone (EPL), both of which are MR antagonists currently used in a clinical setting, are widely recognized to be beneficial for patients with hypertension and heart failure (21-23). A growing body of evidence suggests that MR antagonists also exert a renoprotective effect in type 2 diabetes independently of the antihypertensive effect (24-27). These results suggest the involvement of RAAS in some types of CRS, however, few reports have focused on this point in the animal study.

In Chapter I, to elucidate the detail of the mechanism of CRS, I created the rat model combining myocardial infarction with unilateral

nephrectomy, and confirmed the potencies as a CRS model by the measurement of several cardiac, renal and neurohumoral parameters. In addition, the effects of OLM and SPL on the pathology in this model were also examined.

Both SPL and EPL are MR antagonists with a similar steroid structure, but they are known to have distinct pharmacological/pharmacokinetic profiles; EPL shows greater MR selectivity instead of lower MR blocking potency and has non-genomic properties, and SPL has active metabolites (28). However, there are few studies to compare two drugs directly, and pharmacological differences remains to be completely clarified. Recently, Yamaji et al. have shown that SPL, but not EPL, increases HbA1c in patients with mild chronic heart failure (29). Although the mechanism remains to be elucidated, blood glucose elevation is a major risk of cardiovascular disease. Therefore, I considered that it was important to clarify the mechanism in detail.

In Chapter II, to elucidate the effect of MR antagonists on blood glucose, I administered SPL or EPL to metabolic syndrome rats and examined several blood parameters.

CRS	Primary disorders	Secondary disorders
Type 1	Acute heart disease	Acute renal injury
Type 2	Chronic heart disease	Progression of Kidney damage
Type 3	Acute kidney injury	Acute heart dysfunction
Type 4	Chronic kidney disease	Progression of cardiac damage
Type 5	Systemic diseases Diabetes etc..	Heart failure Renal insufficiency

Figure 1. Cardiorenal syndrome

Cardiorenal syndrome (CRS) is categorized into five types according to the pathophysiology, time-frame, and nature of the concomitant cardiac and renal dysfunction.

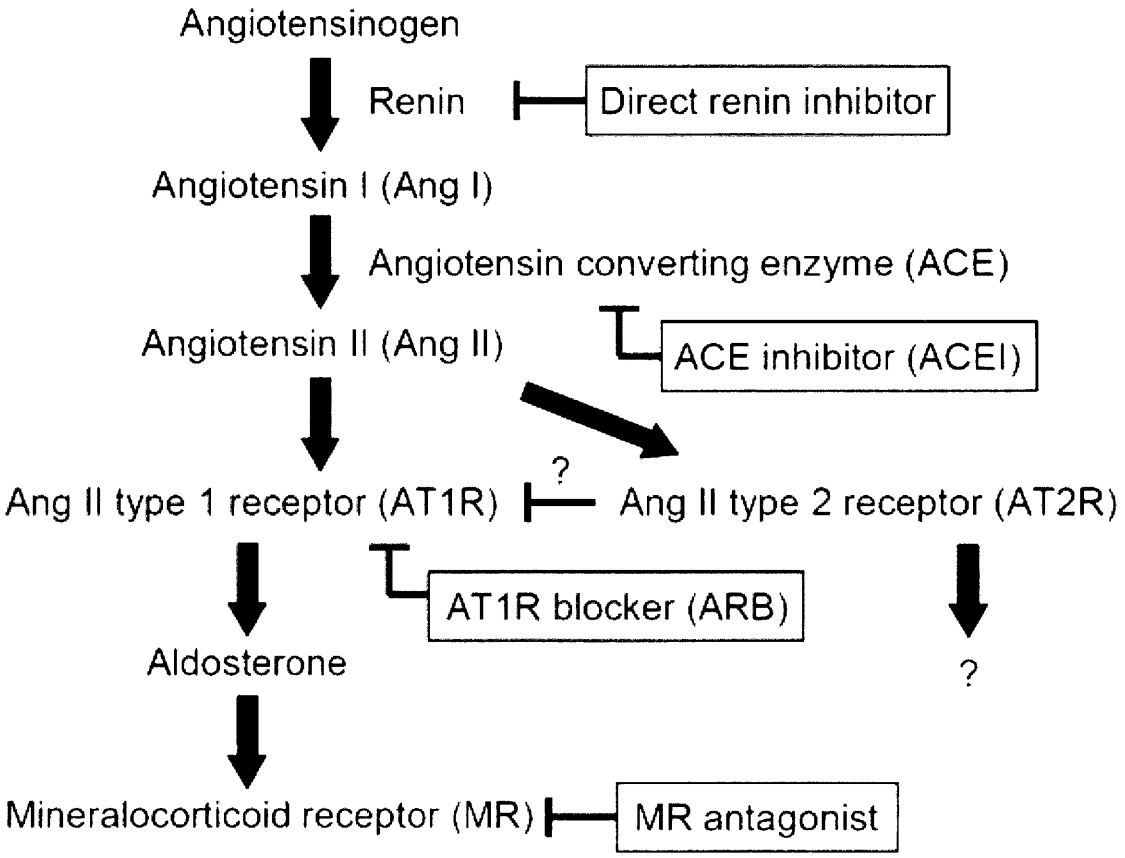

Figure 2. Renin-angiotensin-aldosterone system

The renin-angiotensin-aldosterone system (RAAS) and clinically available classes of agents that can block RAAS.

Chapter I:

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

(1) Introduction

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, 30-38). These findings have led to the recognition that there is an interaction between heart and kidney diseases, a condition referred to as CRS (1, 38, 39). Ronco et al. categorized CRS into five types according to the pathophysiology, time-frame, and nature of the concomitant cardiac and renal dysfunction (1, 39). 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 known as 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 (1, 36).

The RAAS is well known to play a role in the pathogenesis of both cardiac and renal injury (40-42). Blockade of the RAAS using ACEI and ARB effectively attenuates both forms of injury (3, 5, 43-45). Moreover, accumulating evidence indicates that aldosterone, an agonist for the MR, plays a pathogenetic role in tissue injuries that are characterized by fibrosis, inflammation, and oxidative stress (46-48), and that MR antagonists exert protective actions on both the heart and the kidney (21, 22, 23, 49).

The van Dokkum group has shown that surgically induced myocardial infarction (MI) after unilateral nephrectomy (NX) results in progressive renal injury in rats, reproducing the features of patients with Type 2 CRS (36). Subsequently, based on results obtained using an ACE inhibitor, they suggested that the renin-angiotensin system (RAS) was involved in the pathogenesis of the progressive renal injury (50). In that study, however, the ACE inhibitor caused a dramatic decrease in blood pressure and an improvement of cardiac parameters. Therefore, at present, the pathogenetic role of RAS in that model is still unclear. Furthermore, the contribution of aldosterone to the pathogenesis in that model has yet to be clarified.

In order to reveal some of the detail of the mechanism of renal injury in an animal model corresponding to human Type 2 CRS, I 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.

(2) Materials and Methods

(i) Experimental materials

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

(ii) Experimental protocol and surgical procedures

All experiments were carried out in accordance with *the Animal Experimentation Guidelines of Daiichi-Sankyo Co., Ltd., and the Law Concerning the Protection and Control of Animals* (Japanese Law No. 105, October 1, 1973, revised on June 22, 2005), *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).

Male Slc:Wistar rats (6 weeks of age) were purchased from Japan SLC Inc. (Shizuoka, Japan). The rats underwent surgical procedures for unilateral nephrectomy at 7 weeks of age and myocardial infarction at 8 weeks of age. At two weeks after the myocardial infarction surgery, rats were assigned to 4 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 OLM groups in Study 2 or Control

and SPL groups in Study 3. Under anesthesia with sodium pentobarbital (40 mg/kg, i.p.), rats were subjected to unilateral right nephrectomy after ligating 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 (LAD). Briefly, rats under anesthesia were intubated, connected to a ventilator (SAR-830/AP Small Animal Ventilator, CWE Inc., The Netherlands), and underwent left-sided thoracotomy. The LAD was ligated about 3 mm from its origin with an 8-0 silk suture. The sham group only underwent left-sided thoracotomy.

(iii) Functional parameters

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

At the end of the study (Week 36 in Study 1 and Week 16 in Study 2), a pressure transducer (Micro-Tip 3French; Millar Instruments, Houston, TX) was inserted from the right carotid artery for the measurement of cardiac function under sodium pentobarbital anesthesia. After stabilization, the left ventricular systolic pressure (LVSP), LVEDP, heart rate (HR) and ±dP/dt were recorded using a polygraph system (Nihon Kohden Corporation, Tokyo, Japan). To determine cardiac reserve, changes of 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 in 10% neutral buffered-formaldehyde solution (Wako Pure Chemical Industries Ltd., Japan) for histopathological examination and another piece was stored at -80°C. For the heart, the left and right ventricular weights were measured and the left ventricle was also fixed in 10% neutral buffered-formaldehyde solution for measurement of the myocardial infarct size. In Study 2, before fixation, the LV pressure-volume relationship was obtained using isolated hearts as described previously (51).

(iv) Analyses of blood and urine samples

ANP and BNP (DRG Instruments GMBH, Germany), and PRA (Renin RIA beads, TFB Inc., 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 an Aution Master system (UM-3410; ARKRAY Inc., Kyoto, Japan).

(v) Real-time PCR

Total RNA was isolated from frozen kidney using an RNeasy Plus Mini Kit (QIAGEN, Tokyo, Japan). Five μg 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 Inc., Shiga, Japan). The reverse-transcribed cDNA was mixed in a final volume of

20 μL with specific primers as shown in Table 1. GAPDH was used as an internal control. Real-time PCR and data analyses were performed using a 7900HT system (Applied Biosystems, Life Technologies).

(vi) Myocardial infarct size and renal histology evaluation

For measurement of the myocardial infarct size, the hearts were cut into five transverse slices. The images of the sections were captured and digitized, and then the lengths of the infarcted wall and non-infarcted wall (epicardial surface) were measured in 4 slices through the apical side using Photoshop 4 (Adobe Systems Inc.). The infarct size was expressed as a ratio of the sum of infarct length relative to the entire LV length. The infarcted myocardium was identified by the histology of the scar tissue.

Histological and immunohistochemical analyses of the kidneys were performed as described previously (52). The kidneys were fixed in a 10% formaldehyde solution, and then the tissue was paraffin-embedded, cut into 2 μm thick, deparaffinized, and rehydrated. Renal sections were stained with hematoxylin and eosin (HE) or periodic acid–Schiff (PAS). To determine the incidence of glomerulosclerosis (FGS), 50 glomeruli in each PAS-stained section were examined, and the severity of glomerulosclerosis was scored into 4 grades (0-3) in a blinded manner. The degree of mononuclear cell infiltration was quantified in areas across the kidney specimen, as described previously (53). 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-AT1R antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and immunoreactivity was visualized using Alexa Fluor® 488 anti-rabbit IgG (Molecular Probes, Inc., Eugene, OR). Semiquantitative analysis was performed by measuring the intensity 155 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, more than 10 fields in each section were examined. When a conventional immunohistochemical analysis was performed, endogenous peroxidase was consumed using 3 % H_2O_2 solution, and then immunoreactivity was visualized using Envision System Labelled Polymer reagent (DAKO Japan, Tokyo, Japan), followed by treatment with 3, 3'-diaminobenzidine tetrahydrochloride.

(vii) Statistical analysis

Data are presented as mean ± SE unless specified otherwise. The differences from the Control group were compared by 2-way ANOVA followed by Dunnett's tests for parameters determined in the multi-group study. For comparison between the Vehicle and OLM groups, or the Control and SPL groups, Student's t-test was used. In all tests, $P < 0.05$ was considered statistically significant.

(3) Results

(i) Basic characterization of model rats with myocardial infarction after unilateral nephrectomy

In the first series of experiments (Study 1), rats were divided into 4 groups: the control group (subjected to a double sham operation), MI group (subjected to MI surgery alone), NX group (subjected to NX surgery alone), and MI+NX group (subjected to both MI and NX surgery). At one week after NX or sham operation, MI or sham surgery (2nd surgery) was performed. The time point at two weeks after the 2nd surgery was assigned as Week 0, and I 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 4 groups (control: 467 ± 10, n = 12; MI: 451 ± 179 11, n = 10; NX: 479 ± 8, n = 12; MI+NX: 443 ± 11 g, n = 14), indicating that the procedures employed in this study did not produce any non-specific toxicological effects. Figure 3 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. In order to estimate the renal function, I 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 ± 0.1, n = 12; MI: 0.4 ± 0.1, n = 10; NX: 0.5 ± 0.1, n = 12, $P<0.05$; MI+NX:

0.6 ± 0.2 mg / dl, N = 13, $P<0.001$), but the values were within the normal range, indicating that the renal function was little affected by the surgery.

Figure 4 and 5 show the time course of the heart rate (HR) and systolic blood pressure (SBP), respectively. The HR did not differ among the groups except the control group at Week 12. Only in the MI group, the SBP was significantly lower at Week 23 or later, in comparison with the control group.

Table 2 summarizes the cardiac parameters and myocardial infarction size measured at the end of the observation period (Week 37). A marked decrease in the maximum rate of increase or decrease in left ventricular pressure (±dP/dt) and an increase in left ventricular end-diastolic pressure (LVEDP) were observed in both the MI and MI+NX groups, accompanied by significant increases in the levels of atrial natriuretic peptide (ANP) and B-type natriuretic peptide (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. Comparison between the MI and MI+NX groups revealed virtually no difference in cardiac parameters. Along with cardiac dysfunction, myocardial infarction 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.

(ii) Renal histology

At Week 37 the renal histology was evaluated, and the results are shown in Figure 6 and 7. In comparison with the Control group, there were no significant changes of 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).

(iii) Evaluation of renal inflammation and fibrosis

As renal inflammation and fibrosis were marked in the MI+NX group, I examined the renal expression of mRNAs for interleukin (IL)-1β, an inflammatory cytokine, and transforming growth factor (TGF)-β1, a profibrogenic cytokine (54). As shown in Figure 8 and 9, when compared with in the Control group, the mRNAs of both renal IL-1β and TGF-β1 were significantly up-regulated in the MI+NX group.

(iv) Plasma renin activity and expression of rennin-angiotensin system-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 (50). Therefore we examined plasma renin activity (PRA) and renal expression of mRNAs for RAS-related genes, such as angiotensin II type 1a receptor (AT1aR), Type 2

receptor (AT2R), and renin in the experimental groups. As shown in Figure 10, PRA was significantly lower only in the MI+NX group relative to the controls. On the other hand, the gene expression level of AT1aR in the kidney was significantly higher only in the MI+NX group (Figure 11). Expression of mRNA for both AT2R tended to be increased in the MI+NX group relative to the controls, but not to a significant degree (Figure 12). There was no difference between the groups in the level of renin mRNA (Figure 13).

(v) Immunohistochemical localization of AT1aR in the kidney of MI+NX group

Next, I examined the renal expression level and distribution of AT1R protein using an immunohistochemical technique. As shown in Figure 14, up-regulation of AT1R was clearly observed in the renal cortex in the MI + NX group, relative to the control group (Figure 14, 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 up-regulation in the glomerulus was modest (Figure 14, F and G). Higher magnification (Figure 14E) 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 I carefully observed serial sections from the MI+NX group stained with HE

and anti-AT1R antibody (Figure 15), most 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 (Figure 14H). On the other hand, in the inner medulla, up-regulation 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.

(vi) Effect of olmesartan medoxomil on the renal injury in MI+NX group

The above results suggested the involvement of renal AT1R signaling in the progressive renal injury seen in MI+NX rats. Therefore, I examined the effect of olmesartan, an ARB (16), at a dose that would not be expected to cause hypotension in normotensive rats (55), on proteinuria in the MI+NX group. For this series of experiments (Study 2), rats were divided into two groups: Vehicle group (0.5% methylcellulose, p.o.) and OLM group (olmesartan medoxomil, 5 mg/kg/day, p.o.). At one week after NX, MI surgery was performed. At two weeks after the MI surgery, drug administration was started and continued for 16 weeks, after which necropsy was performed. The start of drug administration was designated as Week 0. As shown in Figure 16, 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 (Figure 18 and 19). On the other hand, when we measured HR and SBP at Week 12, there was no significant difference between the two groups (Figure 20 and 21). Figure 17 and Table 3 show the LV-pressure-volume relationship and cardiac parameters at Week 16, respectively, and neither of the parameters differed significantly different 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 reduced proteinuria was mediated by direct inhibition of renal AT1R signaling.

(vii) Effects of spironolactone on the renal injury in MI+NX rats

Finally, in order to clarify the involvement of aldosterone, I investigated the effect of spironolactone (47) on the proteinuria in MI rats after NX (Study 3). Rats were divided into two groups: Control group (normal diet) and SPL group (0.05% spironolactone-mixed diet). The treatment schedule was the same in Study 2. As shown in Figure 22, the level of proteinuria did not differ significantly between the two groups. When I compared the urinary volume between the two groups at Week 11, the SPL group showed greater urinary volume excretion than the Control group (the Control group: 9.8 ± 0.6, n = 7; the SPL group: 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.

(4) Discussion

In this study, MI surgery alone induced a reduction of cardiac function, but did not affect the renal function or histology in comparison with the control rats. NX alone caused a moderate degree of renal injury as evidenced by a mild increase in urinary protein excretion, in comparison with rats subjected to sham or MI surgery. Combined MI and NX notably induced progressive renal injury in comparison with the other experimental groups, but did not induce further cardiac dysfunction in comparison with rats that underwent MI alone, at least during the experimental period I employed. Moreover, cardiac contraction was markedly inhibited at two weeks after MI surgery (on the basis of M-mode echocardiography; data not shown), and proteinuria was evident at 10 weeks 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 (1), 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 (38). 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 I employed.

The model used in this study was first established by van Dokkum's group (36). 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 (50). However, in their study, the ACE inhibitor also caused a 30% reduction of SBP just after the 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, in comparison with vehicle-treated rats. 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, I observed that combined MI and NX induced up-regulation of the renal AT1R gene and protein, along with proteinuria and renal injury, and down-regulation of PRA. Moreover, treatment with olmesartan medoxomil was dramatically effective in reducing both the proteinuria and renal injury of rats after both MI and NX, without either hypotension or improvement of cardiac parameters. In contrast to the effect of ARB, the 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, rate-limiting enzyme of the RAAS, and PRA has been reported to increase in heart failure after MI due to renal hypoperfusion (56). 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 (57). 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 a reduced renal cortical renin content (58), 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. observed that the degree of proteinuria increased in parallel with MI size (36). MI is known to cause heart failure, leading in turn to hypoperfusion of the kidney, and thereby systemic activation of the RAS (36, 56). It has also been shown that Ang II infusion causes renal mononuclear cell infiltration, and that the mononuclear cells contain

angiotensin converting enzyme, renin, renin receptors, angiotensinogen, and AT1R (59-65). 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 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 rats that have been unilaterally nephrectomized, 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 (66). However, in this study I 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 I employed in this study was quite different from that of the other study. Here, drug administration was started two weeks after MI surgery, whereas it was started 1 week before surgery in the other study. In general, pre-treatment with drugs is known to be more effective than post-treatment, 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 5/6 nephrectomy (67) and chronic inhibition of nitric oxide synthesis (68), AT1R is over-expressed 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 data overall suggest that activation of AT1R in the renal interstitium 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 (69), 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 (70). Given that AT1R is intensely expressed in the renal vasculatures (71), inhibition of RAS in the efferent glomerular arterioles 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 of 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 (59-65, 72). 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 (73, 74), while the results obtained with AT1R-deficient mice have suggested a protective role of macrophages against kidney injury resulting from unilateral ureteral obstruction (61). 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 (75). In order to further examine the mechanisms underlying the 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 AT2 mRNA tended to have increased in the NX+MI group, in comparison with the control group. Furthermore, I also showed that ARB was effective in ameliorating the progression of renal injury in rats MI+NX. It has been

thought that the beneficial effect of ARB is largely linked to the 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. (76) have shown that the beneficial effect of ARB on renal injury induced by 5/6 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. (77) using the same 5/6 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, I 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/day). 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; respective areas under the curve during 24 h: 4,900 ng x h/ml and 5,200 ng x h/ml). In addition, weI 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 that has examined whether cardiovascular events are increased after kidney donation (78). 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, 30-38). However, among the studies available, the CATS randomized trial has examined the effect of RAS inhibition by captopril, an ACE inhibitor, in the 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 / year in the placebo group but by only 0.5 mL / min / year 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. In order to verify this possibility, further clinical evaluations in humans will be required.

