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Combination of exercise and losartan enhances renoprotective and peripheral effects in spontaneously type 2 diabetes mellitus rats with nephropathy

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Objectives We assessed the renal and peripheral effects of chronic exercise in a rat model of diabetic nephropathy and the benefits of combined exercise and losartan.

Methods Heminephrectomized Goto–Kakizaki rats were divided into four groups: (i) no exercise (control); (ii) exercise with treadmill running; (iii) losartan; (iv) exercise plus losartan, and the rats were treated for 12 weeks.

Results Losartan and exercise plus losartan significantly decreased systolic blood pressure (SBP). Exercise, exercise and losartan, and losartan blunted the increases in proteinuria. The index of glomerular sclerosis (IGS) and the relative interstitial volume of the renal cortex were significantly improved in the exercise, exercise and losartan, and losartan groups. The IGS, expressions of ED-1 and α-smooth muscle actin in the glomerulus were the lowest, and the number of Wilms' tumour was the highest in the exercise plus losartan group. The endurance, the proportion of type I fibre and capillarization in the extensor digitorum longus muscle were greater in the trained groups.

Conclusion These results suggest that both exercise and losartan have renoprotective effects, and the combination of exercise and losartan provided greater renoprotective effects than losartan alone, and may affect macrophage infiltration, mesangial activation, and podocyte loss in this model of diabetic nephropathy. It is also suggested that exercise has a specific renoprotective effect that is not related to SBP reduction, and can enhance endurance without renal complications. *J Hypertens* 26:312–321
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Keywords: chronic exercise, combination therapy, diabetic nephropathy, endurance, losartan, rat

Abbreviations: ANOVA, analysis of variance; BUN, blood urea nitrogen; BW, body weight; C, no exercise; CD, capillary density; C/F, capillary-to-fiber ratio; DN, diabetic nephropathy; EDL, extensor digitorum longus; EX, chronic exercise; D, fiber density; GCS, glomerular cross section; GK, Goto-Kakizaki; Glu, blood glucose; IGS, index of glomerular sclerosis; LOS, losartan; mATPase, myofibrillar adenosine triphosphatase; NX, heminephrectomy; RIV, relative interstitial volume of the renal cortex; SBP, systolic blood pressure; Scr, serum creatinine; SEM, standard error of the mean; SMA, smooth muscle actin; TRD, total running distance; UP, 24-h urinary excretion of protein; VO_2, oxygen consumption; WT-1, Wilms' tumor-1

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Introduction

The incidence of type 2 diabetes mellitus has been increasing worldwide. Individuals with type 2 diabetes mellitus are at risk of developing diabetic nephropathy, and the number of cases progressing to end-stage renal disease from diabetic nephropathy is increasing continuously. It is predicted that globally patients with overt nephropathy will overwhelm healthcare resources and finances by the year 2025 [1]. Therefore, the establishment of an effective therapy to prevent the progression of diabetic nephropathy is a matter of great urgency.

Patients with end-stage renal disease have low exercise tolerance and experience progressively worsening disability and quality of life [2]. Although advances in dialysis treatment have extended the lifespan of patients with end-stage renal disease, this treatment alone does not ensure preservation of the quality of life. In particular, muscle strength and endurance are decreased in such patients [3]. The presence of type 2 diabetes mellitus has a negative effect on these problems and may increase the risk of cardiovascular disease in patients with chronic renal failure [4]. The markedly low exercise capacity of patients with diabetic nephropathy [5] indicates that there is a need to increase physical function, and the benefits of exercise training in type 2 diabetes mellitus on glycemic control and its protective effect on cardiovascular disease in such patients are well known [6]. It is also necessary, however, to consider the influence of exercise on renal function because acute exercise causes proteinuria and decreases the renal blood flow and glomerular filtration rate [7].

There are few reports on the influence of chronic exercise on renal function and there is little information about the effect of exercise on diabetic nephropathy in humans. Castaneda *et al.* [8] reported in a clinical trial of 26 patients with renal disease, including 10 patients with diabetic

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nephropathy treated for 12 weeks, that resistance training and a low protein diet significantly improved the glomerular filtration rate.

In addition, there are few reports about the effect of exercise on renal function in animal models of chronic renal failure. It has been reported that exercise improved the renal function and morphology in rats with subtotal nephrectomy [9,10], and that exercise significantly decreased urinary albumin excretion and the mesangial volume in animals with diabetic nephropathy [11–13].

Various studies have shown that exercise training can be beneficial in patients with chronic renal failure [14]. Most studies have excluded patients with type 2 diabetes mellitus, although there are many patients with end-stage renal disease who have type 2 diabetes mellitus, which may contribute to the limited exercise capacity. The optimal intensity and duration of exercise for patients with chronic renal failure has not yet been formulated.

Similarly, because appropriate animal models are few, there is no definitive conclusion on whether exercise has any renal protective effect and what kind of exercise produces renal benefits and also enhances exercise tolerance. So far, the mechanism of the effects of exercise on renal function has not been established and its effect on kidney lesions using an immunohistological study has not been investigated. Moreover, the effect of exercise on renal parameters and on the muscle morphology and capillarity in a Goto–Kakizaki rat model with nephropathy has not yet been studied. A better understanding of the mechanisms of the effect of exercise on these parameters may facilitate the establishment of an optimal exercise programme for patients with diabetic nephropathy.

Losartan is a potent, highly selective and orally active angiotensin II type 1 receptor antagonist [15]. Besides its antihypertensive effect, many experimental and clinical data have demonstrated that losartan provides significant renal protection and has a slowing effect on the progression of diabetic nephropathy [16,17].

The present study was designed to test the hypothesis that exercise has a renal protective effect, changes muscle morphology and capillarity, and enhances endurance in a diabetic rat model with nephropathy. In addition, we performed an immunohistological study of the renal tissue from rats with diabetic nephropathy to evaluate the cellular mechanism underlying the effect of exercise and the combined effect of exercise and losartan in a rat model of diabetic nephropathy.

Methods

Animals

Forty-four 8-week-old male spontaneously type 2 diabetes mellitus rats (Goto–Kakizaki; CLEA Japan, Tokyo, Japan) were used. In order to hasten the development of diabetic nephropathy, the rats were subjected to heminephrectomy, fed a high-caloric diet (350 kcal/100 g, 0.43 wt% sodium, 0.47 wt% potassium, 13.5 wt% protein; Nosan Corporation, Yokohama, Japan) and had free access to 30% sucrose solution and tap water. Heminephrectomy was performed by removal of the left kidney under ether anaesthesia. The left kidney was exposed via a flank incision, removed in total, and the flank incision closed. Rats were housed in a metabolic cage (model ST; Sugiyamagen, Tokyo, Japan) designed to prevent feces–urine contact and kept in a humidity and temperature-controlled room ($55 \pm 10\%$ and $22 \pm 2°C$) with a 12-h light/dark cycle. Every 2 weeks, 24-h urine samples were collected and proteinuria was assessed semiquantitatively using a dipstick method (Pretest; Wako Pure Chemical Industries, Osaka, Japan). When the concentration of urinary excretion of protein increased more than 300 mg/dl, the rats were 20 weeks old and they were randomly divided into four groups: (i) no exercise (control; $n = 10$); (ii) exercise with treadmill running (KN-73; Natsume Industries Co., Tokyo, Japan) at a speed of 20 m/min, 0 grade incline for 60 min/day, 5 days/week ($n = 10$); (iii) exercise and losartan ($n = 10$); (iv) losartan ($n = 10$). The rats were then treated for 12 weeks.

This running programme was chosen because Kanazawa *et al.* [10] have shown that this amount of exercise had renoprotective and peripheral effects in a 5/6-heminephrectomized Wistar–Kyoto rat.

Losartan (5 mg/kg per day) was administrated continuously using osmotic minipumps (model 2002; Alza Corp., Palo Alto, California, USA), implanted subcutaneously every 2 weeks in the cervical subcutis under light ether anaesthesia.

Measurements

Baseline measurements of body weight, systolic blood pressure (SBP), and 24-h urinary excretion of protein were made when the rats were 20 weeks old. A treadmill test was performed and the oxygen consumption (VO_2) when the rats were running at a speed of 20 m/min, 0 grade incline, the peak VO_2 and total running distance (TRD) were measured using an oxygen/carbon dioxide metabolism measuring system with a sealed chamber treadmill (model MK-5000, MK680AT/02R; Muromachikikai, Tokyo, Japan) [18]. Peak VO_2 was measured by expired gas analysis during progressive exercise testing on a motor treadmill with 5 m/min increments (5–35 m/min) every 3 min and no grade incline until exhaustion. Rats were considered to be exhausted when they were unable to keep running at the treadmill speed. TRD (in metres) was defined as the distance between the beginning of exercise and exhaustion. The treadmill exercise test was repeated at the end of the experimental protocol and TRD was measured.

SBP was monitored every 2 weeks in conscious rats by an indirect tail-cuff method (UR5000; Elquest Corporation, Ciba, Japan) without anaesthesia [19] between 1300 and 1500 h. Urine volume and body weight were measured gravimetrically, and urine for the measurement of urinary protein was collected every 6 weeks and stored at −80°C.

At the end of the experiment, the rats were killed by decapitation. Blood samples from the trunk were collected in polyethylene tubes for the determination of serum creatinine, blood urea nitrogen (BUN) and fed blood glucose. Urinary protein, serum creatinine, BUN and blood glucose were measured by a standard autoanalysis technique (Synchron-CX-3; Beckman Coulter Inc., Fullerton, California, USA). The extensor digitorum longus (EDL) muscles were removed and frozen immediately in liquid isopentane, cooled in dry ice, and stored at −80°C until sectioning.

Index of glomerular sclerosis and relative interstitial volume of the renal cortex

At the end of the experiment, the rats were killed, and portions of the remnant kidneys were removed and fixed in 10% neutral buffered formalin, and the index of glomerular sclerosis (IGS) and relative interstitial volume (RIV) were assessed by the methods previously described [10].

Immunohistochemistry of the remnant kidney

Other portions of the remnant kidneys were fixed in 95% cold ethanol and then embedded in paraffin blocks. Primary antibodies included the following: ED-1: a mouse antirat CD68 monoclonal antibody to a cytoplasmatic antigen present in macrophages (Serotec, Oxford, UK), working dilution 1/100; α-smooth muscle actin (SMA): a mouse monoclonal antihuman SMA (1A4; Dako, Glostrup, Denmark), working dilution 1/200; Wilms' tumour (WT)-1: a rabbit polyclonal anti-Wilms' tumour protein (C19; Santa Cruz Biotechnology, California, USA), working dilution 1/100.

An indirect immunoperoxidase technique was employed as previously described [20,21], with some modifications. In brief, 2 μm-thick paraffin sections were mounted on glass slides for ED-1 and α-SMA staining and deparaffinized. For WT-1 staining, 2 μm-thick paraffin sections were mounted on silane-coated slides (Dako, Kyoto, Japan) and then deparaffinized sections were treated with 10 mmol/l of citrate buffer, pH 6.0 in a microwave for 10 min. After washing in phosphate-buffered saline (pH 7.4), the sections were blocked with 5% normal goat serum, then incubated with individual primary antibodies for 1 h at room temperature. Then they were incubated with peroxidase-conjugated goat antimouse or antirabbit immunoglobulines (Histofine Simple Stain Rat MAX PO (M) or (R); Nichirei Biosciences Inc., Tokyo, Japan) for 30 min at room temperature. Immunoreaction products were developed using 0.02% 3,3′diaminobenzidine tetrahydrochloride (Dako, Japan) in Tris hydrochloride buffer (pH 7.4) containing 0.01% H_2O_2 and 0.3% NaN_3 for 5 min. For WT-1 staining we used the same substrate solution with 0.07% nickel chloride enhancement.

The number of positively stained cells for ED-1 and WT-1 was expressed as the mean cell number per glomerular cross-section for each section. The glomerular expression of α-SMA was graded semiquantitatively according to the area of positive expression from 0 to 4: grade 0, none; grade 1, < 25%; grade 2, 25–49%; grade 3, 50–75%; grade 4, > 75% of the cortex in one section, as previously described [22]. The index of glomerular α-SMA was calculated using the following formula:

$$(1 \times N_1 + 2 \times N_2 + 3 \times N_3 + 4 \times N_4)/(N_0 + N_1 + N_2 + N_3 + N_4),$$

where N is the number of glomeruli at each grade of sclerosis.

Muscle morphology and capillarity

Serial transverse cross-sections (8 μm thick) near the mid-belly portion of the EDL muscle were cut in a microtome cryostat (Micron HM 520; Walldorf, Germany) at −22°C, mounted on silane-coated slides (Dako, Japan), and air dried.

Each muscle fibre type was determined by a myofibrillar adenosine triphosphatase staining method [23,24] after preincubation in alkaline (pH 10.7) and acid (pH 4.4 and 4.7) solution.

For capillarization analysis, another section from each sample was fixed in acetone-chloroform (1 : 1) mixture, and then incubated in dipeptidyl peptidase substrate solution for 90 min at room temperature. The slides were rinsed in distilled water before transfer to a substrate solution specific for alkaline phosphatase for 25 min. The treatment with alkaline phosphatase stains the arterial ends of the capillary segments blue, whereas the dipeptidyl peptidase stains the venous ends of the capillary segments red [25]. The validity of this double staining method for the differentiation of arteriolar and venular capillary portions was confirmed previously [26]. The stained sections for capillarization were photographed, and the blue-stained capillaries in artefact-free 0.271 mm^2 areas were counted and the capillary density (capillaries/mm^2) and capillary-to-fibre ratio (C/F) were determined in each section [27].

Fibre type classification was performed according to the staining intensities [24]. The entire muscle cross-section was digitally imaged with a CCD colour camera CS530MD

connected to an optical microscope. Then, the muscle fibre composition was determined by evaluating more than 500 countable fibres in each section and artefact-free 1.085 mm^2 areas were used for determining the fibre cross-sectional area for the calculation of fibre density.

The measurement system was composed of a computer-assisted image analysis system (SD-510C, Wacom, Tokyo, Japan). The software used for analysing the images was Win ROOF version 5.0 (Mitani Corporation, Tokyo, Japan).

Statistical analysis

Values are expressed as the means ± SEM. With respect to serum creatinine, BUN, blood glucose, TRD, RIV, glomerular ED-1-positive cells, the number of WT-1-positive cells, C/F, capillary density and muscle fibre type, comparisons between the different groups of rats were made using one-way analysis of variance and Bonferroni/Dunn test. For TRD, a comparison between the groups was made using the paired *t*-test. For SBP, urinary protein and body weight comparisons between the different groups of rats were made by analysis of variance with repeated measures over the duration of the study. Statistically significant differences on each day were assessed between groups by the Bonferroni/Dunn test. With respect to the IGS and index of glomerular α-SMA, comparisons between the different groups of rats were made using the Kruskal–Wallis test and Bonferroni/Dunn test. Values of $P < 0.05$ were considered statistically significant. The statistical analysis in the present study was performed using the statistical software package STATVIEW 5.0 (Abacus Concepts Inc., Berkeley, California, USA).

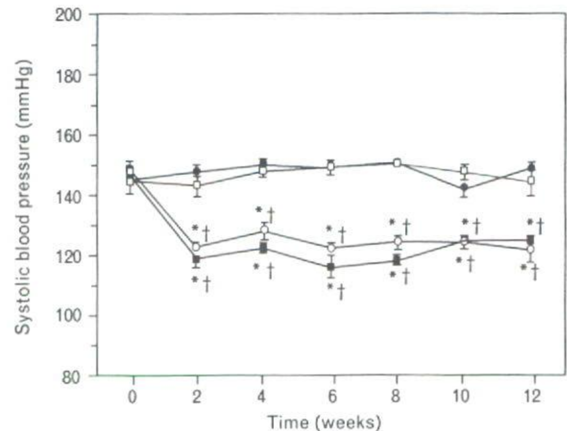
This study conformed to the principles for the use of live animals outlined in the Declaration of Helsinki and those of the ethics committee of Tohoku University Graduate School of Medicine.

Results

The peak VO_2 in the heminephrectomized rats before the start of the experiment was 6.2 ± 0.14 ml/min per 100 g body weight. The VO_2 when the heminephrectomized rats were running at a speed of 20 m/min was 4.7 ± 0.07 ml/min per 100 g body weight and corresponded to approximately 75% of the peak VO_2.

At baseline, the SBP in the heminephrectomized rats was 147 ± 1 mmHg and there were no significant differences among all the groups. At the end of the experiment, the SBP in the losartan and exercise plus losartan groups (121 ± 2 and 124 ± 4 mmHg, respectively) was significantly lower ($P < 0.0001$) than that in the control and exercise groups (149 ± 4 and 144 ± 2 mmHg, respectively) (Fig. 1). Treatment with either losartan or exercise plus losartan had similar antihypertensive effects and

Fig. 1



Sequential systolic blood pressure values in the following groups: no exercise ($n = 10$), losartan (5 mg/kg per day, subcutaneously) ($n = 10$), exercise with treadmill running ($n = 10$), exercise in combination with losartan ($n = 10$) groups during the 12 week experimental period. Values are expressed as the means ± SEM. —●— control; —□— exercise; —○— losartan; —■— exercise plus losartan. *$P < 0.0001$ versus control group, †$P < 0.0001$ versus exercise group.

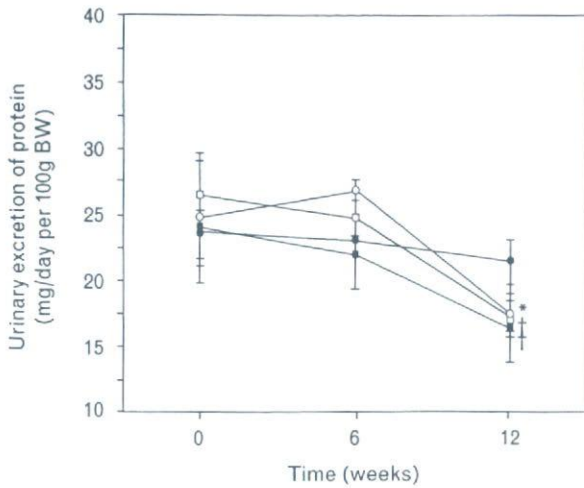
SBP in the exercise group was not significantly different from that in the control group.

After heminephrectomy, the high-caloric diet and sucrose solution, the urinary protein progressively increased in all groups of rats and, at the beginning of the 12-week experimental period, the urinary protein in the control group was 23.5 ± 1.7 mg/day per 100 g body weight and was not significantly different to that in the exercise, exercise plus losartan, and losartan groups (26.4 ± 2.6, 23.9 ± 2.7 and 24.7 ± 4.9 mg/day per 100 g body weight, respectively; Fig. 2). At the end of 12 weeks of treatment, the urinary protein in the exercise, exercise plus losartan, and losartan groups (17.1 ± 1.2, 16.3 ± 2.5, 17.7 ± 2 mg/day per 100 g body weight, respectively) was significantly lower ($P < 0.01$, $P < 0.01$ and $P < 0.05$, respectively) compared with that in the control group (21.4 ± 1.7 mg/day per 100 g body weight). The urinary protein was not significantly different among the exercise, exercise plus losartan, and losartan groups.

The serum creatinine and BUN were not significantly different among all groups of rats (Table 1).

There were no significant differences in body weight before the start of the 12-week experimental period among the groups of rats (360.8 ± 4.9, 379 ± 5, 374 ± 6 and 355.2 ± 3.3 g for exercise, exercise plus losartan, losartan and control groups, respectively). Over the 12-week study period, the body weight increased gradually in all groups of rats. At the end of the experiment, the body weight in the groups with exercise training (exercise and exercise plus losartan) was significantly lower ($P < 0.0001$) in

Fig. 2



Urinary excretion of protein during the 12-week experimental period. Values are expressed as the means ± SEM. —●— control ($n = 10$); —□— exercise ($n = 10$); —○— losartan ($n = 10$); —■— exercise plus losartan ($n = 10$). BW, Body weight. $^*P < 0.05$, $^†P < 0.01$ versus control group at 12 weeks.

comparison with those in the losartan and control groups (Table 1). The body weight was not significantly different between the losartan group and the control group.