Table 1. Primer sequence for real-time PCR

Gene	Sense primer	Anti-sense primer
AT1aR	CCCACTCAAGCCTGTCTACGAA	GTGTGCTTTGAACCTGTCACTCC
AT2R	CTTGGATGCTCTGACCTGGATG	AAGCGGTTTCCAACGAAACAATAC
Renin	AGGCAGGACCTACACTCTCAGCA	ATGAAGGTGGCACCCAGGAC
IL-1β	GCTGTGGCAGCTACCTATGTCTTG	AGGTCGTCATCATCCCACGAG
TGF-β1	TGCGCCTGCAGAGATTCAAG	AGGTAACGCCAGGAATTGTTGCTA
GAPDH	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA

AT1aR: angiotensin II type 1a receptor; AT2R: angiotensin II type 2 receptor;

ACE; angiotensin converting enzyme;

IL-1β: interleukin-1β;

TGF-β1: transforming growth factor-β1;

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

Table 2. Cardiac function, myocardial infarction size and plasma natriuretic peptide concentration in the experimental groups

Parameters	Control	MI	NX	MI+NX
BW (g)	467 ± 10	451 ± 11	479 ± 8	443 ± 11
HR (bpm)	392 ± 17	404 ± 19	397 ± 13	402 ± 19
LVSP (mmHg)	138 ± 5	110 ± 7 **	133 ± 5	124 ± 4
+dP/dt (mmHg/sec)	6120 ± 306	4014 ± 391**	6179 ± 393	4413 ± 251**
-dP/dt (mmHg/sec)	-4619 ± 272	-2891 ± 274**	-4695 ± 308	-3237 ± 197**
LVEDP (mmHg)	3.2 ± 0.7	13.8 ± 3.4*	4.7 ± 1.9	15.1 ± 3.4**
Δ+dP/dt (mmHg/sec)	1691 ± 273	490 ± 127	1938 ± 572	369 ± 90*
Δ-dP/dt (mmHg/sec)	-1110 ± 117	-288 ± 77	-1405 ± 649	-175 ± 50
MI size (%)	-	35.6 ± 1.5	-	32.7 ± 2.5
ANP (pg/mL)	248 ± 57	1108 ± 126**	203 ± 58	1239 ± 113**
BNP (pg/mL)	106 ± 2	198 ± 28**	108 ± 3	213 ± 14**

BW: body weight; 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 values of ±dP/dt after 6 μg/kg dobutamine infusion; MI size: myocardial infarction size; ANP: atrial natriuretic peptide; BNP: brain natriuretic peptide. All parameters were measured at week 36. The values are shown as mean ± SE; n = 10 to 12 in each group. *P <0.05 and **P <0.01 compared with the Control group.

Table 3. Effects of olmesartan medoxomil on cardiac function and myocardial infarction size in rats underwent both unilateral nephrectomy and myocardial infarction surgery

Parameters	Vehicle	OLM	*P*-value
BW (g)	359 ± 18	353 ± 17	0.814
HR (bpm)	373 ± 6	377 ± 6	0.641
LVSP (mmHg)	124 ± 5	110 ± 4	0.069
+dP/dt (mmHg/sec)	7506 ± 446	6402 ± 434	0.112
-dP/dt (mmHg/sec)	-5715 ± 390	-5060 ± 395	0.273
LVEDP (mmHg)	17.8 ± 4.4	19.8 ± 5.4	0.780
Δ+dP/dt (mmHg/sec)	1614 ± 330	1278 ± 377	0.528
Δ-dP/dt (mmHg/sec)	-1277 ± 287	-1153 ± 239	0.746
MI size (%)	38.9 ±- 2.9	38.4 ± 1.6	0.879

BW: body weight; 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 values of ±dP/dt after 6 μg/kg dobutamine infusion; MI size: myocardial infarction size. All parameters were measured at week 16. The values are shown as mean ± SE; n = 5 to 6 in each group.

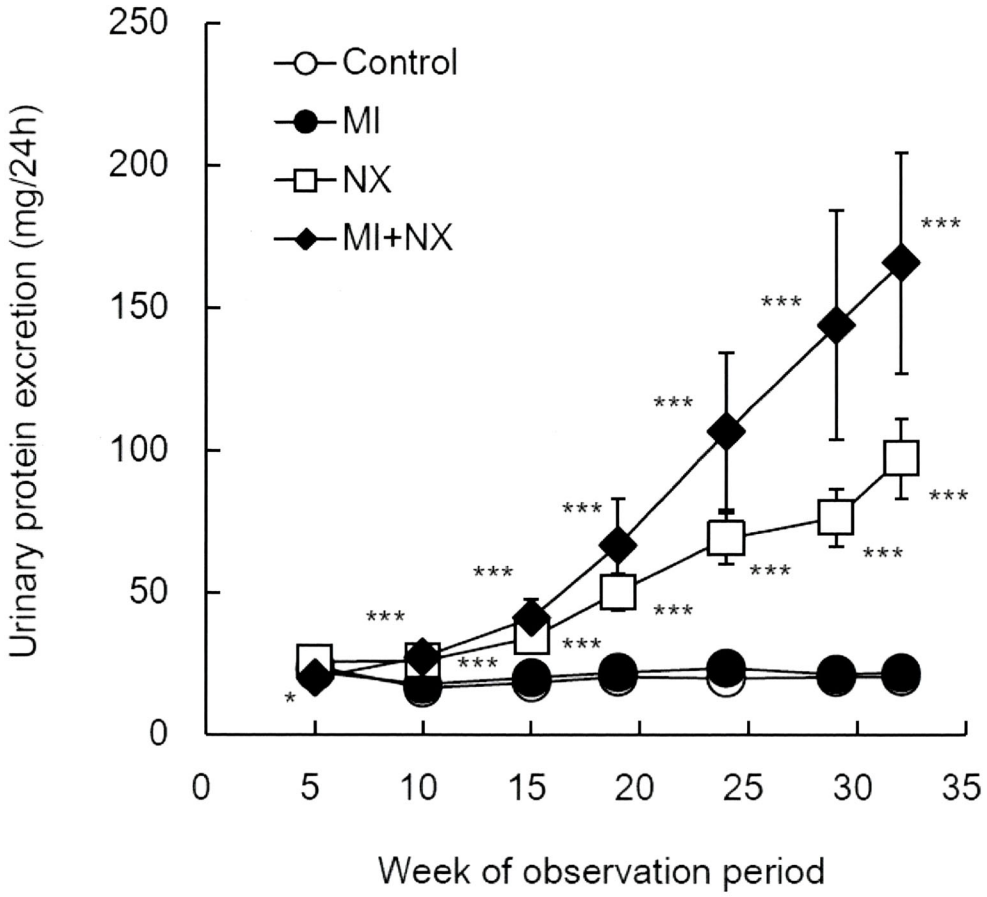

Figure 3. Urinary protein excretion in rats combined NX and MI

Rats were assigned to 4 groups: double sham operation (Control), myocardial infarction (MI), unilateral nephrectomy (NX), and combined surgery (MI+NX). The time point at which MI or sham operation (2nd surgery) was performed was assigned as Week 0. Urinary protein excretion per day is expressed at the indicated time points. The values are shown as mean ± SE; n = 12 to 16 in each group. *P<0.05, **P<0.01, and ***P<0.001 compared with the Control group.

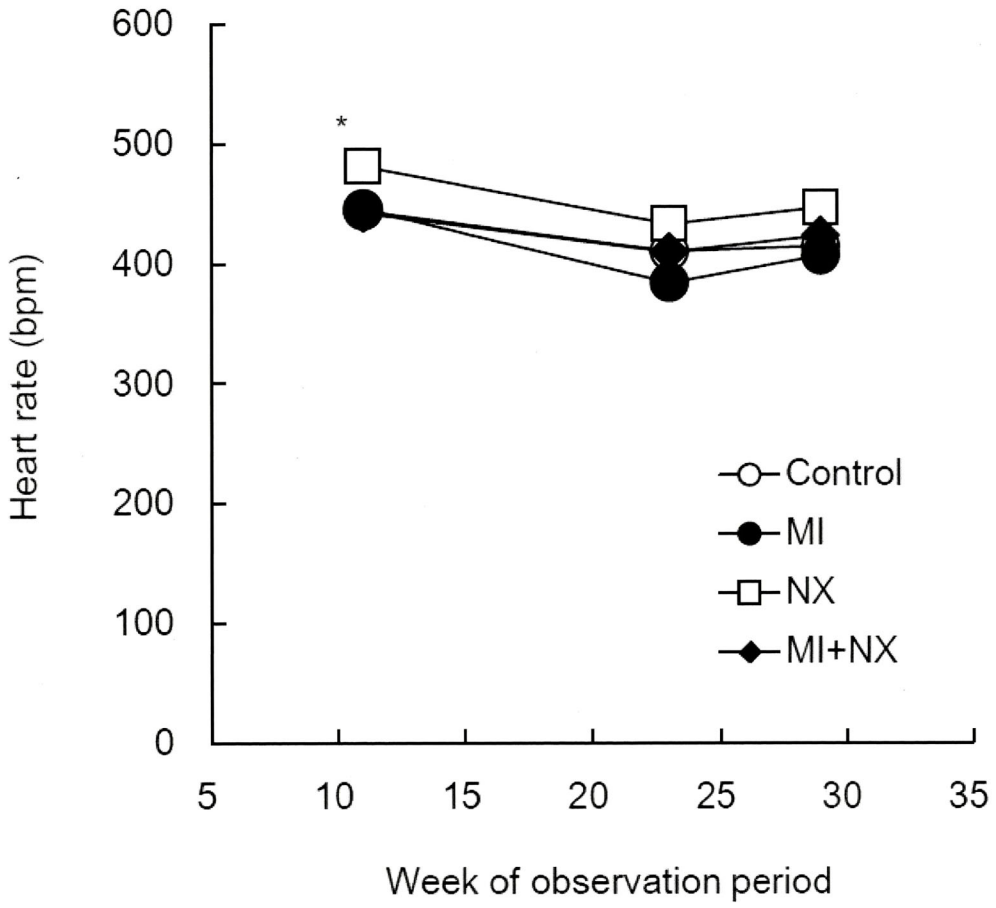

Figure 4. Heart rate in the rats combined NX and MI

Rats were assigned to 4 groups: double sham operation (Control), myocardial infarction (MI), unilateral nephrectomy (NX), and combined surgery (MI+NX). The time point at which MI or sham operation (2nd surgery) was performed was assigned as Week 0. Heart rate measured by tail-cuff method, is expressed at the indicated time points. The values are shown as mean ± SE; n = 12 to 16 in each group. $*P<0.05$ compared with the Control group.

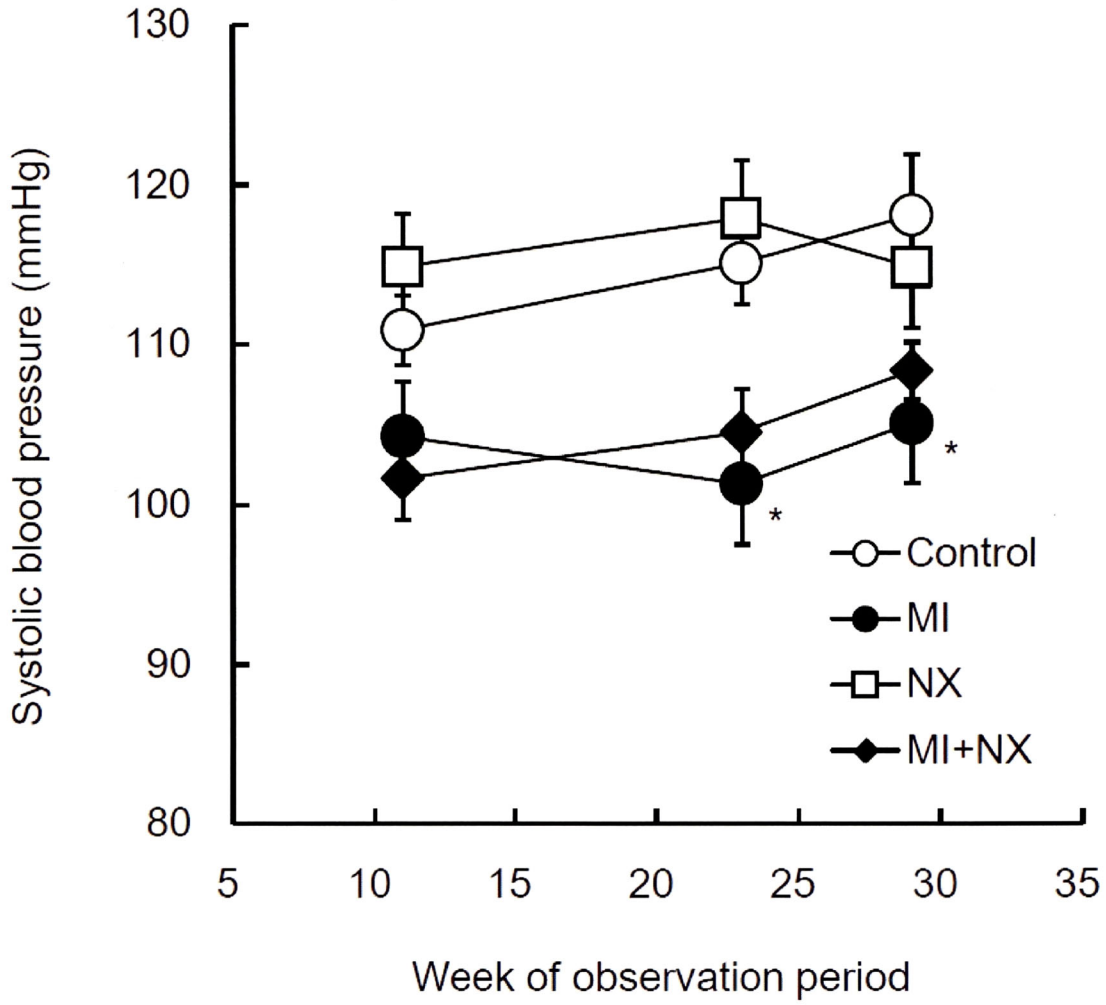

Figure 5. Systolic blood pressure in the rat combined NX and MI

Rats were assigned to 4 groups: double sham operation (Control), myocardial infarction (MI), unilateral nephrectomy (NX), and combined surgery (MI+NX). The time point at which MI or sham operation (2nd surgery) was performed was assigned as Week 0. Systolic blood pressure measured by tail-cuff method, is expressed at the indicated time points. The values are shown as mean ± SE; n = 12 to 16 in each group. $*P<0.05$ compared with the Control group.

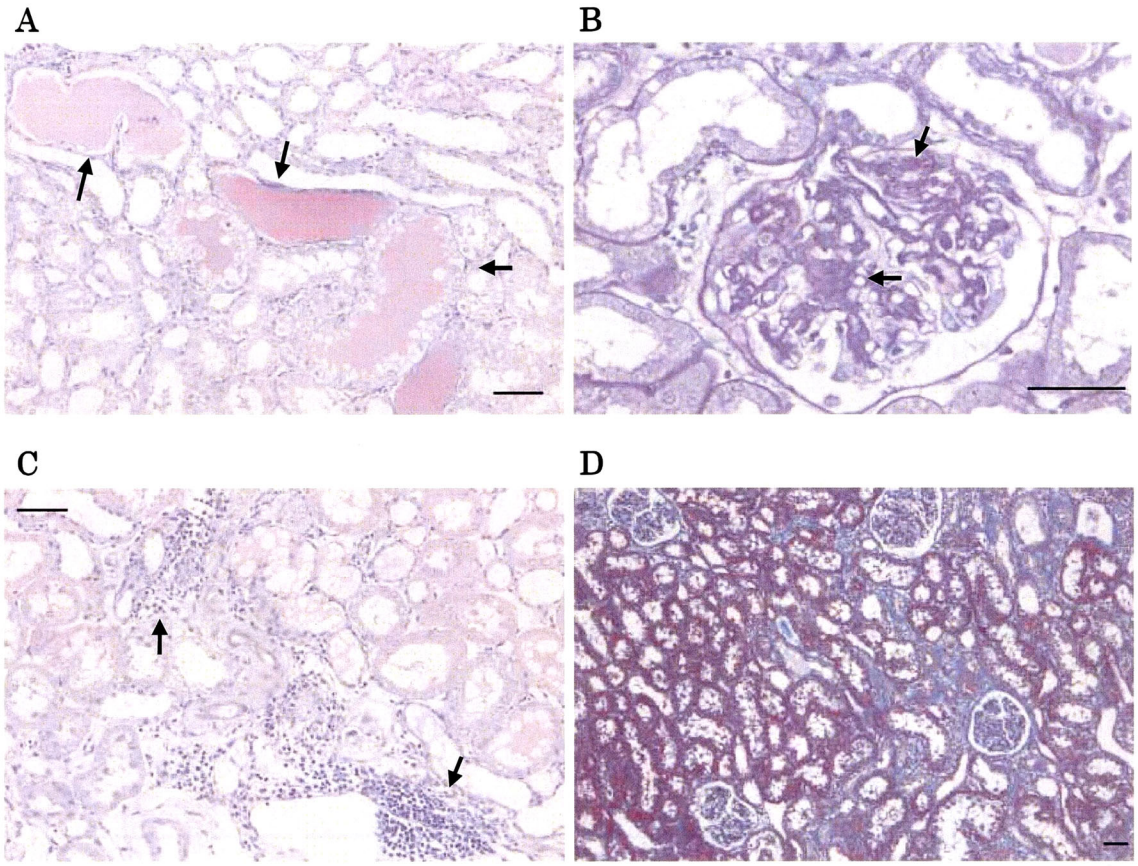

Figure 6. Histopathological observation of kidney in rats combined NX and MI

At the end of the study (Week 36), kidney sections were stained with hematoxylin-eosin (A), periodic acid–Schiff (B, C), and Masson's trichrome (D). All images are from the MI+NX group. Arrows in (A), (B), and (C) indicate cast formation, increase of the mesangial matrix, and infiltration of mononuclear cells, respectively. Scale bar, 50 μm.