The blood glucose level at the end of the 12-week experiment period was significantly higher ($P < 0.05$) in the exercise and exercise plus losartan groups than in the control group (Table 1). The blood glucose level was not significantly different between the exercise and the exercise plus losartan groups.

Exercise endurance

The TRD before the start of the 12-week exercise training (0 week) in all groups was not significantly different. At the end of the experiment (12 weeks), the TRD in the exercise and exercise plus losartan groups was significantly longer ($P < 0.0001$) than that in the losartan and control groups (Fig. 3). The TRD in the exercise group was not significantly different from that in the exercise plus losartan group. In contrast, the TRD at 12 weeks in the control group was significantly shorter than that at 0 weeks ($P < 0.05$). There was no significant difference between the TRD at 0 weeks and that at 12 weeks in the losartan group.

Renal histology

The control group demonstrated focal and segmental glomerular structural lesions and increased cortical interstitial volume. These lesions were milder in the exercise plus losartan and losartan groups than in the control group (Fig. 4).

The IGS in the control group was the highest (Table 1). The IGS in the exercise and losartan groups were significantly lower than that in the control group ($P < 0.0001$, $P < 0.001$, respectively) and there was no significant difference between the exercise and losartan groups. The IGS in the exercise plus losartan group was significantly lower than that in the control, exercise and losartan groups ($P < 0.0001$, $P < 0.05$, $P < 0.01$, respectively).

The RIV in the control group was the highest (Table 1). The RIV in the exercise, exercise plus losartan, and losartan groups was significantly lower than that in the control group ($P < 0.0001$). The RIV in the exercise and exercise plus losartan groups was significantly lower than that in the losartan group ($P < 0.05$). There was no significant difference between the exercise and exercise plus losartan groups.

Immunohistochemistry of the remnant kidney

The number of glomerular ED-1-positive cells was significantly lower ($P < 0.05$) in the exercise plus losartan group than that in the control group (Table 1, Fig. 5). There was no significant difference between the exercise, losartan and control groups.

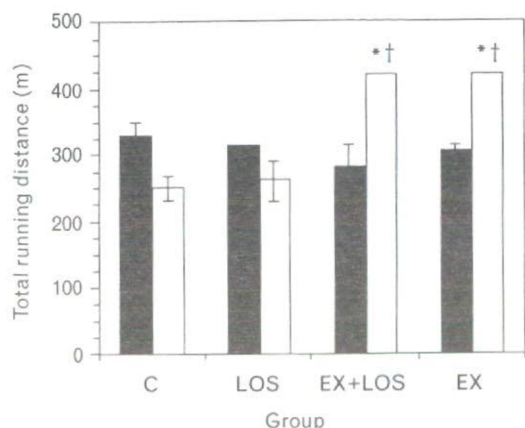
The number of WT-1-positive cells was highest in the exercise plus losartan group and was significantly higher ($P < 0.05$) than that in the control and exercise groups (Table 1, Fig. 5). There was no significant difference between the exercise, losartan and control groups.

Table 1 The serum creatinine, blood urea nitrogen, fed blood glucose, body weight, index of glomerular sclerosis, relative interstitial volume, ED-1, α-smooth muscle actin and Wilms' tumour 1 at 12 weeks

Groups	Serum creatinine (mg/dl)	BUN (mg/dl)	Fed blood glucose (mg/dl)	Body weight (g)	IGS	RIV (%)	ED-1 (cells/GCS)	α-SMA	WT-1 (cells/GCS)
Control ($n = 10$)	0.7 ± 0.02	5.7 ± 0.35	231.5 ± 7.6	434.2 ± 3.0	1.71 ± 0.04	11.2 ± 0.3	3.5 ± 0.2	1.16 ± 0.02	9.2 ± 0.3
Losartan($n = 10$)	0.68 ± 0.02	8 ± 0.5	263 ± 14.4	451.7 ± 8.05	$1.47 \pm 0.04^¶$	$8.2 \pm 0.2^*$	3.5 ± 0.3	1.12 ± 0.04	10 ± 0.3
Exercise ($n = 10$)	0.75 ± 0.01	6.3 ± 0.52	$274.4 \pm 10.9^*$	$414.4 \pm 5.7^{*†}$	$1.39 \pm 0.03^*$	$6.9 \pm 0.2^{*†}$	3.2 ± 0.1	1.15 ± 0.01	9.2 ± 0.2
Exercise + losartan ($n = 10$)	0.71 ± 0.03	8.1 ± 0.7	$278 \pm 9.43^*$	$411.8 \pm 6.8^{*†}$	$1.25 \pm 0.05^{*†‡}$	$6.2 \pm 0.3^{*†}$	$2.5 \pm 0.2^*$	$0.87 \pm 0.02^{*†‡}$	$10.2 \pm 0.2^{*†}$

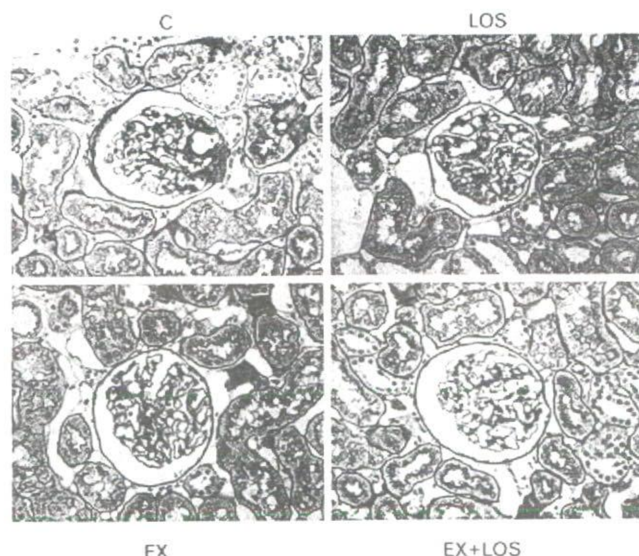
BUN, blood urea nitrogen; Control, no exercise; Exercise, exercise with treadmill running; IGS, index of glomerular sclerosis; RIV, relative interstitial volume; SMA, smooth muscle actin; WT, Wilms' tumour. Values are expressed as means ± SEM. The serum creatinine and BUN were not significantly different among the control, losartan, exercise and exercise plus losartan groups. Glucose: $^*P < 0.05$ versus control group; body weight: $^*P < 0.0001$ versus control group, $^†P < 0.0001$ versus losartan group; IGS: $^*P < 0.0001$ versus control group, $^†P < 0.01$ versus losartan group, $^‡P < 0.05$ versus exercise group, $^¶P < 0.001$ versus control group; RIV: $^*P < 0.0001$ versus control group, $^†P < 0.05$ versus losartan group; ED-1: $^*P < 0.05$ versus control group; α-SMA: $^*P < 0.0001$ versus control group, $^†P < 0.0001$ versus losartan group, $^‡P < 0.0001$ versus exercise group; WT-1: $^*P < 0.05$ versus control group, $^†P < 0.05$ versus exercise group.

Fig. 3



Total running distance before and after the 12-week experimental period. Values are expressed as means ± SEM. ■ 0 weeks; □ 12 weeks. C, Control; EX, exercise; EX+LOS, exercise plus losartan; LOS, losartan. *$P<0.0001$ versus control group, †$P<0.0001$ versus losartan group at 12 weeks. Abbreviations as in Fig. 1.

Fig. 4



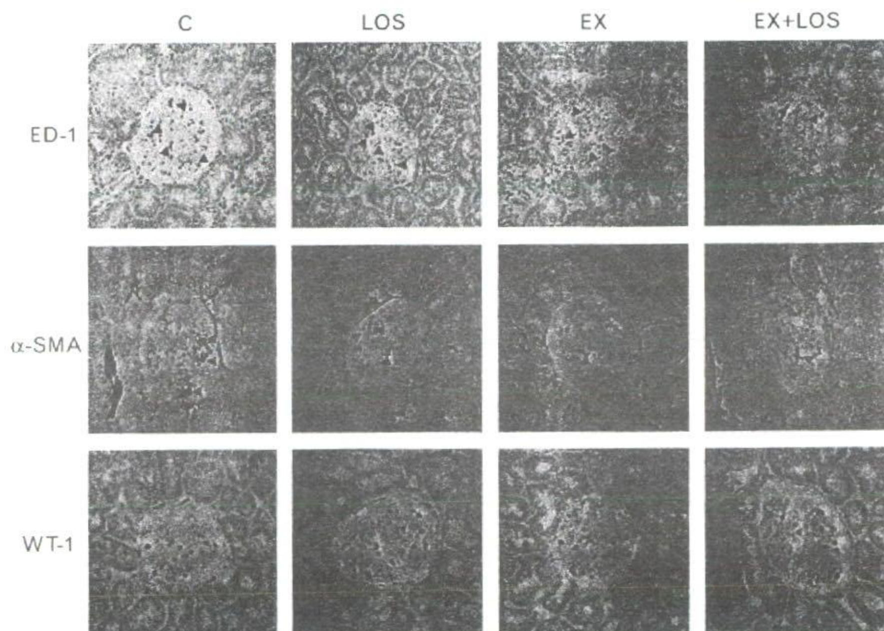
Representative light micrographs of the kidneys in the control ($n=10$), losartan ($n=10$), exercise ($n=10$) and exercise plus losartan ($n=10$) groups. (Periodic acid-Schiff, ×400). C, Control; EX, exercise; EX+LOS, exercise plus losartan; LOS, losartan.

The expression of glomerular α-SMA was lowest in the exercise plus losartan group and it was significantly less extensive than that in the exercise, losartan and control groups ($P<0.0001$; Table 1, Fig. 5). There was no significant difference between the exercise, losartan and control groups.

Muscle morphology and capillarity

The C/F in the exercise and exercise plus losartan groups was significantly higher ($P<0.05$) than that in

Fig. 5



Representative immunoperoxidase staining micrographs of the kidneys in the control ($n=10$), losartan ($n=10$), exercise ($n=10$), exercise plus losartan ($n=10$) groups for ED-1-positive cells (arrowheads), expression of α-SMA (arrowheads) and expression of WT-1 (black staining in nuclei of the podocytes). (Original magnification ×400). C, Control; EX, exercise; EX+LOS, exercise plus losartan; LOS, losartan; SMA, smooth muscle actin; WT, Wilms' tumour.