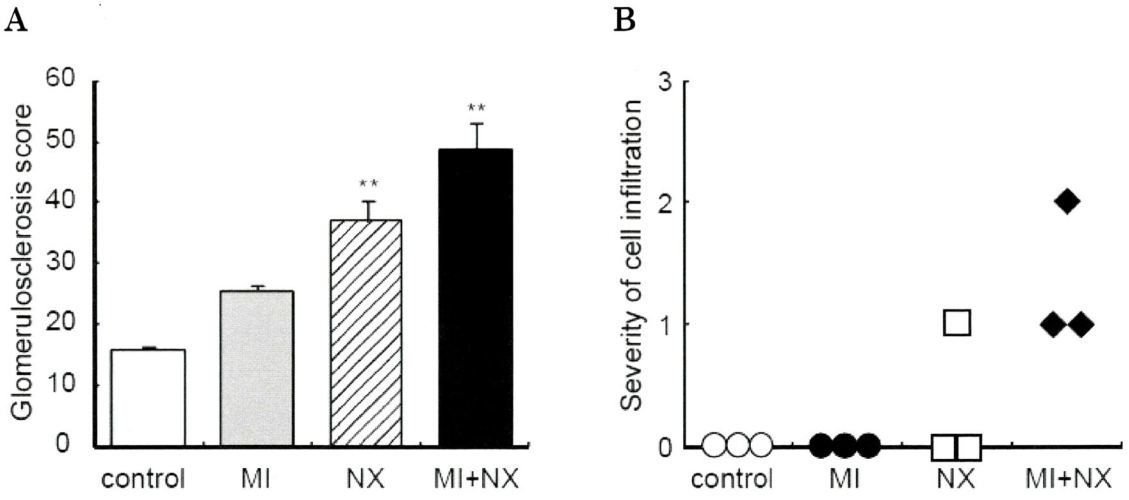

Figure 7. Histopathological analysis of kidney in MI+NX rats

In microscopic observation of renal histopathology, (A) the degree of glomerulosclerosis was scored and the summarized data are shown. The values are shown as mean ± SE. $^{**}P<0.01$ compared with the Control group. (B) The severity of infiltration of mononuclear cells was microscopically semi-quantified and the summarized data is shown. In each group, the specimens from 3 animals were evaluated.

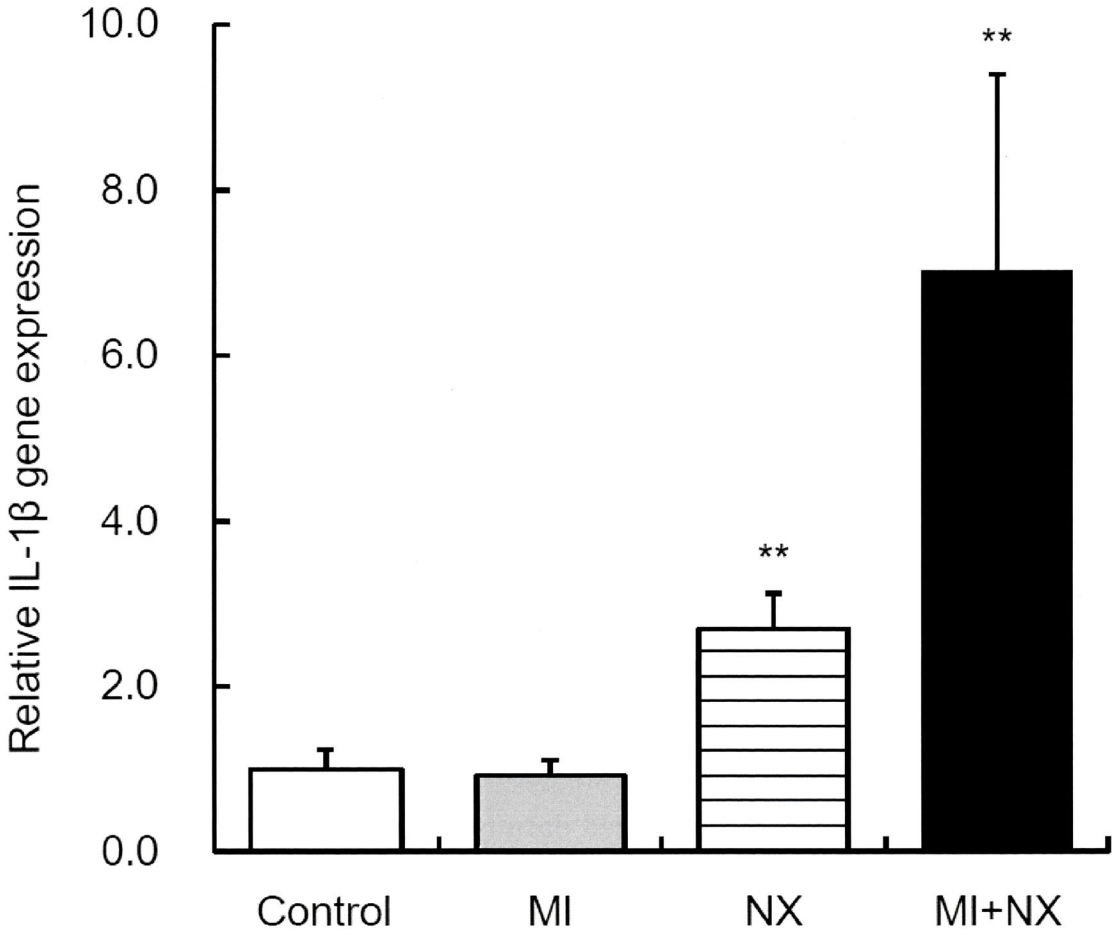

Figure 8. Renal interleukin-1β gene expression in rats combined NX and MI

Renal gene expressions of interleukin-1β (IL-1β) in the kidney of the experimental groups were measured by real-time PCR. The values are shown as mean ± SE; n = 10 to 14 in each group. $^{**}P<0.01$ compared with the Control group.

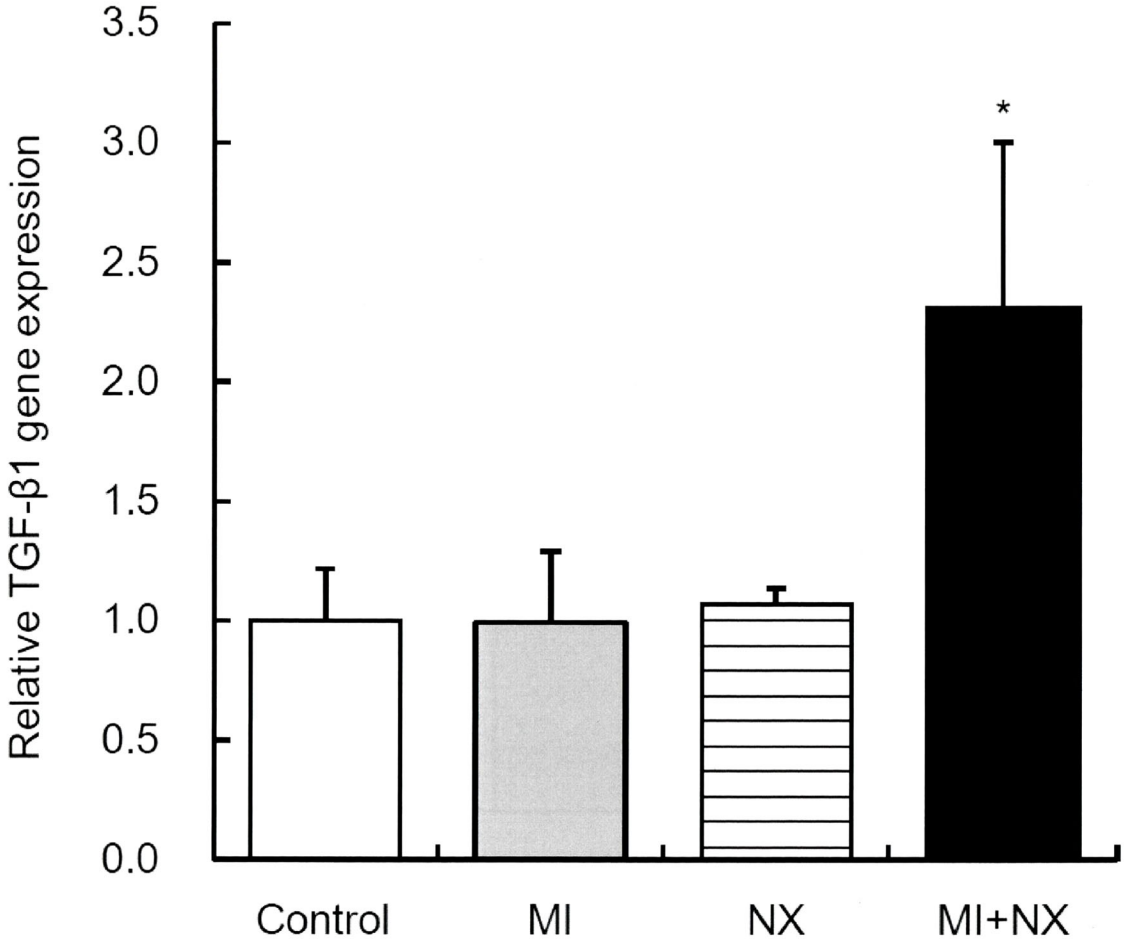

Figure 9. Renal transforming growth factor-β1 gene expression in rat combined NX and MI

Renal gene expressions of transforming growth factor-β1 (TGF-β1) in the kidney of the experimental groups were measured by real-time PCR. The values are shown as mean ± SE; n = 10 to 14 in each group. *P<0.05 compared with the Control group.

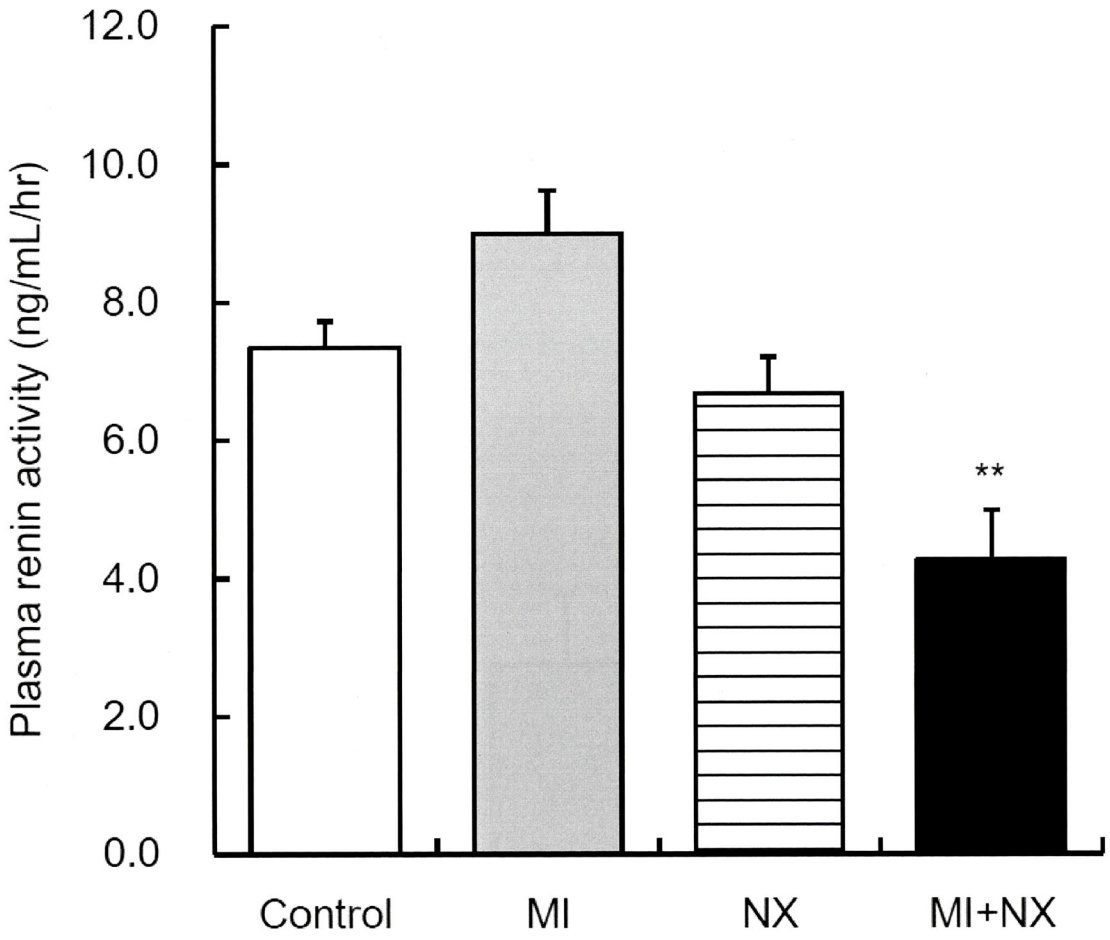

Figure 10. Plasma renin activity in rats combined NX and MI

Plasma renin activity at Week 36 in the experimental groups was measured. The values are shown as mean ± SE; n = 10 to 14 in each group. $^{**}P<0.01$ compared with the Control group.

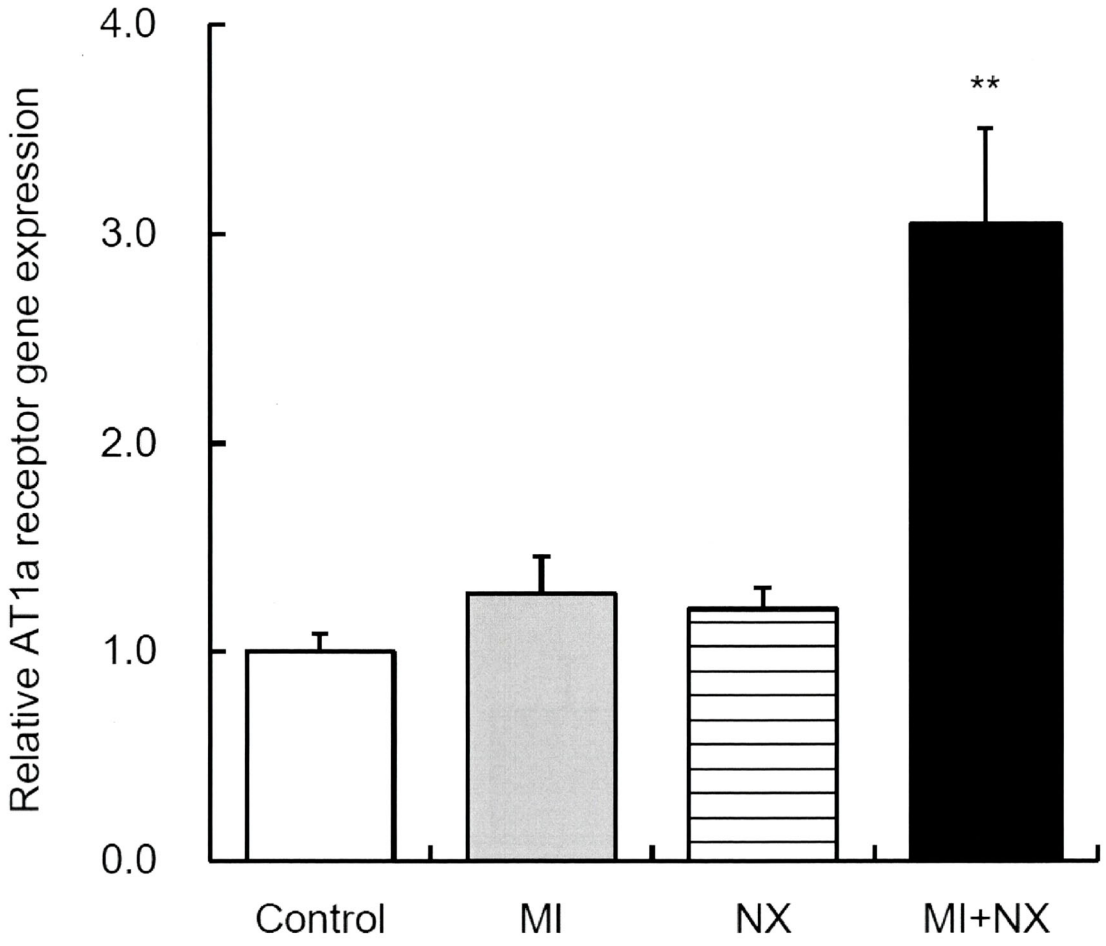

Figure 11. Renal angiotensin II type 1a receptor gene expression in rats combined NX and MI

Gene expression of angiotensin II type 1a (AT1a) receptor in the kidney was measured by real-time PCR. The values are shown as mean ± SE; n = 10 to 14 in each group. $^{**}P<0.01$ compared with the Control group.

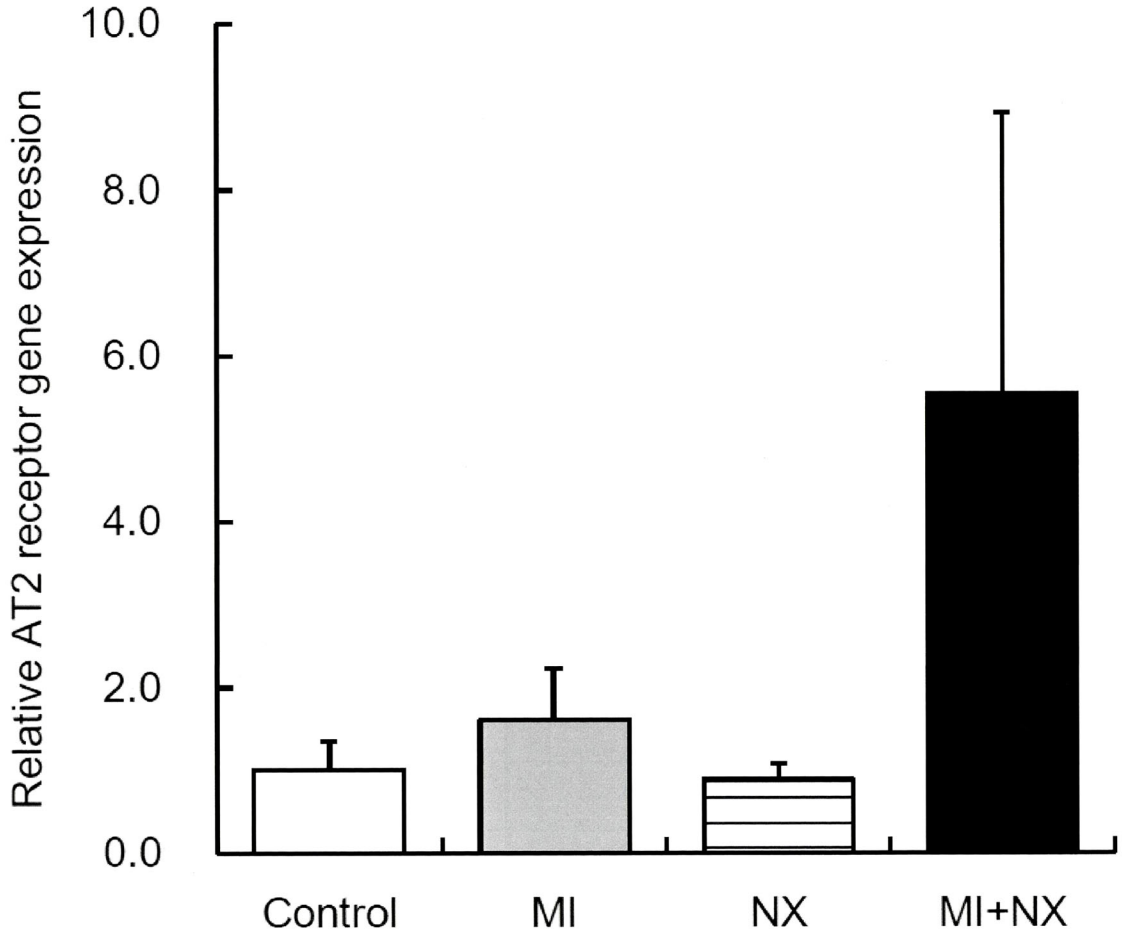

Figure 12. Renal angiotensin II type 2 receptor gene expression in rats combined NX and MI

Gene expression of angiotensin II type 2 (AT2) receptor in the kidney was measured by real-time PCR. The values are shown as mean ± SE; n = 10 to 14 in each group.

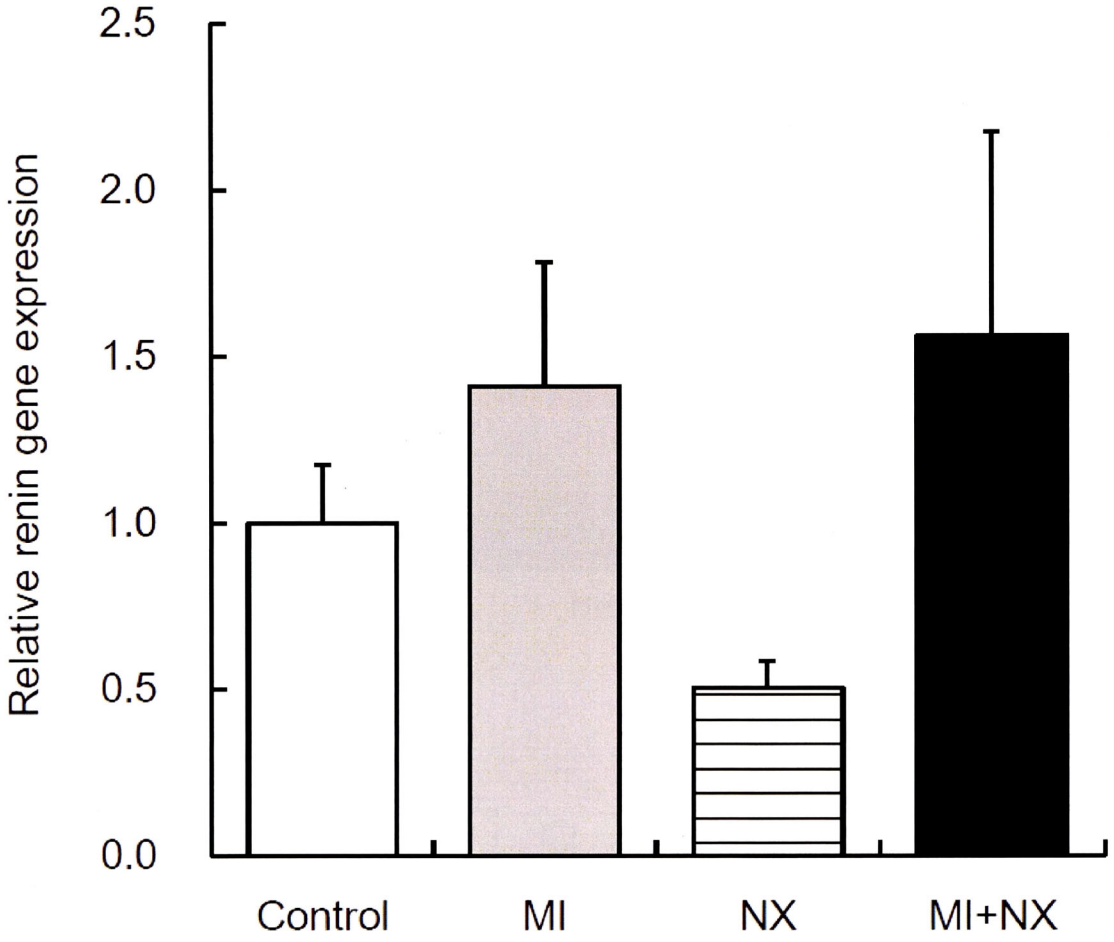

Figure 13. Renal renin gene expression in rats combined NX and MI

Gene expression of renin in the kidney was measured by real-time PCR. The values are shown as mean ± SE; n = 10 to 14 in each group.

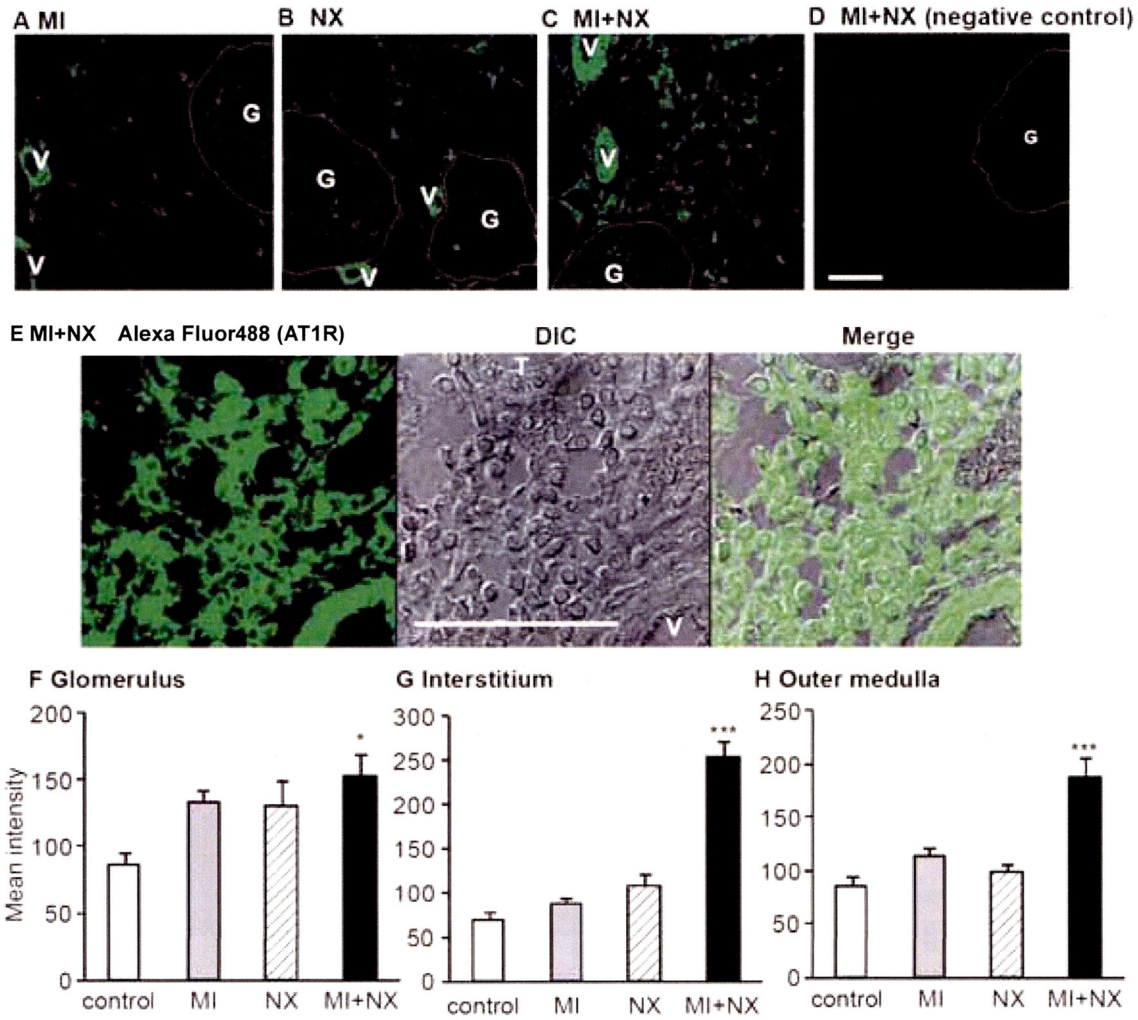

Figure 14. Combined NX and MI increases angiotensin II type 1 receptor protein in the renal interstitium. Kidney specimen from the MI group (A), NX group (B), and MI+NX group (C) were stained for angiotensin II type 1 receptor (AT1R). (D) Negative control without primary antibody. G, glomerulus; V, blood vessel. Scale bar, 50 μm. (E) Staining pattern for AT1R in the renal interstitium with infiltration of mononuclear cells at high magnification. AlexaFluor488 (AT1R), DIC, and Merge show green fluorescence, differential interference contrast, and their merged images, respectively. T, proximal tubule. Scale bar, 50 μm. Summarized quantitative data for fluorescence of AT1R in the glomerulus (F), interstitium (G), and outer medulla (H) in the experimental groups are shown. The values are shown as mean ± SE; n = 3 in each group. $^{*}P<0.05$ and $^{***}P<0.001$ compared with the control group.

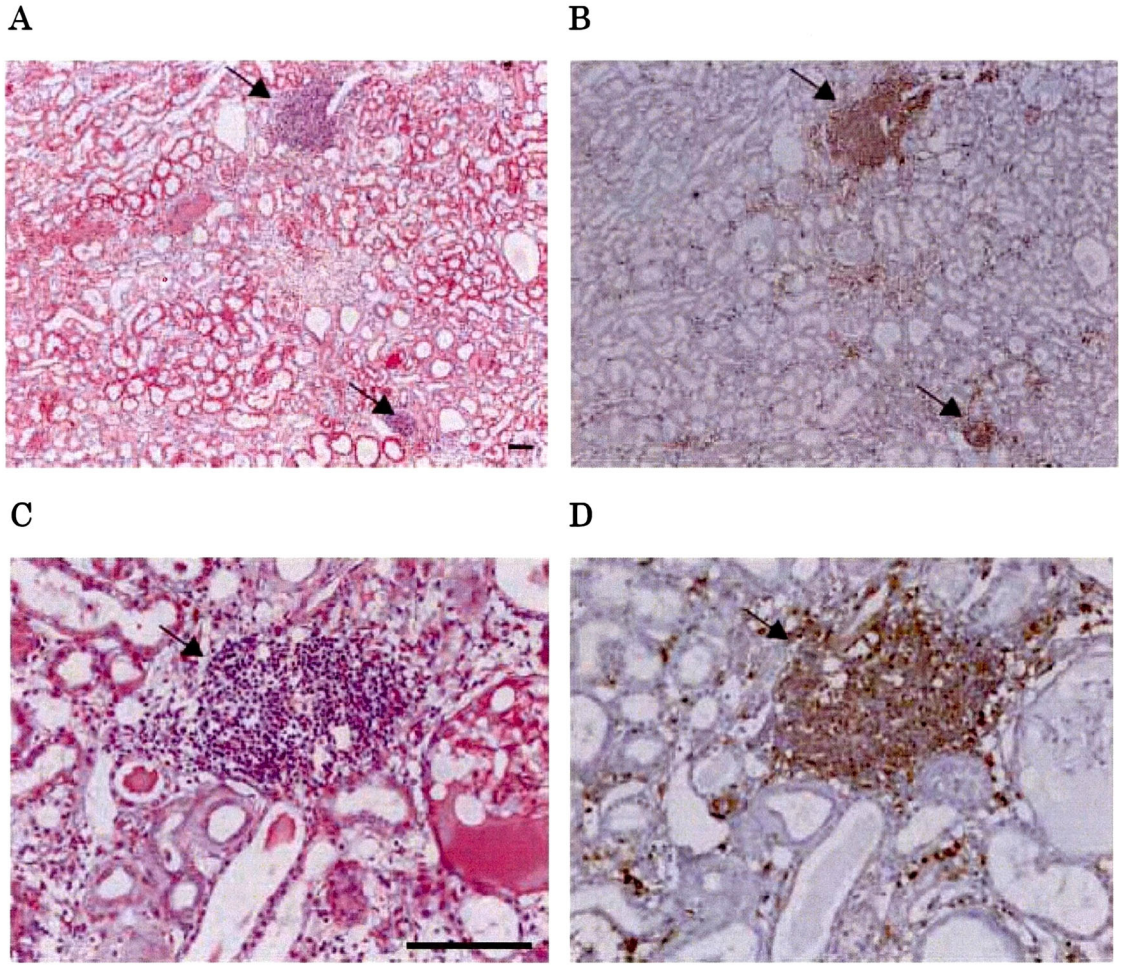

Figure 15. Immunohistochemical analysis of AT1aR using serial sections of kidney in the present model of type 2 cardiorenal syndrome. At the end of the study (Week 37), sections of kidney were stained with hematoxylin-eosin (A and C), and anti-AT1R antibody (B and D). All images are from the MI+NX group. Images at low magnification (A and B) and high magnification (C and D) are shown. Scale bar, 100 μm. Arrows indicate massive infiltration of mononuclear cells.

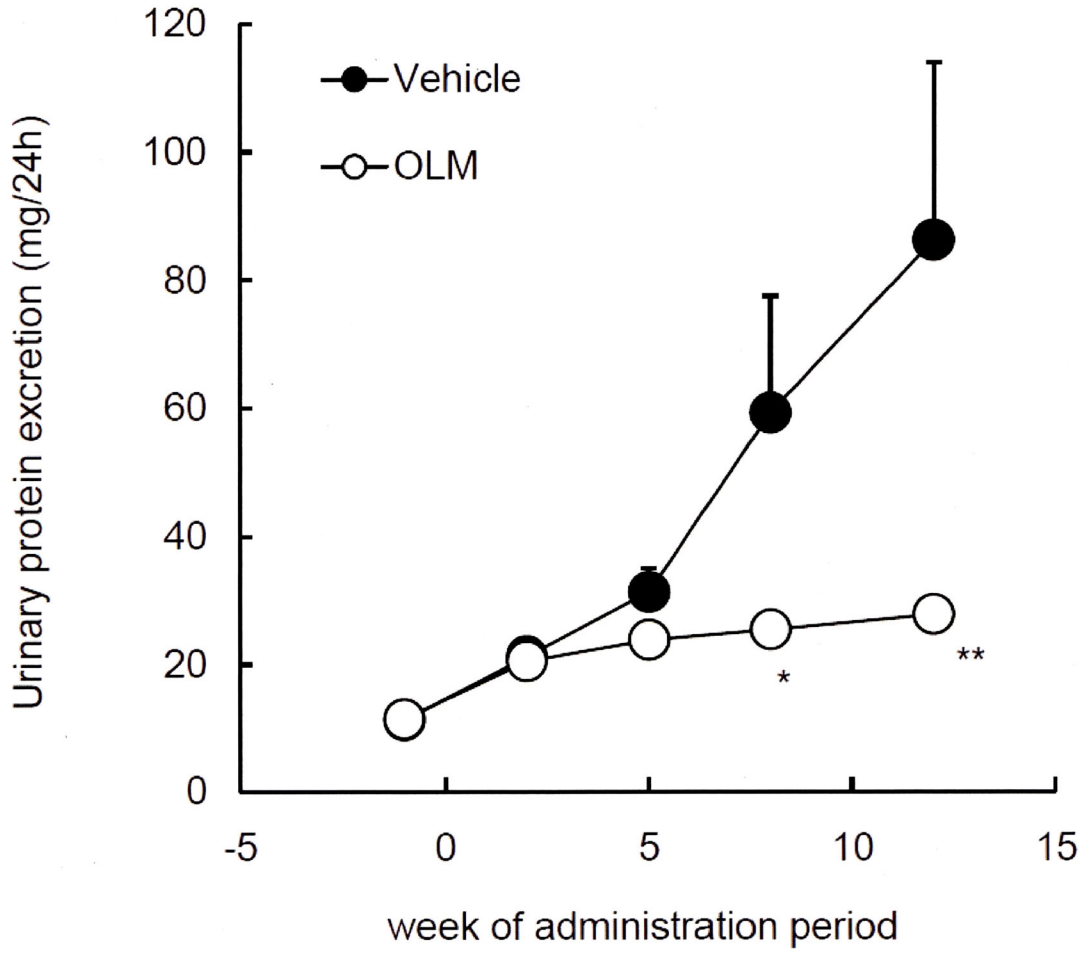

Figure 16. Blockade of AT1R signaling reduces progressive proteinuria in model of Type 2 cardio-renal syndrome. Rats with MI+NX were assigned to 2 groups: Vehicle group (0.5% methylcellulose, p.o.) and OLM group (olmesartan medoxiomil, 5 mg/kg/day, p.o.). The administration started at 2 weeks after the MI surgery. Week 0 indicates the time when administration was started. Urinary protein excretion per day is expressed at the indicated time points. The values are shown as mean ± SE; n = 7 to 11 in each group. $*P<0.05$; $**P<0.01$ compared with vehicle group.

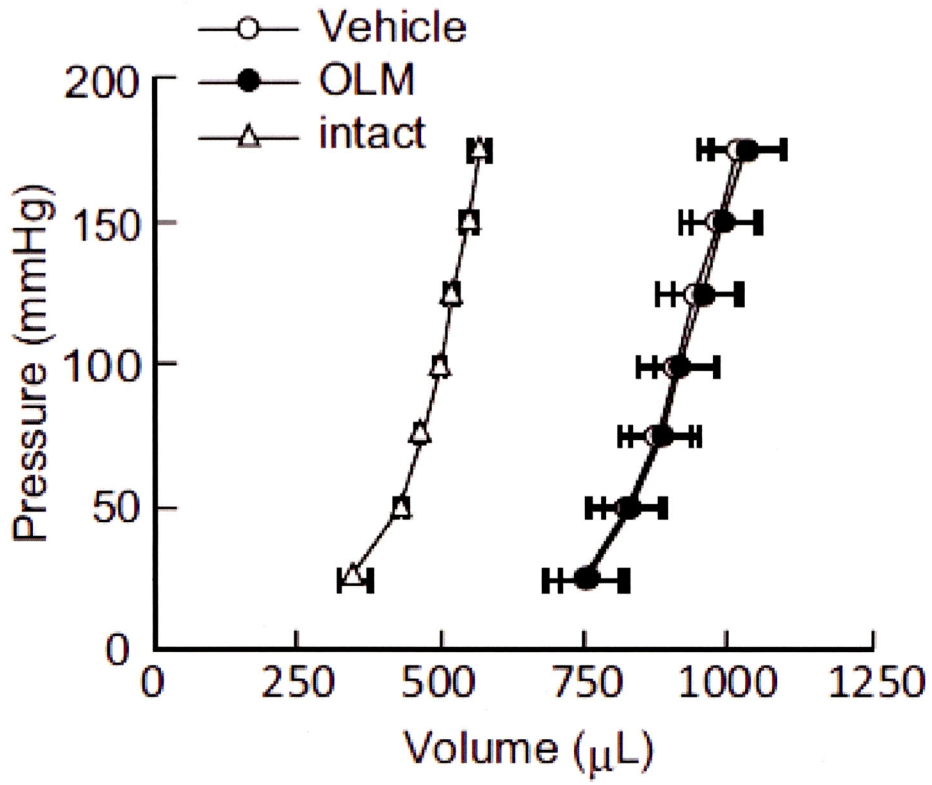

Figure 17. Blockade of AT1R signaling shows no effect on systolic blood pressure and cardiac remodeling in model of Type 2 cardio-renal syndrome. Rats with MI+NX were assigned to 2 groups: Vehicle group (0.5% methylcellulose, p.o.) and OLM group (olmesartan medoxiomil, 5 mg/kg/day, p.o.). The administration started at 2 weeks after the MI surgery. Week 0 indicates the time when administration was started. Left ventricular pressure-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. The values are shown as mean ± SE; n = 3 for the intact group, n = 6 for the Vehicle group and n = 6 for the OLM group.