Table 2 The capillary-to-fibre ratio, capillary density, fibre density, and proportion of type I fibre at 12 weeks

Groups	C/F	Capillary density (no./mm^2)	Fibre density (no./mm^2)	Type I fibre (%)
Control ($n=10$)	1.40 ± 0.08	399.74 ± 1.96	319.05 ± 18.8	2.35 ± 0.58
Losartan ($n=10$)	1.45 ± 0.06	391.52 ± 17.7	306.14 ± 15.9	2.56 ± 0.38
Exercise ($n=10$)	$1.73 \pm 0.06^{*\dagger}$	$513.91 \pm 17.8^{\ddagger\S}$	329.49 ± 17.3	$4.25 \pm 0.68^{*\dagger}$
Exercise + losartan ($n=10$)	$1.7 \pm 0.06^{*\dagger}$	$537.47 \pm 17.8^{\ddagger\S}$	353.93 ± 23.3	$4.64 \pm 0.67^{*}$

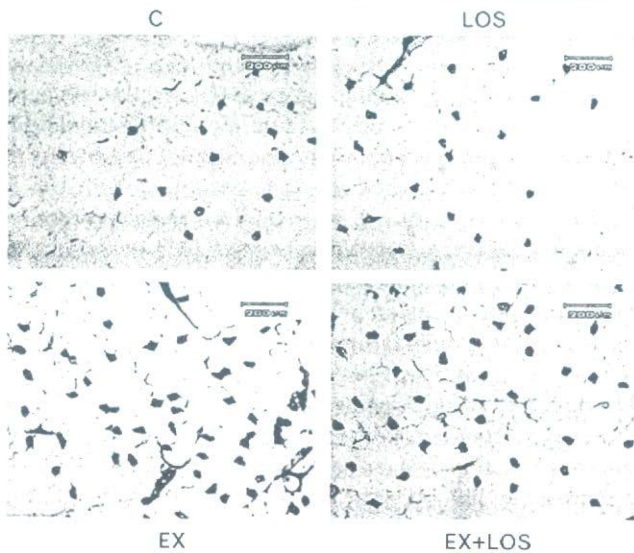
C/F, Capillary-to-fibre ratio; Control, no exercise; Exercise, exercise with treadmill running. Values are expressed as means ± SEM. C/F; $^{*}P < 0.05$ versus control group, $^{\dagger}P < 0.05$ versus losartan group; capillary density: $^{\ddagger}P < 0.0001$ versus control group, $^{\S}P < 0.0001$ versus losartan group; proportion of type I fibre: $^{*}P < 0.05$ versus control group; $^{\dagger}P < 0.05$ versus losartan group.

the control and losartan groups. There was no significant difference between the control and the losartan groups (Table 2).

Similarly, the capillary density in the exercise and exercise plus losartan groups was significantly higher ($P < 0.0001$) than that in the control and losartan groups. There was no significant difference between the control and the losartan groups (Table 2).

The proportion of high-oxidative type I fibre was significantly higher ($P < 0.05$) in the exercise group than that in the control and losartan groups. The proportion of type I fibre in the losartan plus exercise group was significantly higher than that in the control group ($P < 0.05$) and tended to be higher than that in the losartan group ($P = 0.051$; Table 2, Fig. 6).

Fig. 6



Representative cross-sections of extensor digitorum longus muscle in the control ($n = 10$), losartan ($n = 10$), exercise ($n = 10$) and exercise plus losartan ($n = 10$) groups. Sections were assayed for myofibrillar adenosine triphosphatase activity after different preincubation pH treatments. The type I fibre stained black after acid treatment (pH 4.4), whereas the others (i.e., type II fibres) stained light. Scale bar 200 μm. C, Control; EX, exercise; EX+LOS, exercise plus losartan; LOS, losartan.

Discussion

In the Goto–Kakizaki strain, type 2 diabetes mellitus usually develops at 3–4 weeks of age. The blood glucose level ranges from 200 to 300 mg/dl, and the metabolic profiles are similar to the findings in humans with type 2 diabetes mellitus. This strain exhibits glomerular structural lesions, and these characteristics are similar to those of progressive human diabetic nephropathy. Goto–Kakizaki rats, however, do not exhibit obvious alterations in renal morphological changes until at least approximately 8 months of age [28]. Therefore, in order to hasten the development of diabetic nephropathy, the rats were subjected to heminephrectomy, fed a high-caloric diet and had free access to 30% sucrose solution and tap water in this study. At 20 weeks of age, we confirmed that the concentration of urinary protein had increased more than 300 mg/dl. Consequently, we considered that nephropathy had begun to occur, and started treatments for 12 weeks.

In the present study, we observed focal and segmental glomerular sclerosis and an increased cortical interstitial volume associated with a progressive increase in proteinuria in heminephrectomized Goto–Kakizaki rats. Both exercise and losartan blunted the increases in proteinuria and improved the IGS and RIV. Losartan blocked the development of hypertension. Exercise had a specific renoprotective effect that was not related to the reduction in SBP. Exercise plus losartan decreased the number of glomerular ED-1-positive cells and the index of glomerular α-SMA, and increased the number of WT-1-positive cells. In particular, glomerular sclerosis, glomerular macrophage infiltration, glomerular mesangial activation, and podocyte loss were milder in the exercise plus losartan group compared with the other groups.

Moreover, the TRD in both the exercise and exercise plus losartan groups was longer by approximately 39% than that in the losartan and control groups at the end of the experiment. Exercise enhanced the capillarization as well as the proportion of type I fibre in EDL muscle.

These results suggest that the combination of exercise and losartan provided a greater renoprotective effect than that of losartan alone. Moreover, exercise enhanced the exercise endurance without renal complications in this rat model.

The VO_2 when the diabetic nephropathy rats were running at a speed of 20 m/min corresponded to approximately 75% of the peak VO_2 in this study. Therefore, the intensity level of the exercise given to the diabetic nephropathy rats was moderate, and all animals in the exercise groups were able to continue running for 1 h.

The present results indicate that the effects of exercise must be distinguished from the effects of acute exercise. In other words, a decrease in renal blood flow, contraction of renal efferent arterioles, contraction of mesangial cells, and a decrease in the glomerular filtration rate caused by accelerated sympathetic nerve activity are the effects of acute exercise.

There have been a few reports regarding the influence of exercise on the progression of diabetic nephropathy. Ward *et al.* [12] reported a significant decrease in urinary albumin excretion, less glomerular basement membrane thickening and less mesangial volume expansion in trained obese Zucker rats by 1 h/day treadmill running at 10–15 m/min, 17% incline, 5 days a week for 11 weeks. Albright *et al.* [11] reported that treadmill exercise at 18 m/min, 8° incline, 2 h/day, 5 days a week for 12 weeks significantly reduced the increase in the fractional volume of the mesangium but did not show any change in urinary protein excretion, without exacerbating the renal injury in Sprague–Dawley rats made diabetic with streptozotocin. Chiasera *et al.* [13] exercised trained obese Zucker rats and showed that training by 10–15 m/min, 8° incline, 1 h/day, 5 days a week for 20 weeks did not significantly affect the morphometric indices of the renal ultrastructure, but significantly reduced albuminuria. In their studies the renal histological changes were minor, they did not find focal glomerulosclerosis, and urinary protein was mildly elevated. Actually, in all previous studies exercise did not reduce the urinary protein level, but in the studies of Ward *et al.* [12] and Chiasera *et al.* [13] exercise was associated with less albuminuria. In contrast, in the present study, exercise significantly decreased the urinary protein, IGS and RIV. Previous authors did not find any significant differences regarding serum creatinine, BUN and SBP associated with exercise, which is consistent with our results. The possible reason for the discrepant effects of exercise in the present study may be that the animal models used as well as the intensity and duration of exercise were different. Moreover, in the present study, it was also demonstrated that exercise has renoprotective effects as assessed by a range of functional and structural parameters including the results of an immunohistochemical study.

Data on the effects of exercise on diabetic nephropathy are, however, scarce and there is no definitive conclusion as to whether exercise has any renal protective effect in diabetic nephropathy. This is the first report describing the renal effects of exercise using an immunohistological analysis of the remnant kidneys in a diabetic nephropathy rat model.

In order to explore the mechanisms underlying the effects of exercise, losartan and the combined therapy of exercise and losartan on renal function, we examined the remnant kidney of diabetic nephropathy rats immunohistologically using antibodies against ED-1 (a surface marker of the macrophages), α-SMA (an indicator of mesangial activation and renal fibrosis) and WT-1 (a transcriptional factor involved in nephrogenesis and podocyte differentiation, as a marker of the podocyte nucleus). In the progression of renal fibrosis, macrophage infiltration and mesangial activation have been described as important cellular mechanisms [29]. Recent evidence suggests that podocyte loss is also associated with glomerulosclerosis and the progression to end-stage renal disease [30].

In the present study, the suppression of glomerular macrophage infiltration, prevention of glomerular mesangial activation and of podocyte loss were observed in the exercise plus losartan-treated group but not in the exercise or losartan alone groups. In addition, because no differences were seen in the parameters from the immunohistochemical study in exercise and losartan alone groups compared with the control group, it may be suggested that both monotherapies have some effects on the glomerular macrophages, on the mesangial cells and on the podocytes, but not sufficiently. There have been a few recent studies on the anti-inflammatory effect of exercise, and thus the renal protective effect of exercise might be partly caused by suppression of the inflammatory process [31].

The mechanism by which exercise protected the remnant kidney in the present study has not been fully elucidated. Although we did not assess intraglomerular hypertension and hyperfiltration in the present experiment, if the capillary pressure is reduced by exercise, renal protective effects may appear. Bergamaschi *et al.* [32] reported the dilation of renal efferent arterioles and an improvement in glomerular hypertension by exercise in Munich–Wistar rats with chronic renal failure. Provided that these findings are also true for the Goto–Kakizaki rat with diabetic nephropathy, it is conceivable that exercise exerts a protective effect on kidneys by improving glomerular hypertension. Physical exercise decreases the sympathetic nervous tone [33,34]. Although we did not assess the sympathetic nervous activity directly, it is possible that the decreased sympathetic nerve activity may have contributed partly to renal protection. In addition, exercise may have caused a renal protective effect by inhibiting nephropathic risk factors such as proteinuria through some mechanisms that are not related to the reduction in blood pressure. It is possible that exercise may directly influence glomerular cells to reduce glomerular sclerosis independent of its haemodynamic actions. Further investigations

will be needed to determine the mechanisms of the renal protective effects of exercise.