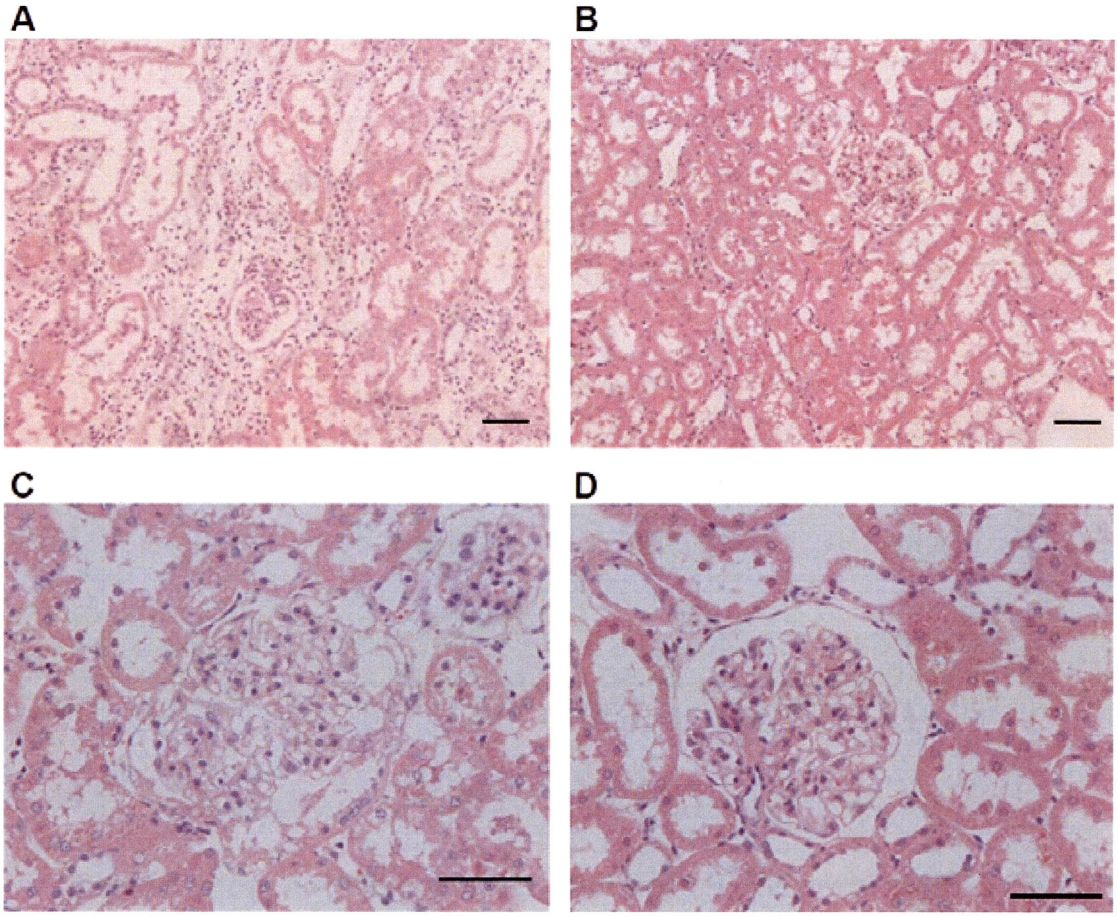

Figure 18. Blockade of AT1R signaling ameliorates histopathological alterations in model of Type 2 cardio-renal syndrome. Kidney sections were stained with hematoxylin-eosin. Typical histopathological images in the Vehicle (A, C) and OLM (B, D) groups were shown. Scale bar, 50 μm. The arrows show infiltration of mononuclear cells (A) and increase in mesangial matrix (C).

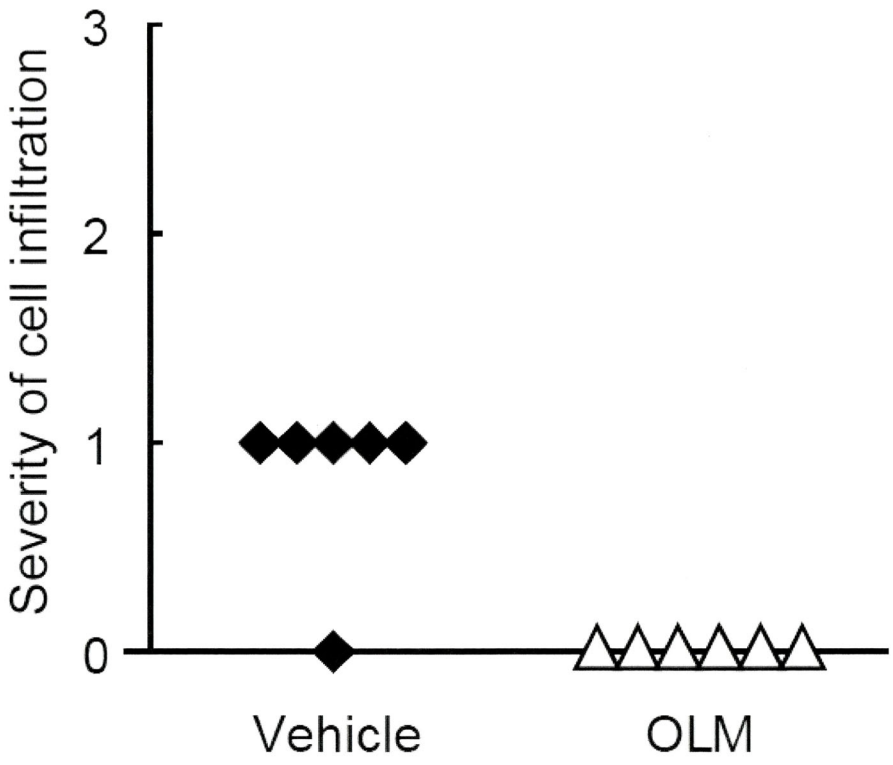

Figure 19. Blockade of AT1R signaling ameliorates histopathological alterations in model of Type 2 cardio-renal syndrome. Kidney sections were stained with hematoxylin-eosin. The severity of infiltration of mononuclear cells was microscopically semi-quantified and the summarized data is shown. In each group, the specimens from 6 animals were evaluated.

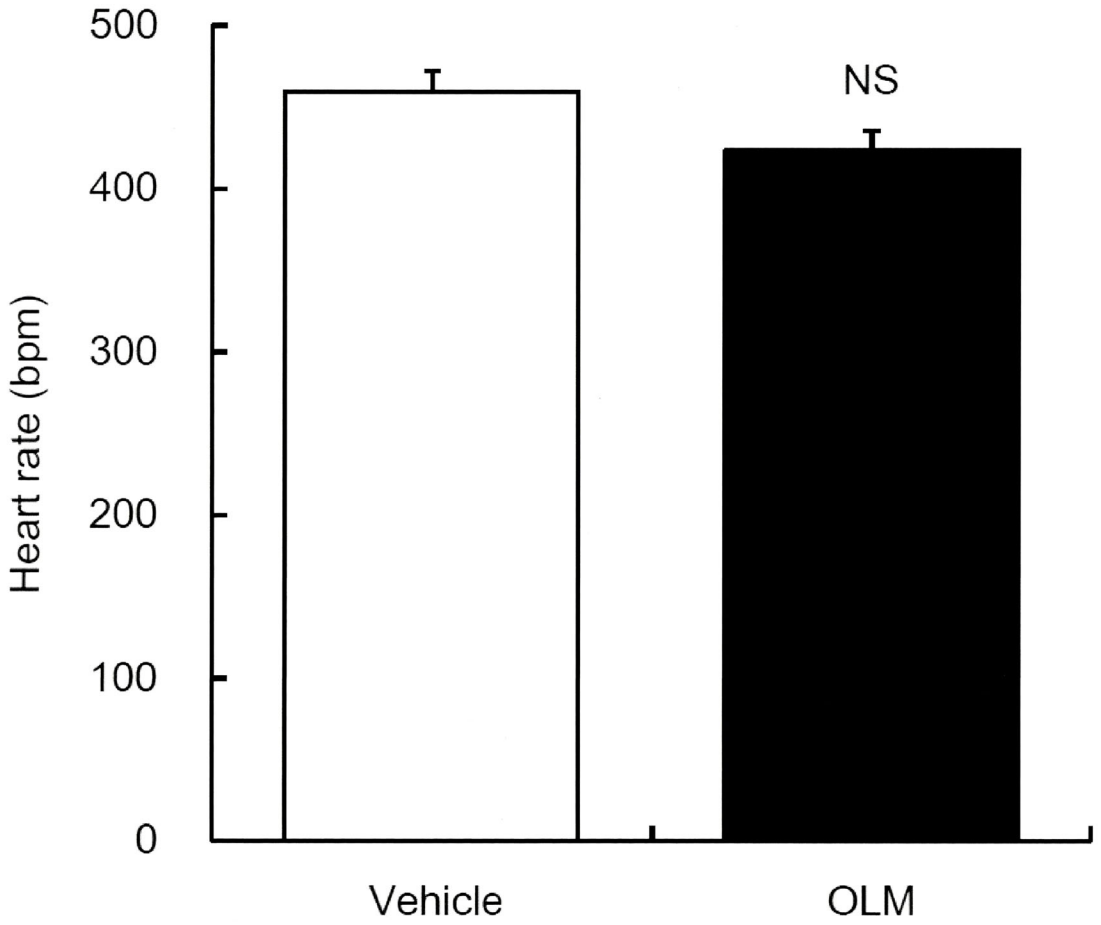

Figure 20. Heart rate in model of Type 2 cardio-renal syndrome. Rats with MI+NX were assigned to 2 groups: Vehicle group (0.5% methylcellulose, P.O.) and OLM group (olmesartan medoxiomil, 5 mg/kg/day, p.o.). The administration started at 2 weeks after the MI surgery. Week 0 indicates the time of starting administration. The heart rate (HR) was measured by the tail-cuff method at 12 weeks after starting administration. The values are shown as mean ± SE; n = 5 to 6 in each group. NS: not significant in comparison with Vehicle group.

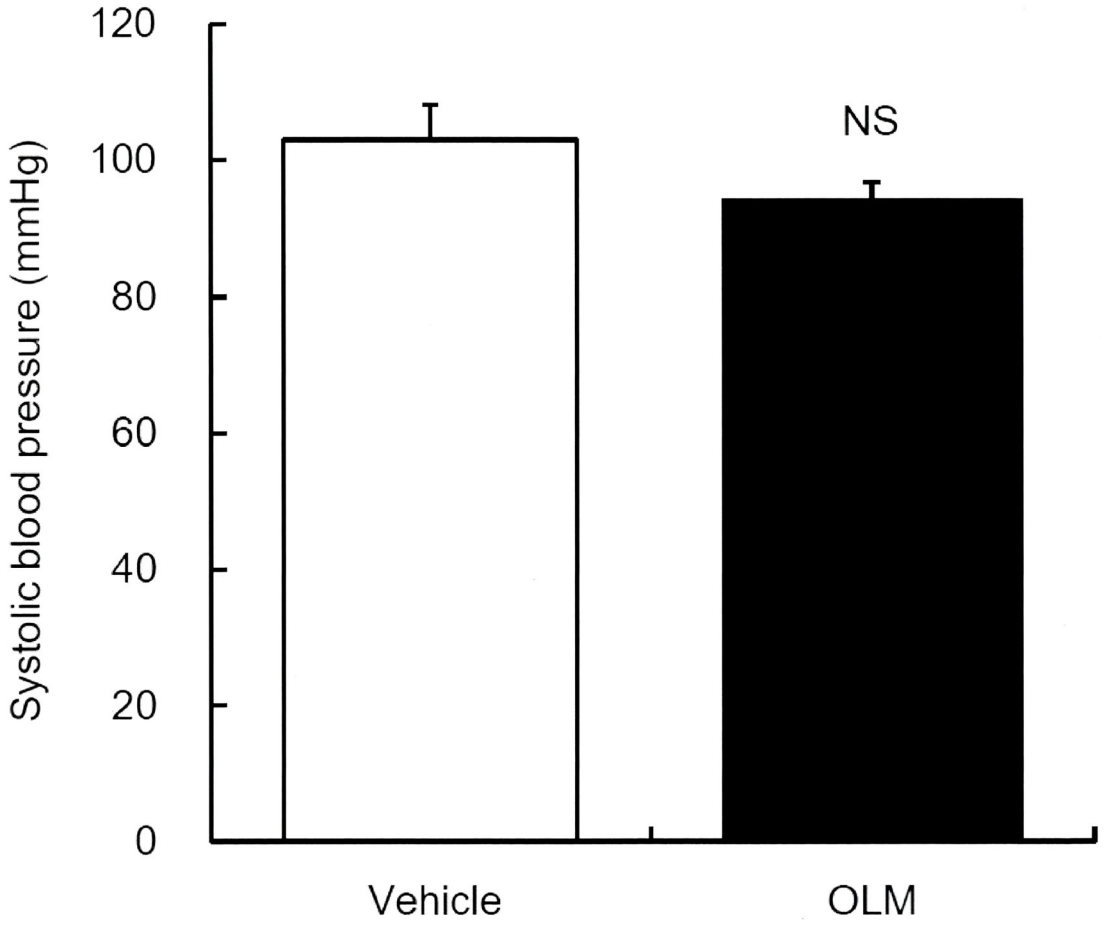

Figure 21. Systolic blood pressure in model of Type 2 cardio-renal syndrome. Rats with MI+NX were assigned to 2 groups: Vehicle group (0.5% methylcellulose, P.O.) and OLM group (olmesartan medoxiomil, 5 mg/kg/day, p.o.). The administration started at 2 weeks after the MI surgery. Week 0 indicates the time of starting administration. Systolic blood pressure (SBP) was measured by the tail-cuff method at 12 weeks after starting administration. The values are shown as mean ± SE; n = 5 to 6 in each group. NS: not significant in comparison with Vehicle group.

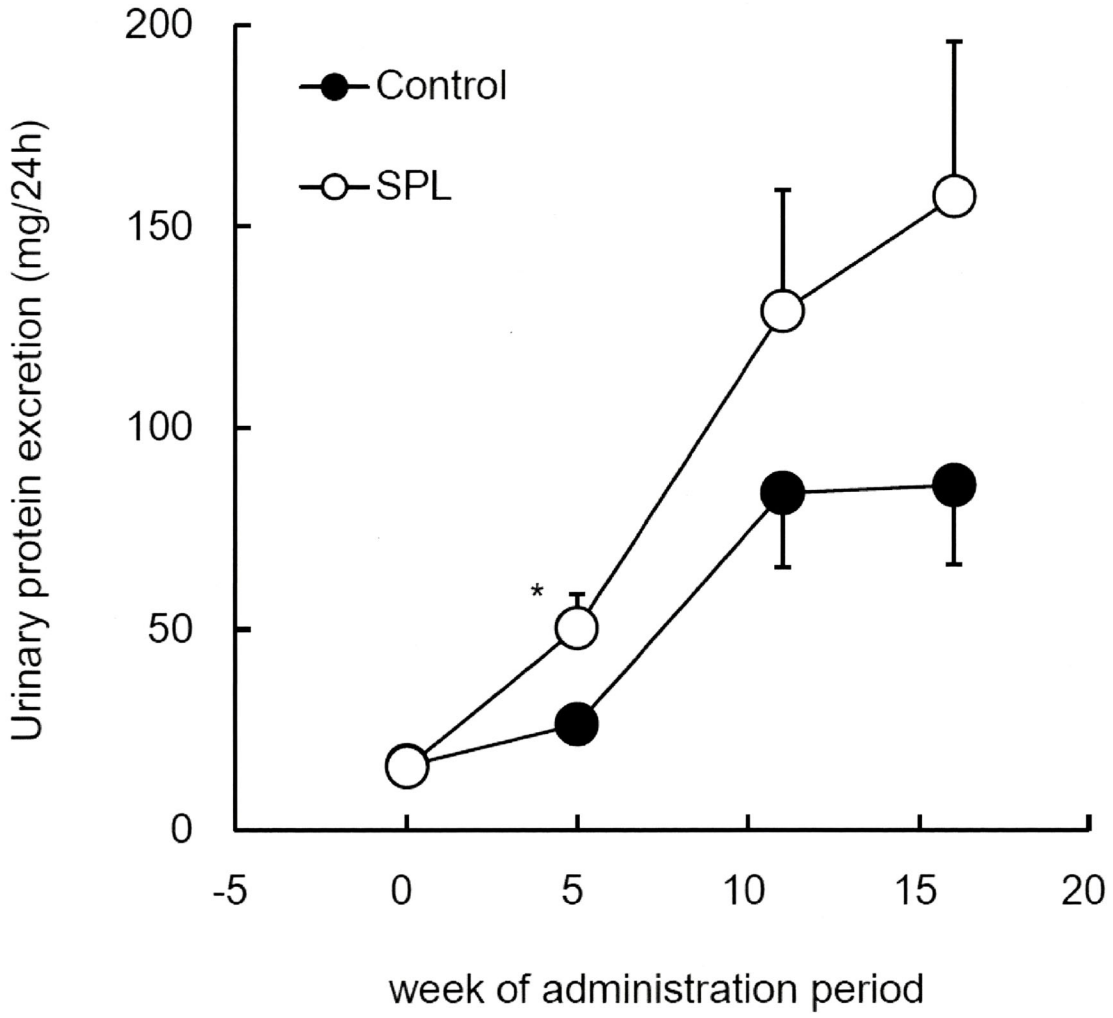

Figure 22. Effect of a mineralocorticoid receptor blocker on proteinuria in model of Type 2 cardio-renal syndrome. Rats with MI+NX were assigned to 2 groups: Control group (normal diet) and SPL groups (0.05 % spironolactone-mixed diet). The administration started at 2 weeks after the MI surgery. Week 0 indicates the time of starting administration. Urinary protein excretion per day is expressed at the indicated time points. The values are shown as mean ± SE; n = 6 to 10 in each group. *P<0.05 compared with Control group.

Chapter II:

Spironolactone, but not eplerenone, impairs glucose tolerance in a rat model of metabolic syndrome

(1) Introduction

Aldosterone, a physiological ligand of the mineralocorticoid receptor (MR), is classically known to regulate electrolyte homeostasis (79), and blood pressure. In addition, several basic and clinical studies have indicated that aldosterone is directly involved in tissue damage in the vasculature such as heart and kidney, through enhancement of oxidative stress, inflammation, and fibrosis.

Much attention has recently been focused on the relation between aldosterone and impairment of glucose metabolism (80). Glucose intolerance and diabetic mellitus are often detected in patients with primary aldosteronism (81) due to adrenal adenoma or hyperplasia (82). The impairment of glucose metabolism is known to be involved in the pathobiology in the metabolic syndrome that comprises of a cluster of several abnormalities, including obesity, insulin resistance, dyslipidemia, and hypertension. Elevated plasma aldosterone levels are detected in metabolic syndrome (83), and moreover, experimental studies have produced a body of evidence for the involvement of aldosterone in the pathogenesis of certain

components of metabolic syndrome, such as adipocyte dysfunction (84, 85) and insulin resistance (80).

Apart from the considerably beneficial effects of MR blockade on metabolic syndrome through improvement of glucose intolerance as well as suppression of blood pressure, no clinical trials have focused on preventing further metabolic deterioration and its complications. Spironolactone (SPL) and eplerenone (EPL), both of which are MR antagonists currently used in a clinical setting, are widely recognized to be beneficial for patients with hypertension and heart failure (21-23). However, the two drugs are known to have distinct pharmacological/pharmacokinetic profiles; EPL shows greater MR selectivity and has non-genomic properties, and SPL has active metabolites (28). Regarding the effect on glucose metabolism, EPL has been demonstrated to improve insulin sensitivity in obese model mice (84, 86). On the other hand, treatment with SPL has been reported to increase the blood glucose levels in patients with resistant hypertension, which is defined as blood pressure that remains above the goal despite concurrent use of three antihypertensive agents of different classes (87). Also, Yamaji et al. have shown that SPL increases HbA1c in patients with mild chronic heart failure. These data indicate that the results of experimental studies have not supported the clinical findings in terms of the effects of MR antagonists on blood glucose level.

I considered that further investigations would be necessary to compare the effect of SPL with that of EPL on blood glucose levels in animal

models. In the present study, therefore, I examined the effect of SPL on blood glucose levels in SHR/NDmcr-cp(cp/cp) (ND) rats, an animal model of metabolic syndrome (known to spontaneously develop hypertension, obesity, and hyperlipidemia), in comparison with that of EPL, another MR antagonist.

(2) Materials and Methods

(i) Experimental materials

SPL was purchased from Shanghai FWD Chemicals Co., Ltd. (Shanghai, China). EPL was extracted from Selara® tablets (purchased from Pfizer Japan Inc., Tokyo, Japan). 0.5% (w/v) methylcellulose (MC) solution was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

(ii) Experimental protocol

All experiments were carried out in accordance with the Animal Experimentation Guidelines of Daiichi-Sankyo Co., Ltd. The rats were kept in a room at 55±5% relative humidity and 23±1°C under a 12 hr light/dark cycle, and allowed free access to diet (FR-2, Funabashi Farm Co., Ltd., Chiba, Japan) and water.

Male SHR/NDmcr-cp(cp/cp) rats (ND, 15 weeks of age) and WKY/Izm rats (WKY, 15 weeks of age) as non-obese controls were purchased from Japan SLC Inc. At 18 weeks of age, the rats were divided into 6 groups: ND treated with MC solution (ND-CONT, N = 6), ND treated with 30 mg/kg SPL (ND-SPL30, N = 6), ND treated with 100 mg/kg SPL (ND-SPL100, N = 6), ND treated with 100 mg/kg EPL (ND-EPL100, N = 6), WKY treated with MC solution (WKY-CONT, N = 6) and WKY treated with 100 mg/kg SPL (WKY-SPL100). I selected these doses of SPL, because 30 - 100 mg/kg of SPL showed antihypertensive effect in ND rats in our preliminary study. SPL and

EPL were suspended in 0.5% (w/v) MC solution and administered at 2 ml/kg body weight by gastric gavage once a day for 3 weeks from the grouping day. I defined the grouping day as Day 1.

(iii) Measurement of urinary parameters and blood chemical parameters

The rats were placed individually in metabolic cages, and 24-hr urine was collected on Day 15. Urinary sodium and potassium concentrations were measured using the STAX-2 system (Techno Medica Co. Ltd., Kanagawa, Japan) and urinary sodium-to-potassium (uNa^+/K^+) ratios were calculated. Water intake and food intake were also measured at the time of urine collection.

Blood was collected from the jugular vein on Day 17. Serum aldosterone (DPC Aldosterone RIA Kit, Mitsubishi Chemical Medience Corporation, Tokyo, Japan), corticosterone (COAT-A-COUNT® RAT CORTICOSTERONE, Siemens Medical Solutions Diagnostics, CA, USA) were measured by radioimmunoassay. Insulin (REBIS Insulin Kit, Shibayagi, Gunma, Japan) and adiponectin (CircuLex™ Rat Adiponectin ELISA Kit, CycLex Co., Ltd., Nagano, Japan) were measured by ELISA. Blood chemical parameters such as triglyceride (TG), total cholesterol (T-Cho), high density lipoprotein cholesterol (HDL-C) and blood glucose, were measured using an automated biochemical serum analyzer (JCA-BM2250, JOEL Ltd., Tokyo, Japan).

(iv) Oral glucose tolerance test

An oral glucose tolerance test (OGTT) was performed after 16 hours of fasting on Day 12. Glucose solution at 2 g/kg (OTSUKA GLUCOSE INJECTION 50%, Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) was administered by gastric gavage. Blood glucose levels were measured at 0, 30, 60, 120, 180, 240 and 360 min after glucose administration using a blood glucose meter (GLUTEST®, Sanwa Kagaku Kenkyusho Co., Ltd., Nagoya, Japan). The area under the curve (AUC) of blood glucose was calculated for each group.

(v) Statistical analysis

Data are expressed as mean ± SE. Comparisons between WKY-CONT and WKY-SPL100, and also between WKY-CONT and ND-CONT were performed by Student's t-test. Statistical comparison between ND-CONT and the drug-treated ND groups, and WKY-CONT and the drug-treated ND groups, were performed by Dunnett's test. Differences were considered to be statistically significant at $P<0.05$.

(3) Results

(i) Increase of uNa^+/K^+ ratio in ND-SPL100 is similar in extent to that in ND-EPL

Once aldosterone binds to MR on renal epithelial cells, urinary Na^+ reabsorption and K^+ excretion occur, and therefore the uNa^+/K^+ ratio becomes lower (88, 89). Table 4 summarizes the uNa^+/K^+ ratio as well as body weight, urinary volume, food intake, and water intake in the experimental groups on Day 15. In comparison with the WKY-CONT group, the uNa^+/K^+ ratio sin the ND-CONT group was somewhat higher. Although the reason for this is still unclear, sodium diuresis might occur in ND rats because of their hypertensive state. In comparison with the ND-CONT group, a significantly higher uNa^+/K^+ ratio was observed in both the ND-SPL100 and ND-EPL100 groups, the degree of elevation being comparable, suggesting that both drugs have similar inhibitory potency on MR signaling in renal epithelia.

(ii) SPL, but not EPL, increases the blood glucose level and improves lipid profiles in ND rats

On Day 17, blood glucose and lipid parameters were measured under normal feeding conditions, and the results have been summarized in Figure 23 - 26. In comparison with WKY-CONT, the ND-CONT group showed a significantly higher blood glucose level (Figure 23). Although SPL had no effect on the

blood glucose level in WKY rats, administration of SPL to ND rats dose-dependently and significantly increased the levels. In contrast, EPL had no effect on the blood glucose level (Figure 23) in ND rats. The TG level in ND rats also increased, and this increase was significantly reduced by treatment with SPL, and tended to be reduced in the ND-EPL100 group (Figure 24). In comparison with WKY rats, the T-Cho level was significantly higher and HDL-C was lower in ND rats, respectively, although the values were within the normal ranges (Figure 25 and 26). Treatment of ND rats with SPL decreased T-Cho to the level in WKY rats (Figure 25), but EPL had no significant effect on T-Cho. Neither SPL nor EPL had a significant effect on HDL-C (Figure 26). These data indicate that SPL characteristically increases blood glucose in ND rats.

(iii) SPL, but not EPL, induces impairment of glucose tolerance in ND rats

Next, I performed the OGTT on Day 12. The fasting blood glucose levels (at 0 min after administration) were comparable among all experimental groups. The OGTT showed that the blood glucose concentration increased quickly, peaked at around 60 min, and then returned to the basal level within 300 min after glucose administration in each experimental group except for the ND-SPL100 group (Figure 27 and 28). On the other hand, in the ND-SPL100 group, the blood glucose level peaked at around 120 min and then gradually returned toward the original level, although the level was still higher than that at the baseline at 360 min after administration (Figure 27). The AUC

value calculated from Figure 27 and 28, an index of glucose tolerance, was significantly increased by SPL in both WKY and ND rats (Figure 29). In contrast, EPL had no significant effect on the AUC value in ND rats (Figure 29). These results indicate that ND rats have impaired glucose tolerance, and that this impairment is dramatically exacerbated by administration of SPL.

(iv) SPL does not affect serum insulin or adiponectin concentration in ND rats

On Day 17, I measured serum insulin in the experimental groups. As shown in Figure 30, ND rats showed marked hyperinsulinemia, compared with that of WKY rats. Administration of neither SPL nor EPL exerted any significant effects on the serum insulin levels in ND rats (Figure 30).

The serum level of adiponectin, a reduction of which is known to cause insulin resistance (90), was also measured. The level in the ND-CONT group was higher than that in the WKY-CONT group (Figure 31). Although a slight increase was evident in the ND-SPL30 group, the adiponectin levels in both the ND-SPL100 and ND-EPL100 groups did not differ from that in the ND-CONT group (Figure 31).

(v) SPL increases the serum aldosterone concentration and reduces the serum corticosterone concentration in ND rats

On Day 17, the serum aldosterone and corticosterone concentrations were

also measured. The level of aldosterone in the ND-CONT group was significantly higher than that in the WKY-CONT group (Figure 32). The serum aldosterone concentration in the ND-SPL100 group was significantly increased relative to that in the ND-CONT group (Figure 32). On the other hand, EPL had no effect on the serum aldosterone level even at the same dosage as that of SPL. SPL also increased the serum aldosterone level in WKY rats, but the extent of the increase was smaller than that in ND rats (Figure 32).

The serum concentrations of corticosterone, a major functional glucocorticoid in rodents, in the experimental groups have been summarized in Figure 33. In comparison with the WKY-CONT group, the level of corticosterone in the ND-CONT group was significantly higher (Figure 33). In the ND-SPL100 group, the level was significantly lower than that in the ND-CONT group (Figure 33). On the other hand, EPL did not significantly affect the serum corticosterone level (Figure 33).

(4) Discussion

ND rats are congenic animals harboring a nonsense mutation introduced into the leptin receptor (91). ND rats are known to spontaneously develop symptoms of metabolic syndrome, including obesity, hypertension, hyperlipidemia and diabetes (92, 93), accompanied by age-related renal injury (94). In comparison with non-obese genetic control animals, aldosterone excess has also been reported in ND rats (94). These symptoms are known to be frequently observed in patients with metabolic syndrome, and therefore I considered ND rats to be an appropriate animal model of metabolic syndrome.

In the present study, administration of SPL significantly elevated the levels of blood glucose with impairment of glucose tolerance in ND rats, whereas EPL at a similar renally effective dosage did not. On the other hand, serum insulin levels in rats treated with SPL were not significantly different from those in rats treated with EPL, suggesting that SPL impaired insulin signaling. Previously, the CHARM study has indicated that treatment of patients with SPL is associated with the development of diabetes mellitus (95). Furthermore, SPL has been reported to increase blood glucose levels in patients with type 2 diabetes mellitus (96). Also, Yamaji et al. have shown that SPL increases the level of HbA1c in patients with chronic heart failure, whereas EPL does not (29). Taken together, these data suggest that SPL, but not EPL, increases the blood glucose level in association with glucose

intolerance.

There are some plausible reasons for the differences in the effects of SPL and EPL on the blood glucose level. Since MR activation by aldosterone plays a well-recognized role in insulin resistance (80), increased aldosterone levels in response to treatment with SPL would be a notable effect. If higher aldosterone levels resulted in insufficient blockade of MR, then insulin resistance would be expected to occur. Alternatively, non-specific binding of SPL and its metabolites to the sex steroid hormone (97) and glucocorticoid receptors could exacerbate insulin resistance. EPL was originally identified during the screening of SPL analogues for more selective binding to MR with minimal binding to other steroid receptors (98). Therefore, in comparison with EPL, SPL shows 100- to 1000-fold higher binding affinity for glucocorticoid, androgen, and progesterone receptors (98). Since glucocorticoid receptor activation is widely known to increase blood glucose levels and progesterone has also been reported to contribute to insulin resistance (97, 99), and the lower selectivity of SPL to MR might be attributed to the difference. The decrease in the serum corticosterone concentration resulting from treatment with SPL, as observed in this study, may reflect a feedback mechanism between the serum glucocorticoid level and activation of its receptor.

In the present study, I found that SPL increased blood aldosterone levels, whereas EPL did not. Although the reason for this is still unclear, a difference in the resulting level of aldosterone might be explained by the

view of the differences in the physicochemical and pharmacokinetic properties of SPL and EPL. EPL does not produce active metabolites, whereas SPL is metabolized quickly, generating various active metabolites. Many pharmacological effects of SPL are thought to be mediated by these metabolites. Higher concentrations of canrenone, a metabolite of SPL, were observed in liver, adrenals, kidney and testis in comparison with that in plasma. Furthermore, the retention of radioactive materials was observed in the adrenals after oral administration of ^{14}C-labeled SPL (100), indicating that active metabolites were practically distributed in the adrenal glands (100). If these active metabolites affect MR in the periphery, aldosterone release may occur as a compensated action. Further studies are required to clarify the mechanisms underlying the differential actions of MR antagonists on the blood level of aldosterone.

The increased blood glucose levels resulting from treatment with SPL in ND rats in our present study contrasted with results obtained from mice with high-energy diet-induced obesity (101), in which SPL reduced blood glucose levels and improved glucose tolerance. However, the two models are quite different in that mice with high-energy diet-induced obesity do not have an increased serum aldosterone level, whereas the serum aldosterone level in our present model rats was four times higher than in the control WKY rats, reflecting the results that have been obtained in humans. Moreover, methods used for oral administration of SPL differed between the two studies. In the mouse study, SPL was administered by a subcutaneously

implanted drug release tablet, whereas in our study the drug was administered orally by gastric gavage, thus resembling the clinical situation.

MR blockade not only lowers blood pressure but also ameliorates cardiac morbidity and mortality in patients with heart failure (21-23). Furthermore, accumulated evidence indicates that MR antagonists reduce albuminuria in patients with chronic kidney disease or diabetic nephropathy (102, 103). These results suggest that the treatment of metabolic syndrome with MR antagonists would have various beneficial effects, reducing the complications and risks of cardiovascular disease. However, our present findings indicate that SPL has the potential to increase the blood glucose level associated with glucose intolerance in metabolic syndrome. Although the influence of blood glucose elevation by SPL on clinical outcome remains unclear, SPL has been reported to increase blood glucose levels in humans with type 2 diabetes and to impair endothelial function due to failure of glycemic control (96). In terms of hyperglycemia, EPL is considered to have a superior profile. However, administration of EPL is contraindicated in diabetic patients with albuminuria, because of the risk of hyperkalemia. Recently, some novel non-steroidal MR antagonists with minimized unfavorable effects have been reported (104-106). These new classes of MR antagonist should be novel therapeutic options for patients with metabolic syndrome.

Conclusion

SPL and EPL exert differential effects on the blood glucose profile in ND rats. Although further studies are necessary to clarify the influence of blood glucose elevation by SPL on clinical outcome, our data suggest that hyperglycemia should be taken into account when prescribing SPL.

Table 4. Body weight, urinary parameters, food and water intake in WKY rats on Day 15

	WKY-CONT (n = 6)	WKY-SPL100 (n = 6)
Body weight (g)	418 ± 4	420 ± 3
Urinary volume (g)	12.2 ± 1.5	18.0 ± 2.6
uNa^+/K^+ ratio	0.55 ± 0.01	0.80 ± 0.08 [a]
Food intake (g/24h)	24.5 ± 1.3	20.3 ± 1.8
Water intake (g/24h)	35.9 ± 0.8	41.2 ± 3.3

	ND-CONT (n = 6)	ND-SPL30 (n = 6)	ND-SPL100 (n = 6)	ND-EPL (n = 6)
Body weight (g)	550 ± 5 [b]	529 ± 10 [c]	509 ± 7 [c]	548 ± 5 [c]
Urinary volume (g)	26.9 ± 2.0 [b]	27.9 ± 3.9 [c]	28.1 ± 5.6 [c]	32.0 ± 4.0 [c]
uNa^+/K^+ ratio	0.65 ± 0.03 [b]	0.78 ± 0.08 [c]	0.88 ± 0.08 [c, d]	0.92 ± 0.03 [c, d]
Food intake (g/24h)	26.3 ± 2.6	25.2 ± 0.9	20.9 ± 1.0 [c, d]	26.1 ± 0.7
Water intake (g/24h)	37.9 ± 2.9	40.6 ± 3.2	42.0 ± 5.4	43.6 ± 2.7

uNa^+/K^+ ratio: urinary sodium-to-potassium ratio. Data are expressed as mean ± SE.

[a]: $P<0.05$ in comparison between WKY-CONT and WKY-SPL100 by t-test.

[b]: $P<0.05$ in comparison between WKY-CONT and ND-CONT by t-test.

[c]: $P<0.05$ vs. WKY-CONT by Dunnett's test.

[d]: $P<0.05$ vs. ND-CONT by Dunnett's test.

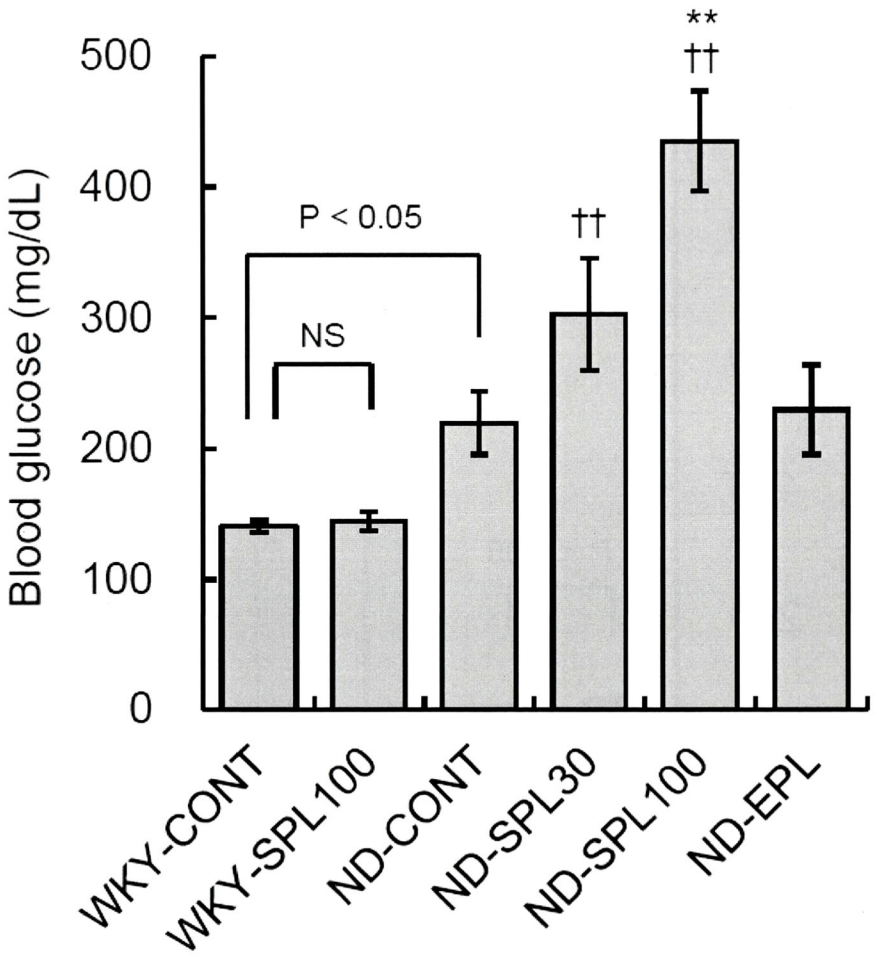

Figure 23. Blood glucose level on Day 17

SPL, EPL or vehicle was administered orally to ND or WKY rats once a day during the experimental period. On Day 17, blood was collected from the jugular vein and the blood glucose was measured. The values are given as mean ± SE. n = 6 for each group. **, P <0.01 compared with ND-CONT. ††, P<0.01 compared with WKY-CONT. NS, not significant.