The mechanism underlying the renoprotective effects afforded by the combination of exercise plus losartan are not clear in the present study. It is, however, possible that both exercise and losartan reduced the glomerular capillary pressure more effectively than either of the monotherapies, even though exercise had no effect on the systemic blood pressure. Both exercise and losartan could have renal protective effects, directly influencing glomerular cells through some mechanism independent of their haemodynamic actions. The combination of exercise and losartan promoted a significant regression of inflammatory signs such as macrophage infiltration, decreased mesangial activation and prevented podocyte loss in the glomerulus, and these could have prevented the progression of glomerulosclerosis in this diabetic nephropathy rat model.

Human and rodent skeletal muscles largely consist of slow twitch oxidative fibre (type I) and fast twitch nonoxidative fibre (type II) [35]. Type I fibre has a high capacity for oxidative metabolism; they are resistant to fatigue, and they are specialized for the performance of repeated strong actions over prolonged periods [35].

In the present study, the proportion of type I fibre, capillary density and C/F ratio were increased in the trained groups. Our results are consistent with previous studies, which demonstrated that the adaptations often seen in both humans and animals in response to endurance training are a shift in fibre type towards a greater percentage of slow-twitch oxidative (type I) fibre and increases in the capillary density of the skeletal muscle [35,36].

In the present study exercise improved the endurance only in the trained groups and to the same level. The mechanism behind the increased endurance capacity in the trained groups of rats in the present study is not clear. Allowing for previous findings that showed that functional peripheral adaptation in rats selectively bred for high-endurance capacity was caused by increases in skeletal muscle capillarity [37], one possibility is that the increased capillarization in the muscle in the trained groups of rats, which would increase the transport of oxygen within the muscle and thereby increase the capacity to oxidize the fuel, could improve their ability to continue running. Another possible mechanism producing the increased endurance capacity could be the increased proportion of type I fibre with increased oxidative capacity, which may be important in allowing the muscle to sustain higher aerobic activity necessary during prolonged exercise [38]. Further studies are, however, needed to elucidate the exact mechanism behind the increased endurance in the trained rats in this rat model.

The present results suggest that the higher percentage of type I fibre with the concomitant increase in capillary density and C/F ratio in the trained groups could contribute to the enhanced endurance capacity in this rat model.

The plasma glucose level of all groups was exceeding 200 mg/dl, and was higher in the exercise and exercise plus losartan groups than that in the control group in the present study. The higher fed plasma glucose level in the trained groups may be partly explained by their higher food and sucrose intake before decapitation. As the blood glucose level was analysed only at the end of the experiment, it is not clear whether a higher blood glucose level in the trained groups was persisting through the whole experimental period. Further investigations will be needed to determine the relationship between the renoprotective effects of exercise and the blood glucose level.

In conclusion, the renal protective effects of both exercise and losartan were demonstrated. These results also suggest that the simultaneous treatment of exercise and losartan provided greater renoprotective effects than those of losartan alone, and may affect glomerular macrophage infiltration, glomerular mesangial activation, and podocyte loss in a rat model of diabetic nephropathy. Furthermore, exercise has a specific renal protective effect that is not related to the arterial blood pressure reduction and also enhances exercise endurance. Exercise alone and the combination treatment of exercise and losartan enhanced the capillarization as well as the proportion of type I fibre in EDL muscle. These findings provide evidence that the combination of losartan and exercise may provide a new therapeutic approach to optimal renoprotection in diabetic nephropathy, and exercise could be a safe and effective therapy for improving exercise intolerance in diabetic nephropathy.

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There are no conflicts of interest.

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Increased expression of adrenomedullin 2/intermedin in rat hearts with congestive heart failure

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Abstract

Adrenomedullin 2/intermedin (AM2/IMD) is a novel member of the calcitonin/calcitonin gene-related peptide family. To investigate the pathophysiological role of AM2/IMD in heart failure, we examined the expression of AM2/IMD, adrenomedullin (AM) and receptor complex components (calcitonin receptor-like receptor, three types of receptor activity-modifying proteins) by quantitative RT-PCR and immunohistochemistry in the hearts and kidneys of rats with congestive heart failure (CHF). Significantly increased levels of AM2/IMD mRNA were found in the atrium, right ventricle, non-infarcted part of the left ventricle and the infarcted part of the left ventricle of CHF rats, compared with sham operated rats (about 2.8-fold, 1.7-fold, 1.7-fold and 2.5-fold, respectively). Expression levels of mRNA encoding AM and the receptor complex components were also increased in the hearts of CHF rats. In a separate experiment, AM2/IMD mRNA levels in the heart did not differ between Wistar Kyoto and spontaneously hypertensive rats. In both sham operated and CHF rats, the myocardium was diffusely immunostained with AM2/IMD. The fibrotic infarcted layer was not immunostained with AM2/IMD but was surrounded by positively immunostained myocardial layers. These findings suggest that the expression of AM2/IMD is enhanced in the failing heart, and AM2/IMD has a certain pathophysiological role in heart failure.

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Keywords: Adrenomedullin; Adrenomedullin 2/Intermedin; Heart failure; RT-PCR; Immunohistochemistry

1. Introduction

Adrenomedullin 2/intermedin (AM2/IMD) is a novel member of the calcitonin/calcitonin gene-related peptide (CGRP) family, which includes calcitonin, CGRP, amylin

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and adrenomedullin (AM) [1–4]. The calcitonin/CGRP family of peptides, which are widely distributed in various peripheral tissues as well as in the central nervous system, induce multiple biological effects including potent vasodilation (CGRP and AM), reduction in nutrient intake (amylin) and decreased bone resorption (calcitonin) [5]. Two research groups recently discovered the peptide almost simultaneously by searching the genome database, and named it intermedin (IMD) [1] and adrenomedullin 2 (AM2) [2], respectively. Human AM2/IMD and rat AM2/IMD consist of 47 amino acids. Rat AM2/IMD has 34% similarity with rat AM. Reverse-transcriptase polymerase chain reaction (RT-PCR) has shown that AM2/IMD mRNA is widely distributed in various tissues of mice [2]. Immunohistochemical investigations in mice and human have revealed that AM2/IMD immunoreactivity was detected in the heart and kidney [3,6].

CGRP, AM and AM2/IMD activate the complex of calcitonin receptor-like receptor (CRLR) and one of the three types of receptor activity-modifying proteins (RAMPs) to transfer their signals [1,2,7]. AM2/IMD interacts nonselectively with all three CRLR/RAMP complexes, whereas CGRP preferentially interacts with CRLR/RAMP1, and AM with CRLR/RAMP2 or CRLR/RAMP3 [1]. AM2/IMD stimulates cAMP production and has a potent vasodilator action like AM and CGRP [1,2]. Intravenous injection of AM2/IMD decreased arterial blood pressure [1,3,8], and this effect was partially blocked with CGRP and AM receptor antagonists [1]. AM2/IMD was reported to have a positive inotropic action on murine cardiomyocytes [9]. In addition, AM2/IMD reduced pulmonary vascular resistance via nitric oxide dependent mechanism in a rat model with increased pulmonary vasoconstrictor tone [10]. Moreover, intrarenal infusion of AM2/IMD caused diuresis and natriuresis without significant decrease in systemic blood pressure in rats [11]. These previous reports suggest that AM2/IMD is a possible novel modulator of systemic circulation and blood pressure homeostasis, and plays an important role in the pathophysiology of cardiovascular diseases like AM.

The importance of AM as an organ protective peptide has been extensively studied, and AM is now a therapeutic target in cardiovascular and renal diseases. Plasma concentrations of AM are elevated in many cardiovascular disorders, including heart failure, renal dysfunction and diabetes mellitus, and AM acts as an autocrine or paracrine factor to prevent organ damage [12–14]. The pathophysiological roles of AM2/IMD in congestive heart failure (CHF) and hypertension, however, have not previously been investigated. In this study, we therefore examined the expression of AM2/IMD, AM and receptor complex components (CRLR, RAMP1, RAMP2 and RAMP3) in the hearts and kidneys of rats with CHF induced by coronary ligation. We also studied the expression of AM2/IMD in the hearts of spontaneously hypertensive rats (SHR).

2. Materials and methods

2.1. Animals

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). Animal experiments were approved by the Animal Care Committee of Tohoku University Graduate School of Medicine.

Eight week old male Wistar–Kyoto rats (WKY) (Charles River Japan Inc., Tsukuba, Japan) underwent either coronary ligation or sham operation under sodium pentobarbital anaesthesia (50 mg/kg), as previously described [15,16]. In short, in the coronary ligation group (CHF rats, $n=35$), a left thoracotomy was performed, the heart was exteriorized, and the left coronary artery was ligated between the pulmonary trunk and the left auricle. The heart then returned to the thoracic cavity, and the thorax was closed. Sham operated (SO) rats ($n=6$) were treated similarly, but without the suture around the coronary artery.

Rats received standard rat chow and water *ad libitum* for the 8 weeks after surgery. All rats underwent blood pressure measurement and echocardiography one day before organ collection under anaesthesia. Blood samples were obtained during the organ collection, and plasma brain natriuretic peptide (BNP) levels were measured by SRL, Inc. (Tokyo, Japan).

Rats which met the following criteria were defined as the CHF group: rats which survived eight weeks after coronary ligation, with infarct size over 30%, and echocardiographic data and plasma BNP concentrations out of the range of mean $\pm$ 2-standard deviation of the SO rats. Using these criteria, we excluded 27 of 35 rats which underwent the coronary ligation (eleven died, fourteen had infarct size less than 30% and normal echocardiographic data, and two had only slightly elevated plasma BNP levels). The remaining eight rats were included in the CHF group. The rats used in this study were also used in a previous study by the same authors [16].

The cardiac atria, right ventricles, non-infarcted and the infarcted part of the left ventricles and the kidneys were harvested, snap-frozen in liquid nitrogen and maintained at −80 °C until RNA extraction. In CHF rats, the thin fibrotic infarcted region was carefully dissected from the viable non-ischaemic myocardium, and used as the infarcted tissue sample. Tissues for histological examination were excised, fixed with 10% neutral buffered formalin, and embedded into paraffin. The central part of the ventricle was microscopically examined using the section stained with haematoxylin-eosin (HE) to measure the infarct area. The infarct area was manually measured using Scion Image for Windows (Scion Corporation, USA).

In another experiment, twelve week old male WKY rats ($n=6$) and SHR (Charles River Japan Inc., Tsukuba, Japan) ($n=6$) were killed under anaesthesia, and the cardiac atria

Table 1
The sequence of the primers for polymerase chain reaction (PCR)

Gene	Primer sequence (5′-3′)	Position	PCR products	GenBank accession no.
AM2/IMD				
Sense	CAGACAACAGACGCAGCCCAG	146–166	648 bp	**NM201416**
Antisense	GGCCCCTGTTGGTGCTGAATC	773–793		
AM				
Sense	TCAGCAGGGTATCGGAGCATC	143–163	806 bp	**NM012715**
Antisense	GGCAAGGCAGTGGCTCAGAC	929–948		
CRLR				
Sense	GCAGCAGAGTCGGAAGAAGG	469–488	533 bp	**NM012717**
Antisense	GCCACTGCCGTGAGGTGA	984–1001		
RAMP1				
Sense	GCTGCTGGCTCATCATCTCT	84–103	415 bp	**NM031645**
Antisense	CACCTGGGACACCTACACGA	479–498		
RAMP2				
Sense	GCAACTGGACTTTGATTAGCAG	286–307	194 bp	**NM031646**
Antisense	GGCCAGAAGCACATCCTCT	461–479		
RAMP3				
Sense	ACCTGTCGGAGTTCATCGTGTA	202–223	147 bp	**NM020100**
Antisense	CCACAGTGCAGTTGGAAAAGAA	327–348		
RPL32				
Sense	AAGTTCATCAGGCACCAGTC	110–129	279 bp	**NM013226**
Antisense	GATGGCTTTTCGGTTCTTAG	369–388		

AM2/IMD, adrenomedullin 2/intermedin; AM, adrenomedullin; CRLR, calcitonin receptor-like receptor; RAMP, receptor activity-modifying protein; RPL32, ribosomal protein L32.

and ventricles were harvested. Before anaesthesia, tail systolic blood pressure was measured, and significantly elevated blood pressure was observed in the SHR (202.8±7.1 mmHg) compared with the WKY rats (156.2±6.6 mmHg).

2.2. Reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted by the guanidinium isothiocyanate/cesium chloride method and were reverse transcribed with 400 units of Moloney murine leukaemia virus reverse transcriptase (PrimeScript, TaKaRa, Otsu, Japan) using an oligo(dT) primer. Expression levels of AM2/IMD, AM, CRLR, RAMP1, RAMP2, RAMP3 and ribosomal protein L32 (RPL32) mRNAs were determined using competitive, quantitative RT-PCR methods, as previously described [16–19]. In our preliminary study, expression levels of RPL32 mRNA (mol/1 μg of total RNA) were the most stable among the several housekeeping genes tested including glyceraldehydes-3-phosphate dehydrogenase and β-actin. RPL32 was therefore selected as an internal control.

Primers used for RT-PCR analysis are shown in Table 1. To determine the equivalent concentration point, the competitive reference standard (CRS-) DNA for AM2/IMD, AM, CRLR, RAMP1, RAMP2, RAMP3 and RPL32 was prepared, and a constant amount of wild-type cDNA and increasing amounts of CRS-DNA were added to each PCR tube, as reported previously [16–19]. AM2/IMD, AM, CRLR, RAMP1, RAMP2 and RAMP3 mRNA concentrations were normalized by RPL32 mRNA expression levels.

The obtained PCR products were purified by agarose gel, sequenced by an autosequencer (Model 3100, Applied Biosystems), and confirmed 100% identity with the respective nucleotide sequence registered in the NCBI Data Bases.

2.3. Immunohistochemistry

Rat heart tissues were fixed in 10% neutral buffered formalin and embedded into paraffin for immunohistochemistry. Immunohistochemistry of AM2/IMD was performed by the ABC method using the Vector ABC kit (Vector Laboratories, Burlingame, CA, USA), as previously reported

Table 2
Characteristic data for sham operated (SO) rats and congestive heart failure (CHF) rats

Rats	SO ($n=6$)	CHF ($n=8$)
BW (g)	328±5	318±4
HW (g)	1.23±0.08	1.54±0.06*
LW (g)	1.44±0.05	2.39±0.26*
MAP (mmHg)	108±2	93±4*
LVIDs (mm)	4.7±0.4	7.7±0.4*
LVIDd (mm)	7.7±0.4	9.5±0.3*
EF (%)	78±2	44±4*
FS (%)	39.7±1.8	18.1±2.0*
BNP (pg/ml)	75±3	146±12*

BW, body weight; HW, heart weight; LW, lung weight; MAP, mean arterial blood pressure; LVIDs, end-systolic left ventricular inner diameter; LVIDd, end-diastolic left ventricular inner diameter; EF, ejection fraction of left ventricle; FS, fractional shortening of left ventricle; BNP, plasma brain natriuretic peptide concentration.
Values are mean±SEM. *$P<0.01$ vs sham operated rats by unpaired Student's t-test.

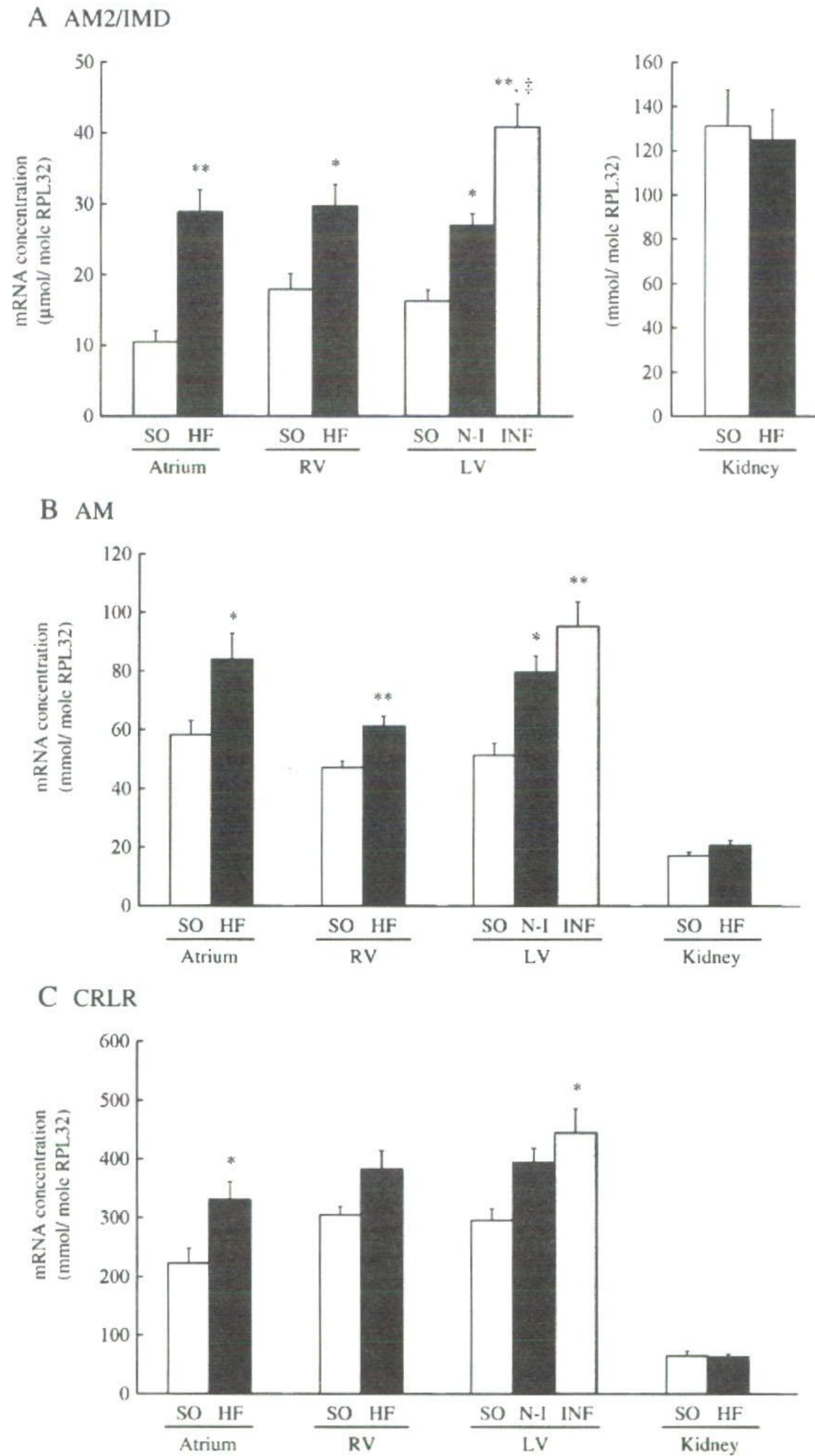
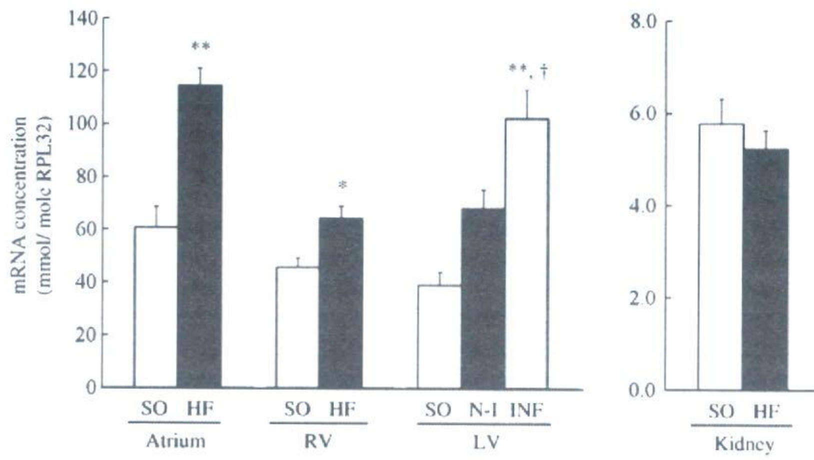
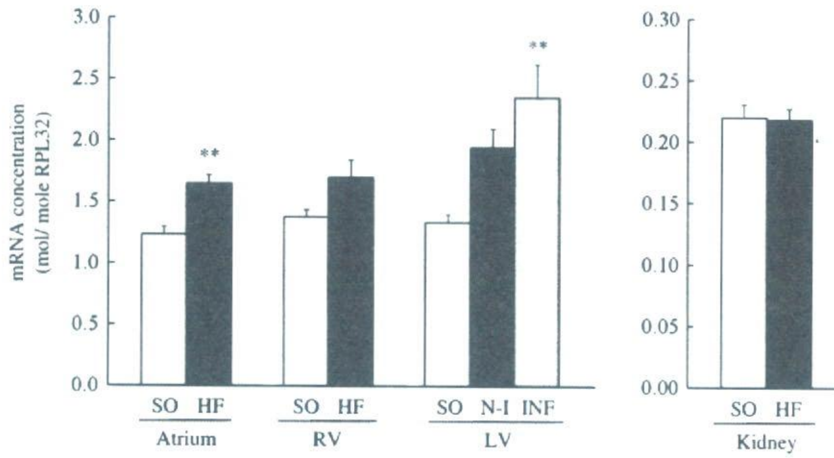