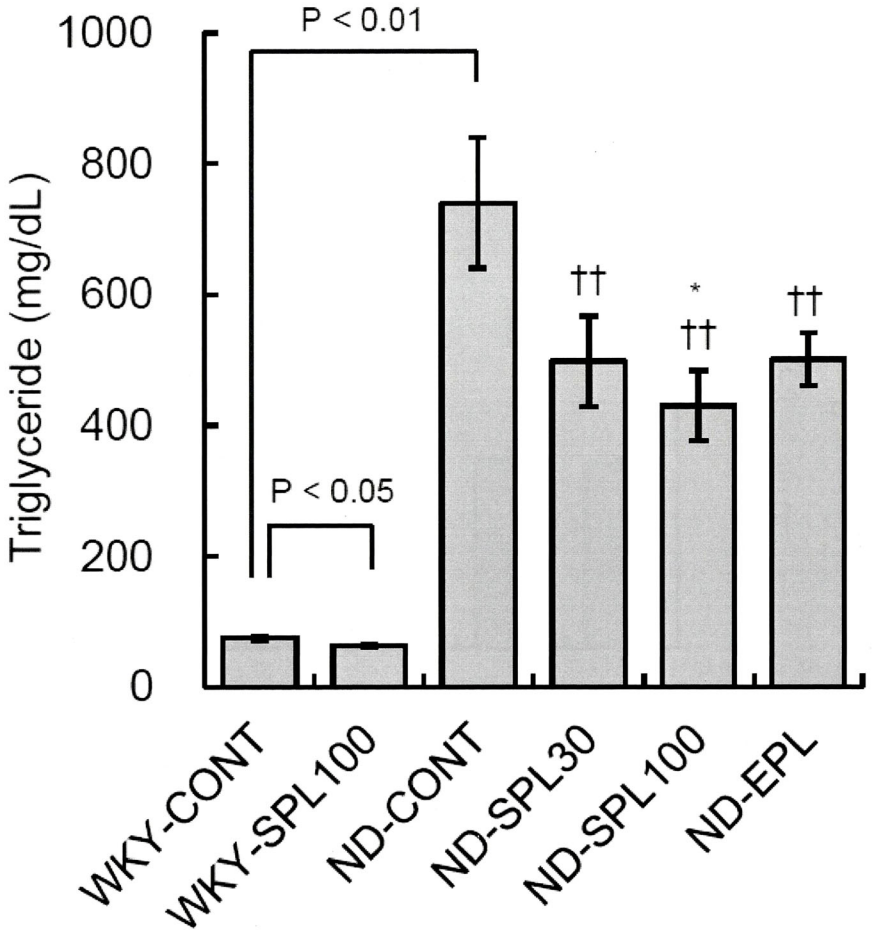

Figure 24. Blood triglyceride level on Day 17

SPL improves lipid parameters, but increases blood glucose in ND rats. SPL, EPL or vehicle was administered orally to ND or WKY rats once a day during the experimental period. On Day 17, blood was collected from the jugular vein and the triglyceride was measured. The values are given as mean ± SE. n = 6 for each group. *, P <0.05 compared with ND-CONT. ††, P <0.01 compared with WKY-CONT. NS, not significant.

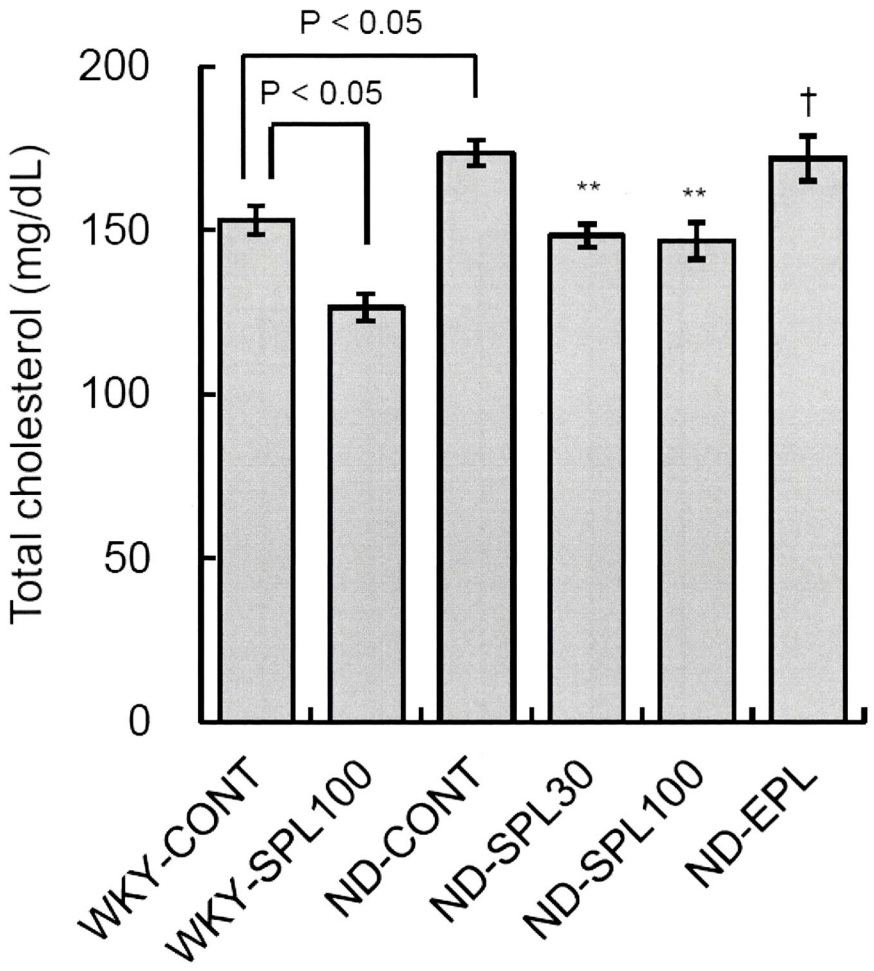

Figure 25. Blood total cholesterol level on Day 17

SPL, EPL or vehicle was administered orally to ND or WKY rats once a day during the experimental period. On Day 17, blood was collected from the jugular vein and the total cholesterol was measured. The values are given as mean ± SE. n = 6 for each group. **, P <0.01 compared with ND-CONT. †, P<0.05 compared with WKY-CONT. NS, not significant.

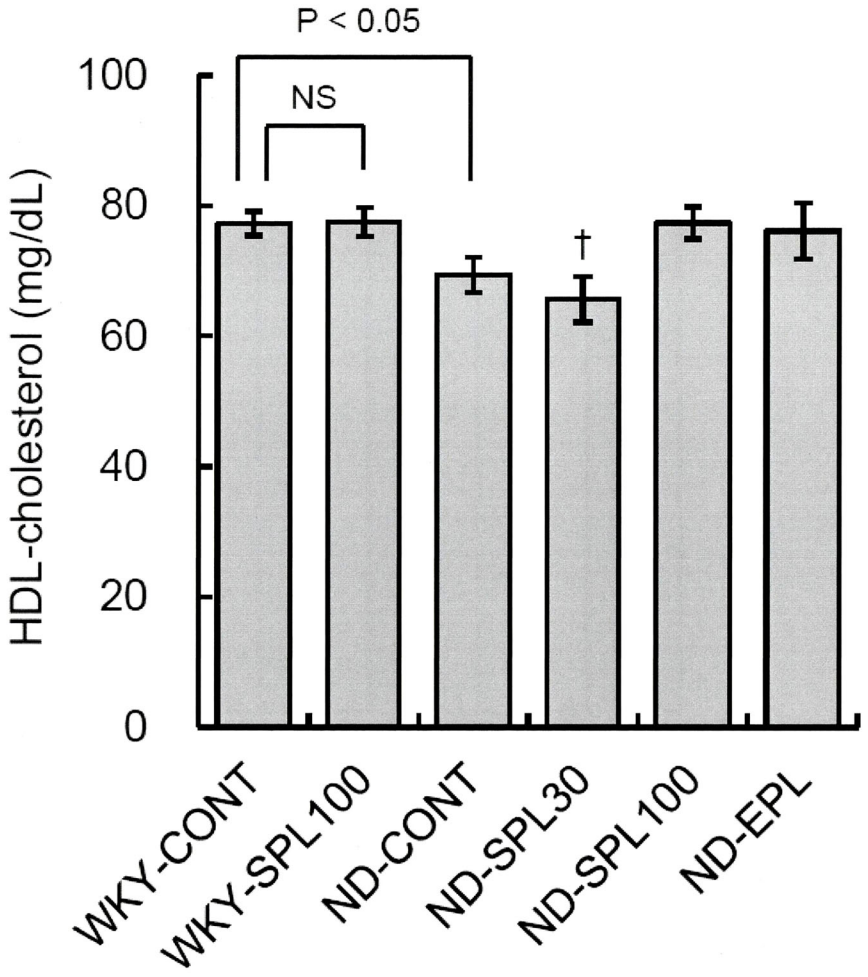

Figure 26. Blood high-density lipoprotein cholesterol level on Day 17

SPL, EPL or vehicle was administered orally to ND or WKY rats once a day during the experimental period. On Day 17, blood was collected from the jugular vein and the HDL-cholesterol was measured. The values are given as mean ± SE. n = 6 for each group. †, $P<0.05$ compared with WKY-CONT. NS, not significant.

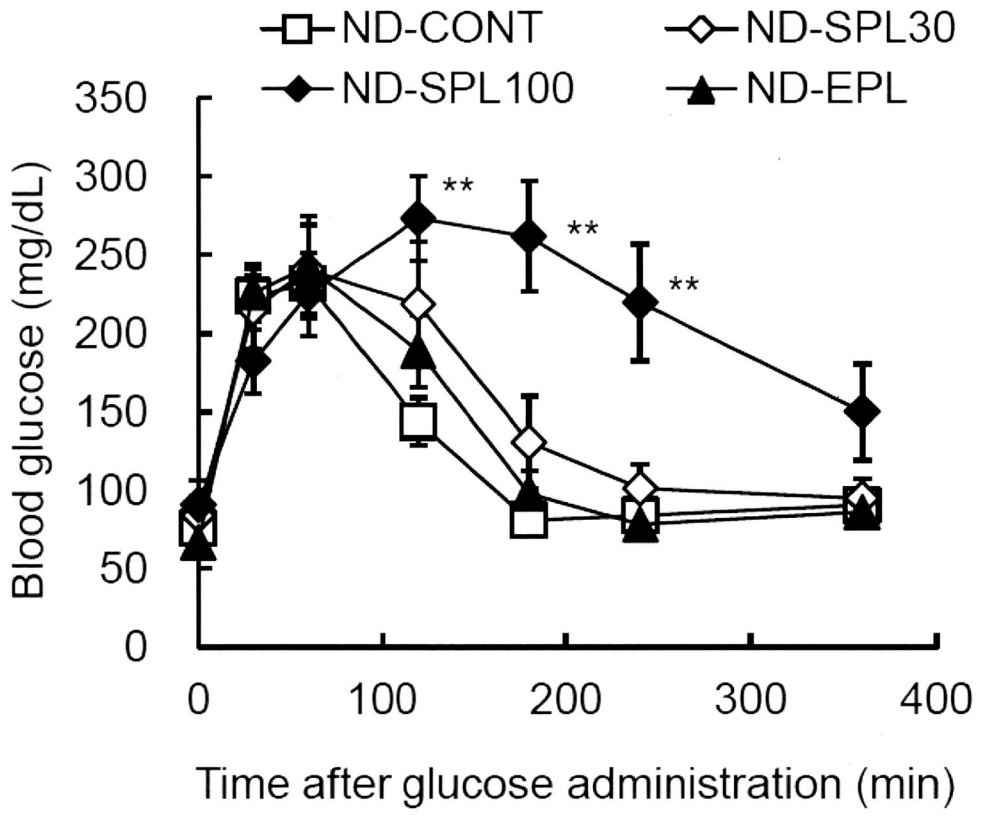

Figure 27. Oral glucose tolerance test in ND rats on Day 12

SPL, EPL or vehicle was administered orally to ND rats once a day during the experimental period. On Day 12, the rats were fasted for 16 hours and then glucose solution was administered at 2 g/kg by gastric gavage. Blood glucose in each group of ND rats was measured before dosing (0), and at 30, 60, 120, 180, 240, and 360 min after oral administration of glucose solution, The values are given as mean ± SE. n = 6 for each group. **, P <0.01 compared with ND-CONT.

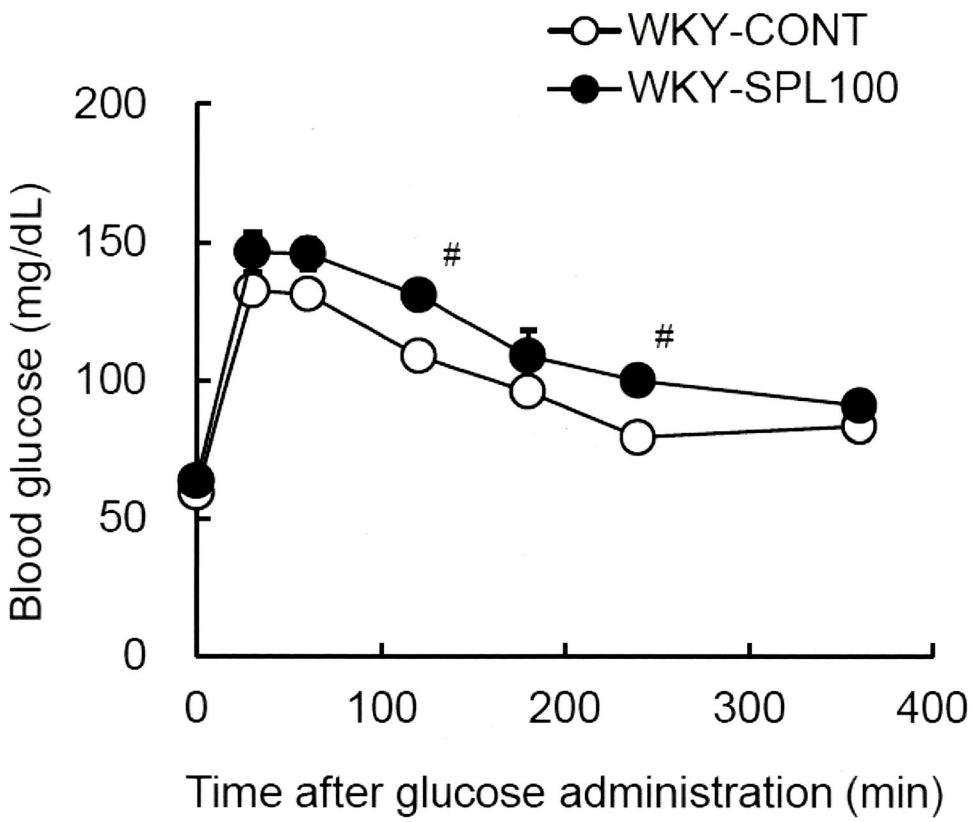

Figure 28. Oral glucose tolerance test in WKY rats on Day 12

SPL or vehicle was administered orally to WKY rats once a day during the experimental period. On Day 12, the rats were fasted for 16 hours and then glucose solution was administered at 2 g/kg by gastric gavage. Blood glucose in each group of WKY rats was measured before dosing (0), and at 30, 60, 120, 180, 240, and 360 min after oral administration of glucose solution, The values are given as mean ± SE. n = 6 for each group. #, P <0.05 for comparison between WKY-CONT and WKY-SPL100.

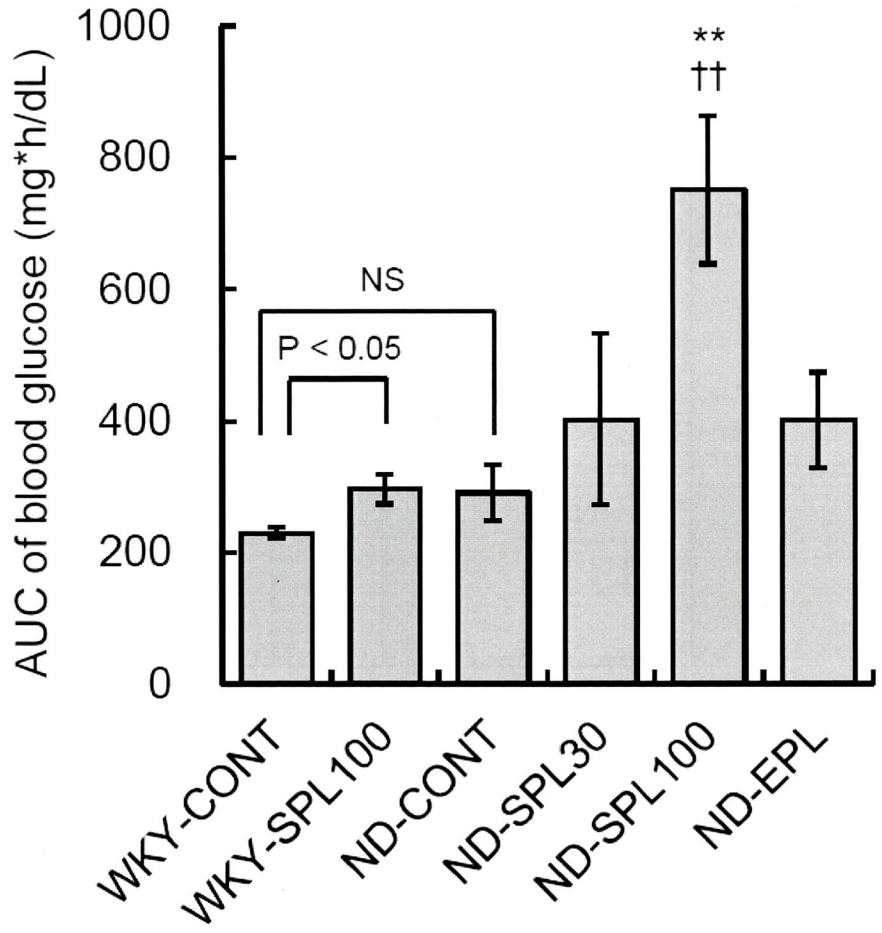

Figure 29. The area under the curve of blood glucose in oral glucose test on Day 12

The AUC of blood glucose was calculated. The values are given as mean ± SE. n = 6 for each group. **, P <0.01 compared with ND-CONT. ††, P <0.01 compared with WKY-CONT.

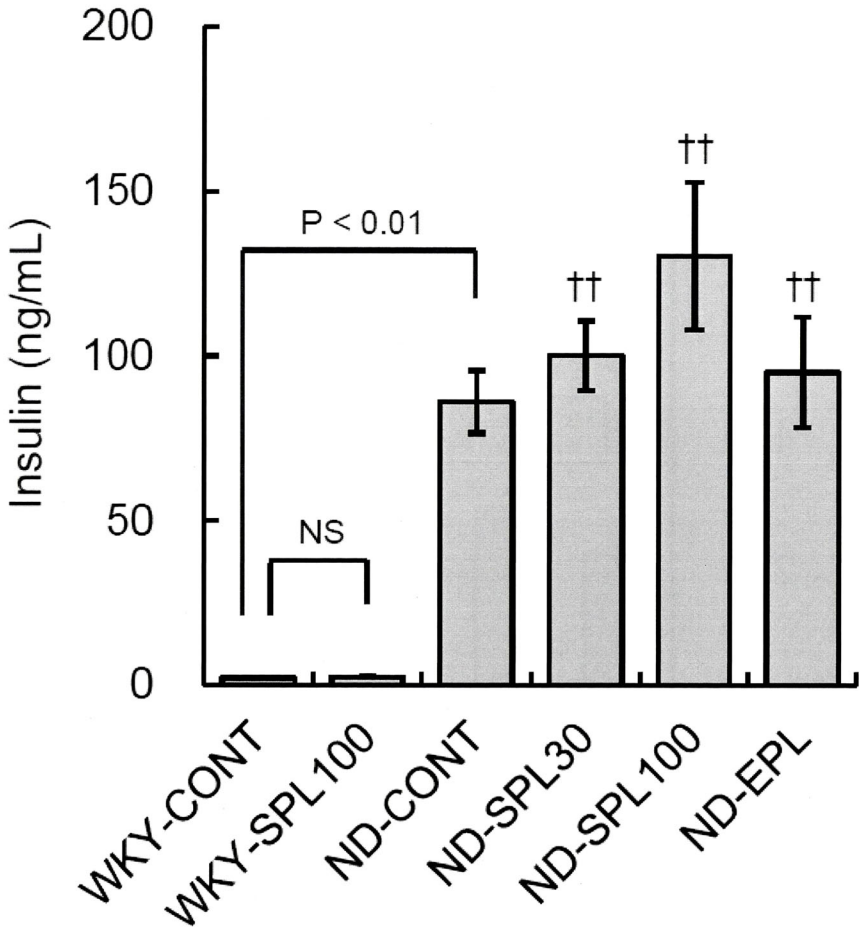

Figure 30. Serum insulin level on Day 17

SPL, EPL or vehicle was administered orally to ND or WKY rats once a day during the experimental period. On Day 17, serum insulin was measured by ELISA. The values are given as mean ± SE. n = 6 for each group. ††, $P<0.01$ compared with WKY-CONT. NS, not significant.

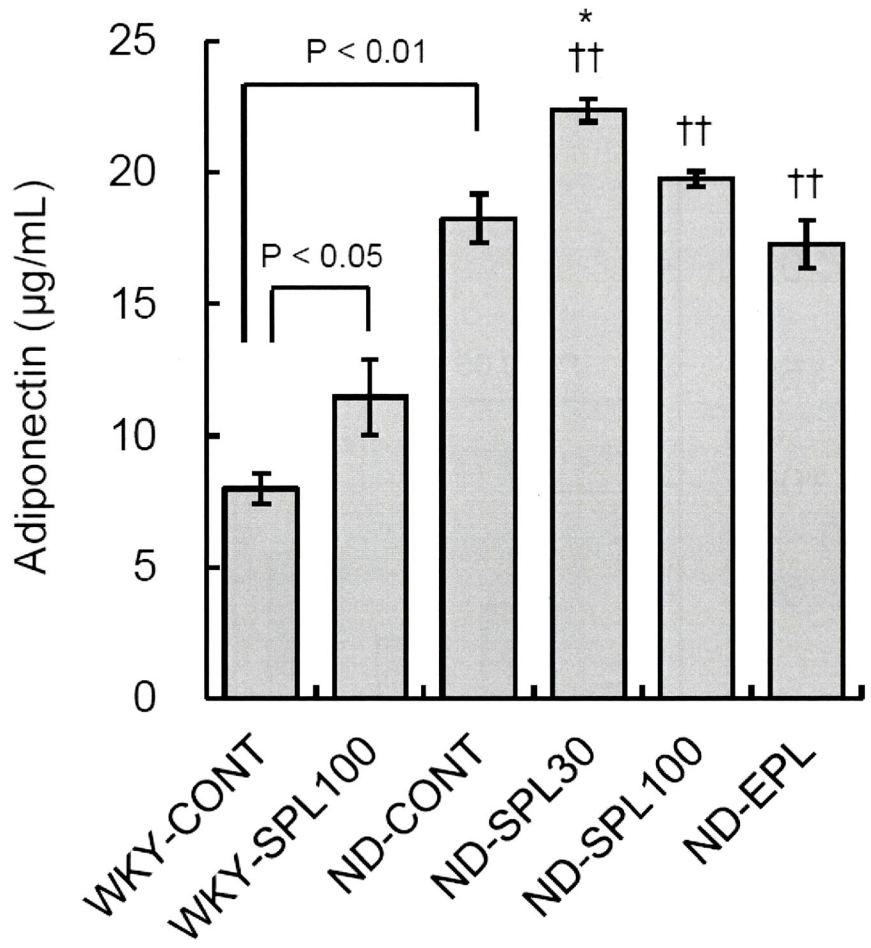

Figure 31. Serum adiponectin level on Day 17

SPL, EPL or vehicle was administered orally to ND or WKY rats once a day during the experimental period. On Day 17, serum adiponectin was measured by ELISA. The values are given as mean ± SE. n = 6 for each group. *, $P<0.05$ compared with ND-CONT. ††, $P<0.01$ compared with WKY-CONT. NS, not significant.

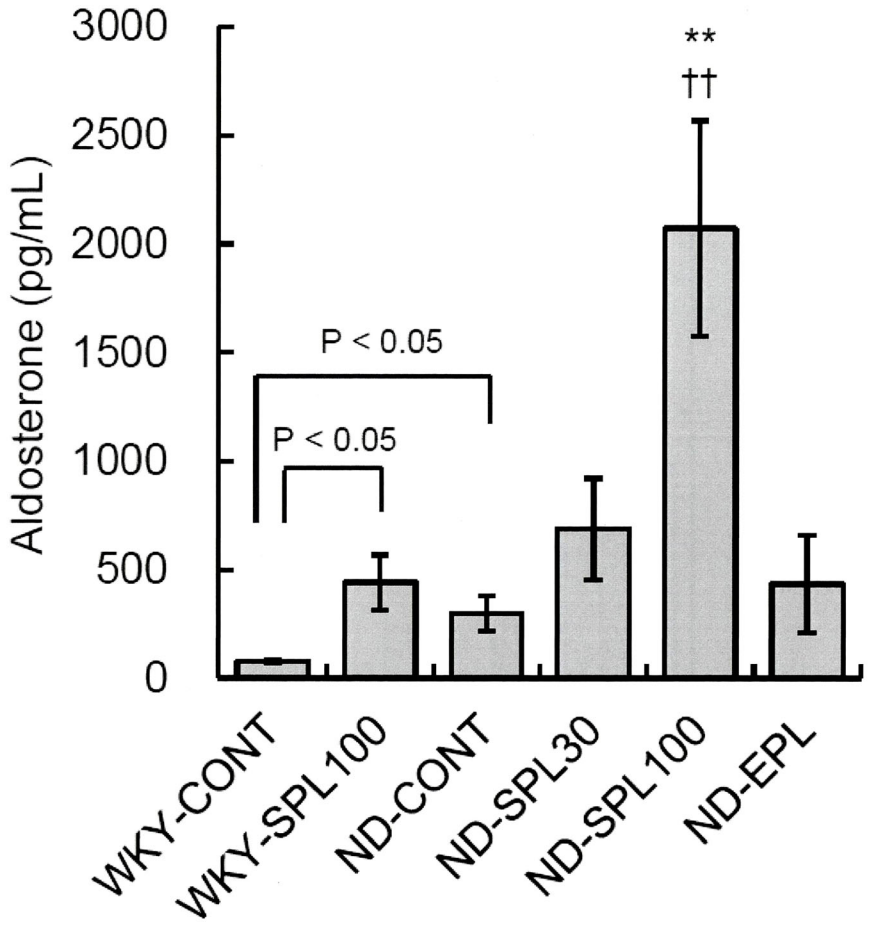

Figure 32. Serum aldosterone level on Day 17

SPL, EPL or vehicle was administered orally to ND or WKY rats once a day during the experimental period. On Day 17, serum aldosterone concentrations were measured by RIA. The values are given as mean ± SE. n = 6 for each group. **, P <0.01 compared with ND-CONT. ††, P<0.01 compared with WKY-CONT. NS, not significant.

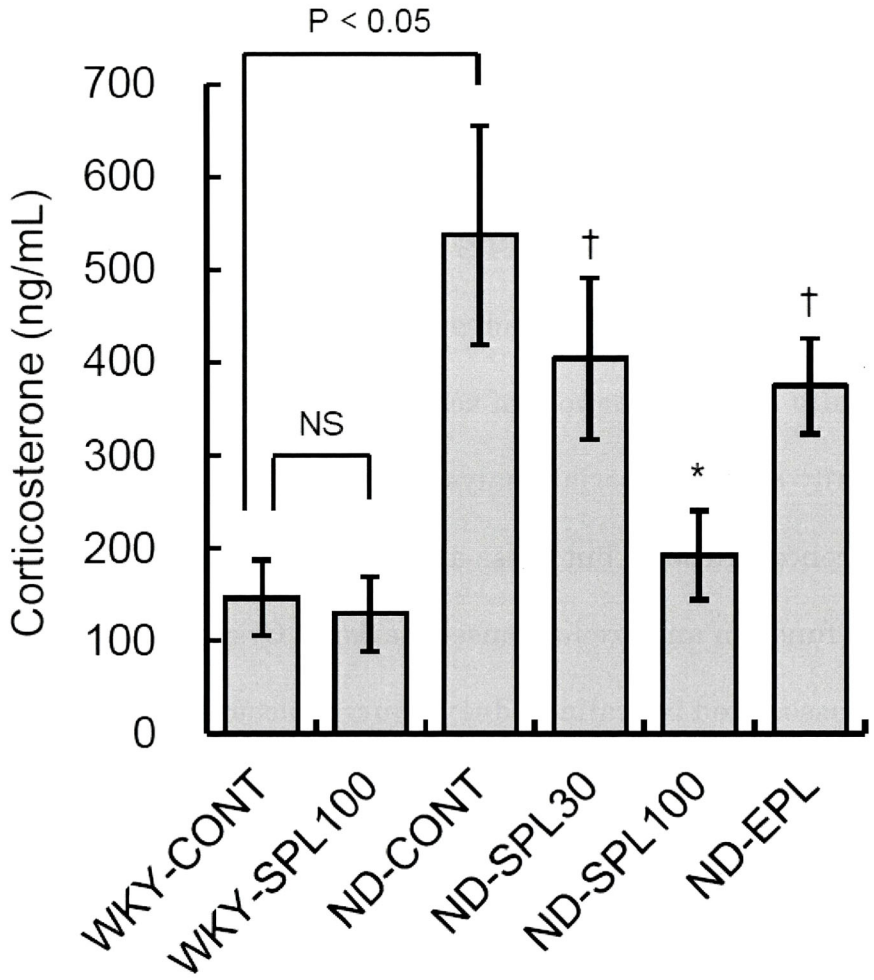

Figure 33. Serum corticosterone level on Day 17

SPL, EPL or vehicle was administered orally to ND or WKY rats once a day during the experimental period. On Day 17, serum corticosterone concentrations were measured by RIA. The values are given as mean ± SE. n = 6 for each group. *, P <0.05 compared with ND-CONT. †, P <0.05 compared with WKY-CONT. NS, not significant.

Further discussion

CRS is a novel concept to understand the crosstalk between heart disease and kidney disease (1), however, there actually remains some challenges in diagnosis and treatment of each CRS due to lack of good biomarkers. For example, in acute type of CRS, such as Type 1 and Type 3, there remains large difficulty in early diagnose of acute kidney injury (AKI). The diagnosis of AKI usually hinges on serial analyses of blood urea nitrogen and creatinine concentrations, but these are relatively insensitive indices of the glomerular function and are late onset markers. Cystatin C, neutrophil gelatinase-associated lipocalin, kidney injury molecule 1, and some cytokines etc., have been proposed as novel biomarkers. In addition, Sonoda et al. has reported that urinary exosomal aquaporin-1, a water channel protein, could be a novel biomarker of AKI (52).

The lack of established animal model is another reason for the limitation of CRS researches, however, in Chapter I, I concluded that the rat model combined myocardial infarction with unilateral nephrectomy, might be a useful animal model of type 2 CRS. In this model, cardiac dysfunction accelerated progressive renal injury, and blockade of Ang II signal using ARB reduced this renal injury independently of both blood pressure and cardiac function. It is well known that inflammation is associated with various types of renal injury, and the mononuclear cells infiltrating into renal interstitium, I could not yet identify the exact type of cell, strongly expressed AT1R, in this

CRS model. Therefore, I speculate that the cells might have a critical role in renal injury.

In clinical, worsening renal function in the context of heart failure is associated with adverse outcomes and prolonged hospitalization (107), although there is very limited understanding of the pathophysiology of renal dysfunction induced by cardiac dysfunction. According to my research results in the CRS model, blockade of the RAS is considered to be a promising strategy for renal protection in patients with heart failure. To establish the adequate strategies of diagnosis and treatment of CRS, it is necessary to drive further studies of CRS both in animal and clinical.

The metabolic syndrome is a growing health concern due to its high risk of CVDs. A growing body of evidence suggests that not only Ang II but also aldosterone are contributed to both the high risk of CVDs and the pathogenesis of the components of metabolic syndrome, such as hypertension, insulin resistance and obesity (83). Therefore, MR blockade can be expected to be a therapeutic option for the treatment of metabolic syndrome. Contrary to the expectation, in Chapter II, I concluded that SPL potentially induced further insulin resistance in metabolic syndrome rats, which was associated with excess elevation of aldosterone, although EPL at a similar effective dosage on urinary electrolytes did not. In terms of hyperglycemia, EPL is considered to have a superior profile compared with SPL.

There are some further speculations for underlying mechanisms of SPL-induced blood glucose elevation. It is well-established that blockers of

the RAAS prompt upstream activation, and MR blockers increase not only aldosterone, but also plasma renin activity and Ang II, which is another risk factor of insulin resistance (11). In my study, the marked increase in aldosterone concentration by SPL was detected, therefore, compensatory increase of Ang II should also happen simultaneously. Recently, Garg et al. have reported that low-salt diet increases insulin resistance in healthy subject, which is possibly associated with RAAS activation (108). Therefore, an excessive compensatory activation of RAAS could be a cause of glucose intolerance in SPL-treated rats.

Interestingly, SPL-induced glucose elevation was not detected in non-obese control rats. Not only systemic but also the local renin-angiotensin-system (RAS) in adipose tissue is closely related with the complications in obesity (109). Adipose tissue expresses all the components of RAS, and locally produced Ang II may impair the adipose tissue insulin sensitivity via both autocrine and paracrine mechanisms. Importantly, this adipose RAS is positively regulated by Ang II (110). It is possible that compensatory activated systemic RAAS by SPL could upregulate the adipose RAS followed by insulin resistance in adipose tissue. In fact, neither blood glucose elevation nor marked increase of aldosterone was detected in the non-obese control animal. The accumulated adipose tissue in obese rats could accelerate SPL-induced glucose intolerance via adipose RAS activation.

As shown in Chapter II, the drugs even in the same class each have individual features (potency, specificity, pharmacokinetics, toxicology, etc.)

and these differences can lead to distinct pharmacological and toxicological profiles. Therefore, it is important to know the profiles of each agent in detail for adequate medication.

Although many drugs are available in clinical, the patients with hypertension, diabetes mellitus, hyperlipidemia, and obesity, have remained largely unchanged and in some cases have even been increasing. For development of novel and appropriate therapeutic strategies for each patient, it is important to know in detail about both the pathophysiology of each disease and the features of each drug. I hope that the results of my research would provide more opportunities to find novel pharmaceutical targets and therapeutic strategies for the medication of CVDs.

Conclusion

The rats with combined uninephrectomy and myocardial infarction could be a good animal model of Type 2 CRS, and cardiac dysfunction could accelerate progressive renal injury through activation of RAS in these rats. Moreover, the blockade of Ang II signal using ARB would be a good therapeutic or preventive strategy of Type 2 CRS.

In addition, SPL, but not EPL, potentially impaired glucose tolerance in an animal model of metabolic syndrome, in association with a higher blood level of aldosterone.

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Summary

Introduction:

Although a number of drugs have been developed and are available for treatment to improve various risk factors of cardiovascular diseases (CVDs), the prevalence of risk factors of CVDs including hypertension, diabetes mellitus, hyperlipidemia, and obesity, has remained largely unchanged and in some cases has even been increasing. For development of appropriate therapeutic strategies for each patient, it is important to know in detail about both the pathophysiology of each disease and the features of each drug.

The renin-angiotensin-aldosterone system (RAAS) is well-known as a major risk factor of CVDs, and blockade of RAAS is attractive therapeutic strategy. Four classes of RAAS blockers, direct renin inhibitor, angiotensin converting enzyme inhibitor, angiotensin II type 1 receptor (AT1R) blocker (ARB), and mineralocorticoid receptor (MR) blocker, are currently available in clinical setting as antihypertensive/cardiorenal protective agents. I focused on the ARB and MR blocker, and the pharmacological effects on various disease models were evaluated to know about the pathophysiology of each disease and the features of each drug.

Chapter I:

Importance of renal angiotensin II type 1 receptor activation in progressive renal injury of a rat model for chronic cardiorenal syndrome

A number of reports have demonstrated a closed linkage between cardiac dysfunction and renal dysfunction. This interaction termed the cardio-renal syndrome (CRS) is a recent, well-recognized definition in clinical. CRS is categorized into five types (Type 1 - 5) according to the pathophysiology, time-frame, and nature of the concomitant cardiac and renal dysfunction. To clarify the pathophysiological mechanisms underlying each subtype will help to understand the clinical derangement and provide the rationale for management strategies for the patients with cardiac or renal dysfunction. However, established animal models for CRS are very limited. Therefore, to elucidate the detail of the mechanism of CRS, I created the rat model combining myocardial infarction (MI) with unilateral nephrectomy (NX), and examined several cardiac, renal and neurohumoral parameters in the rats.

Proteinuria, a biomarker of renal injury, gradually increased in the NX and MI+NX groups, the level being markedly higher in the latter. In contrast, the MI group did not exhibit proteinuria. A marked decrease in cardiac function was observed in both the MI and MI+NX groups, however, both cardiac parameters and MI size were not different between the two groups. In the histological analysis, urinary cast formation, infiltration of mononuclear cells, an increase in the mesangial matrix, adhesion of Bowman's capsule, interstitial fibrosis, and glomerulosclerosis were evident in the MI+NX group. On the other hand, these changes in the NX group were moderate, and were not observed in the MI group. These data clearly indicated that our MI+NX model was characterized by progressive kidney

injury without enhancement of cardiac injury, which is defined as Type 2 CRS.

The renal gene expression of interleukin-1β, an inflammatory cytokine, and transforming growth factor-β1, a profibrogenic cytokine, were significantly up-regulated in the MI+NX group. Plasma renin activity was lower only in the MI+NX, on the other hand, the gene expression of AT1R in the kidney was higher only in the MI+NX group. In the immunohistochemical analysis, up-regulation of AT1R was observed in the renal cortex in the MI+NX group. Higher magnification 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. Also, increased AT1R protein was seen in the outer medulla in the MI+NX group. These results suggest that cardiac dysfunction accelerates renal injury, which is associated with inflammation and fibrosis via AT1R signal. Finally, I examined the effect of olmesartan (OLM), an ARB, and spironolactone (SPL), a MR antagonist. Although SPL showed no effect on urinary protein, OLM significantly suppressed proteinuria as well as the histological alterations resulting from the combined surgery without any effects on hear rate, blood pressure, and the cardiac function. These data clearly indicate that ARB reduced proteinuria was mediated by direct inhibition of renal AT1R signaling.

Chapter II:

Spironolactone, but not eplerenone, impairs glucose tolerance in a rat model of metabolic syndrome

A number of reports have demonstrated the relation between aldosterone and impairment of glucose metabolism. Spironolactone (SPL) and eplerenone (EPL), both of which are MR antagonists currently used in a clinical setting, are widely recognized to be beneficial for patients with hypertension and heart failure. However, the two drugs are known to have distinct pharmacological/pharmacokinetic profiles. Regarding the effect on glucose metabolism, it has been reported that SPL, but not EPL, increased HbA1c in patients with mild chronic heart failure, although the detailed mechanism remains unknown. In the present study, therefore, I examined the effect of SPL on blood glucose levels in SHR/NDmcr-cp(cp/cp) (ND) rats, an animal model of metabolic syndrome, in comparison with that of EPL.

Although SPL had no effect on the blood glucose level in non-obese control rats, administration of SPL to ND rats dose-dependently and significantly increased this. In contrast, EPL, at the dose which exerts the same efficacy on urinary electrolyte, had no effect on the blood glucose level. In the oral glucose tolerance test, SPL dramatically impaired glucose tolerance. Neither SPL nor EPL exerted significant effects on the serum insulin levels in ND rats. The serum level of adiponectin, a reduction of which is known to cause insulin resistance, was not altered by SPL. The serum aldosterone concentration was significantly increased. On the other

hand, EPL had no effect on the serum aldosterone level. These results suggest that the excess compensatory activation of RAAS by SPL induces insulin resistance.

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