Fig. 1. Expression levels of (A) adrenomedullin 2/intermedin (AM2/IMD) mRNA, (B) adrenomedullin (AM) mRNA, (C) calcitonin-receptor-like receptor (CRLR) mRNA, (D) receptor-activity modifying receptor 1 (RAMP1) mRNA, (E) RAMP2 mRNA and (F) RAMP3 mRNA in the atrium, the right ventricle (RV), the left ventricle (LV) and the kidney. These tissues were obtained from sham operated (SO) rats and congestive heart failure (CHF) rats. Non-I: non-infarcted part of LV obtained from CHF rats. INF: infarcted part of LV obtained from CHF rats. *$P<0.05$ vs. SO, **$P<0.01$ vs. SO, †$P<0.05$ vs. Non-I, ‡$P<0.01$ vs. Non-I.





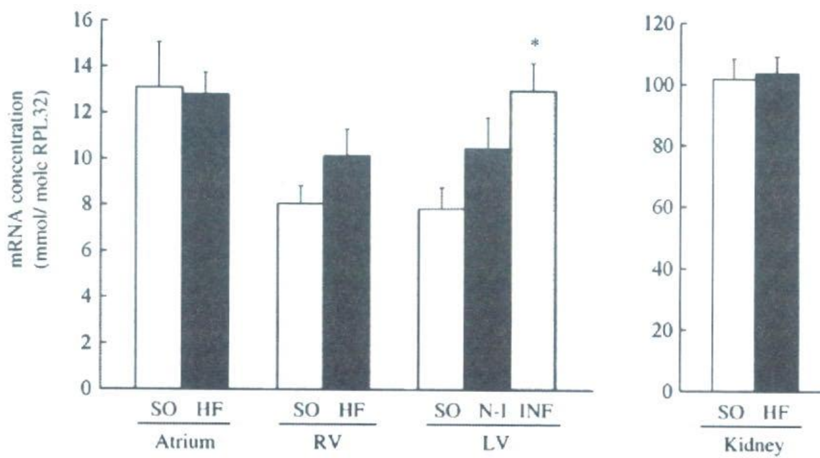


Fig. 1 (*continued*).

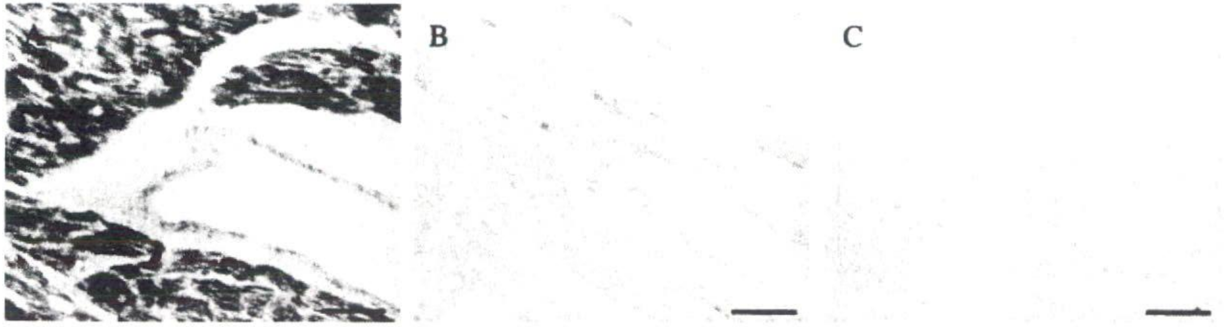


Fig. 2. The absorption test of adrenomedullin 2/intermedin (AM2/IMD) antiserum in rat heart. A: positive control immunostained with AM2/IMD antiserum. B: absorption test using AM2/IMD antiserum preabsorbed with synthetic rat AM2/IMD. C: negative control using normal rabbit serum. Bar = 50 μm.

[6,20]. Briefly, 1.5 μm sections were deparaffinized and incubated in methanol containing 0.3% H_2O_2 for 30 min and then with normal goat serum (1:20) to block non-specific staining. Sections were intensely washed in 0.01 mol/l phosphate buffered saline (pH 7.4) between the procedures. The sections were then incubated in antiserum against AM2/IMD (1:2000) for 20 h at 4 °C. Sections were incubated in biotinylated secondary antibody to rabbit IgG (1:400) for 30 min at room temperature and subsequently incubated with peroxidase-conjugated avidin for 30 min using the Vector ABC kit. These sections were visualized by immersion in 3,3′-diaminobenzidine solution (0.01 mol/l 3,3′-diaminobenzidine in 0.05 mol/l Tris–HCl buffer (pH 7.6) and 0.006% H_2O_2). In negative controls, normal rabbit serum (at a dilution of 1:2000) was used instead of the respective antiserum.

The antiserum against AM2/IMD (No. 0403-721) was raised in a rabbit by injecting human AM2/IMD (17–47) conjugated with bovine serum albumin [6]. The characteristics of this antiserum have been reported previously [6]. Furthermore, the specificity of the AM2/IMD antiserum was examined by the absorption test. The diluted antiserum (1:2000) was incubated with rat AM2/IMD, rat AM, human AM2/IMD or human AM (all these peptides were obtained from Peptide Institute, Minoh, Japan) at concentrations of 10 and 50 nmol peptide/ml of the diluted antiserum for 20 h at 4 °C prior to use.

2.4. Statistics

Data are given as mean ± SEM. mRNA concentration were analyzed by unpaired Student's t-test or one-way analysis of variance (ANOVA) and Scheffe's post hoc test for the multiple comparison of differences among the groups. Statistical significance was accepted at $p<0.05$.

3. Results

3.1. Characteristic data for SO rats and CHF rats

The histological examinations using HE staining confirmed an infarction size of about 35% of the left ventricle in the eight rats with CHF. Furthermore, significantly elevated levels of BNP (about 2.0-fold), heart weight (about 1.3-fold), lung weight (about 1.7-fold), systolic left ventricular inner diameter (about 1.6-fold) and diastolic left ventricular inner diameter (about 1.2-fold) and significantly decreased mean arterial blood pressure (about 86% of SO rats), ejection fraction (about 56% of SO rats) and fractional shortening (about 46% of SO rats) were confirmed in the CHF rats, compared with SO rats (Table 2).

3.2. mRNA expression

In the CHF rats, AM2/IMD mRNA levels were significantly increased by about 2.8-fold in the atrium ($P<0.01$), 1.7-fold in the right ventricle ($P=0.012$), 1.7-fold in the non-infarcted part of the left ventricle ($P=0.024$) and 2.5-fold in the infarcted part of the left ventricle ($P<0.01$), when compared with SO rats (Fig. 1A). AM2/IMD mRNA expression in the infarcted part of the left ventricle was higher than that in the non-infarcted part of the left ventricle of the CHF rats ($P<0.01$) (Fig. 1A). AM2/IMD mRNA levels in the kidney were markedly higher than those in the heart, but there was no significant difference in AM2/IMD mRNA expression levels in the kidney between CHF rats and SO rats ($P=0.789$).

AM mRNA was much more abundantly expressed in the heart than AM2/IMD mRNA. However, the increase of the expression levels of AM mRNA in CHF was not so marked as that of AM2/IMD mRNA. In the CHF rats, AM mRNA levels were significantly increased by about 1.4-fold in the atrium ($P<0.05$), 1.3-fold in the right ventricle ($P<0.01$), 1.6-fold in the non-infarcted part of the left ventricle ($P<0.05$) and 1.9-fold in the infarcted part of the left ventricle ($P<0.01$), when compared with SO rats (Fig. 1B).

CRLR mRNA levels in CHF rats were significantly increased about 1.4-fold in the atrium ($P<0.05$), and 1.5-fold in the infarcted part of the left ventricle ($P<0.05$), when compared with SO rats (Fig. 1C). RAMP1 mRNA levels in CHF rats were significantly increased about 1.9-fold in the atrium ($P<0.01$), 1.4-fold in the right ventricle ($P<0.05$) and 2.6-fold in the infarcted part of the left ventricle ($P<0.01$), when compared with SO rats (Fig. 1D). RAMP2 mRNA levels in CHF rats were significantly increased about 1.3-

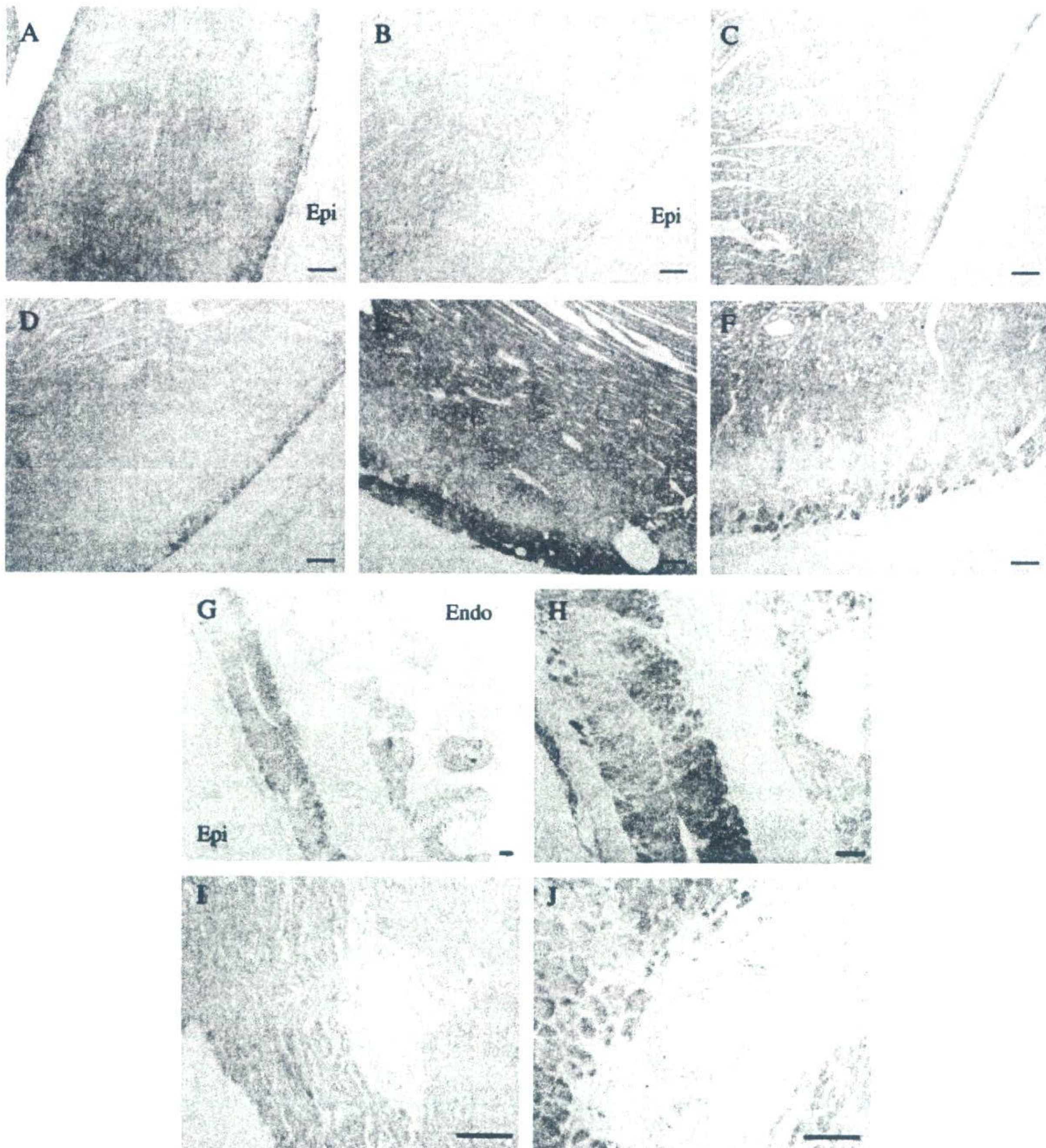


Fig. 3. Immunohistochemistry of adrenomedullin 2/intermedin (AM2/IMD) in rat heart. A and B: the right ventricle of sham operated (SO) rats (A) and congestive heart failure (CHF) rats (B). C and D: the interventricular septum of SO rats (C) and CHF rats (D). E and F: the left ventricle of SO rats (E) and CHF rats (F). G and H: infarcted part of left ventricle of CHF rats. I and J: the blood vessels of SO rats (I) and CHF rats (J). Endo: endocardium site. Epi: epicardium site. Bar = 100 μm.

fold in the atrium and 1.8-fold in the infarcted part of the left ventricle, when compared with SO rats (Fig. 1E). RAMP3 mRNA levels in CHF rats were significantly increased about 1.6-fold only in the infarcted part of the left ventricle, when compared with SO rats (Fig. 1F). In the kidney, there was no significant difference in expression levels of AM, CRLR, RAMP1, RAMP2 and RAMP3 mRNA between CHF rats and SO rats.

There was no significant difference in the expression levels of AM2/IMD mRNA between WKY rats and SHR in either the atrium (WKY: 10.7±1.8 μmol/mole RPL32, SHR: 10.5±1.7 μmol/mole RPL32, $P=0.918$) or the

ventricle (WKY: 17.7±2.9 μmol/mole RPL32, SHR: 19.9±3.3 μmol/mole RPL32, $P=0.618$).

3.3. Immunohistochemistry

Immunohistochemistry of AM2/IMD in rat hearts showed positive immunostaining in normal rat heart (Fig. 2A). The antiserum absorbed with synthetic rat AM2/IMD (Fig. 2B), or human AM2/IMD (data not shown) significantly attenuated positive immunostaining in normal rat heart whereas the antiserum absorbed with synthetic rat AM or human AM (data not shown) did not affect the immunostaining. Normal rabbit serum showed no positive immunostaining (Fig. 2C).

The myocardium was diffusely immunostained with AM2/IMD in SO rats (Fig. 3A, C, E, I) and CHF rats (Fig. 3B, D, F, G, H, J). On the other hand, the subendocardial area and the subepicardial area were barely immunostained with AM2/IMD, and these areas with low AM/IMD immunostaining were widened in CHF (Fig. 3B, D, F) compared with SO rats (Fig. 3A, C, E). In the infarcted region, the fibrotic layer was barely immunostained but was surrounded by positively immunostained myocardial layers (Fig. 3G, H). The vascular smooth muscle cells in the blood vessels of the CHF rats were positively immunostained (Fig. 3J), whereas those of the SO rat were barely immunostained (Fig. 3I).

4. Discussion

The present study shows for the first time increased gene expression of AM2/IMD in the hearts of CHF rats. Although increased gene expression of AM2/IMD has been reported in isoproterenol-induced hypertrophic myocardium in rats [8], there has been no previous report on AM2/IMD expression in the heart and kidney tissues in a CHF model. We have also confirmed the increased cardiac expression of AM and receptor complex components (CRLR, RAMP1, RAMP2 and RAMP3), which is consistent with previous reports [18,21–23]. The increase in AM2/IMD mRNA expression in the failing heart was more marked than that of AM mRNA, whereas the expression pattern of AM2/IMD mRNA in the failing heart was similar to that of AM mRNA. Increased expression of AM2/IMD, AM and receptor complex components in the failing heart seems to be an adaptive response to compensate for failed cardiac function as an autocrine or paracrine factor.

It is known that haemodynamic stress, hypoxia and inflammatory cytokines all induce AM expression. Thus the direct haemodynamic consequences of heart failure, the accompanying myocardial ischaemia or increased levels of cytokines in the cardiac tissues following infarction, might elevate the expression levels of AM mRNA in the heart. In contrast, there have been no reports on the induction of AM2/IMD expression by such stimuli or stresses. We therefore evaluated AM2/IMD mRNA levels in the hearts of WKY rats and SHR to study the effect of pressure overload on AM2/IMD expression, but found no significant change in AM2/IMD mRNA levels in the hearts of the two types of rats. Pressure overload is therefore unlikely to explain the increased expression of AM2/IMD mRNA in the cardiomyocytes in the failing heart.

Our immunohistochemical studies showed immunostaining of AM2/IMD in the myocardium and the vascular smooth muscle cells of the failing heart. The positive immunostaining of AM2/IMD in the vasculature of the rat heart is consistent with previous findings in mice [3] and in humans [6]. Increased expression of AM2/IMD mRNA in the ventricle of CHF rats was shown by RT-PCR, but the subpericardial and subendocardial areas were barely immunostained in our CHF rats. AM2/IMD peptide in these subpericardial and subendocardial areas may be secreted by stresses accompanied by heart failure, and the negative AM2/IMD immunostaining in these areas may reflect the depletion of this peptide from the cytoplasm of the cardiomyocytes. Using immunohistochemistry, Hagner et al. showed that CRLR and RAMPs are immunostained in the entire vasculature and that CRLR is expressed mainly in the endothelial layer [24]. Therefore, AM2/IMD secreted from the myocardium in the failing heart may act as a cardiovascular regulator via the activation of CRLR/RAMPs complexes in vascular endothelial cells.

Increased AM2/IMD mRNA levels were shown in the infarcted part of the left ventricle by quantitative RT-PCR. Immunohistochemical analysis suggested that this increase was due to the upregulation of AM2/IMD expression in the myocardium surrounding the fibrotic layer. However, it is possible that the increase may be due to increased levels of AM2/IMD in the vascular smooth muscle cells or an increased number of vascular vessels in the infarcted part of the left ventricle.

There were no significant changes in the mRNA expression levels of AM2/IMD, AM and receptor complex components in the kidney of CHF rats. It is therefore likely that the renal haemodynamic changes induced by heart failure had no significant influence on the expression of the AM system in the kidney including AM2/IMD.

The pathophysiological role of the upregulated AM2/IMD in the failing heart seems to be related to its cardio-protective effects, like AM and CGRP. There is accumulating evidence, which shows that AM and CGRP are potent endogenous cardio- and reno-protective substances. Exogenous administration of AM and CGRP peptides or their gene delivery is a new preventive and therapeutic strategy for cardiovascular diseases such as hypertension, myocardial ischaemia, heart failure and renal failure [13,14,25]. The cardio-protective effects of AM and CGRP are thought to be mediated by CRLR/RAMPs complexes [26]. Yang and co-workers recently demonstrated the cardio-protective effects of AM2/IMD in rat hearts, both in vitro and in vivo [27,28]. These cardio-protective effects of AM2/IMD are similar to those of CGRP and AM, which share the same receptors consisting of CRLR/RAMPs complexes and the cAMP signalling pathway [1,25]. Thus, AM2/IMD could have cardio-protective effects by cross-reacting on the CGRP receptor and the AM receptor, and

could be a new target for the prevention and treatment of cardiovascular diseases.

Furthermore, there have been some reports suggesting that AM2/IMD might act on receptor systems other than CRLR/RAMPs, particularly in the hypothalamus and/or pituitary [29]. Contrary to previous findings that AM2/IMD elevated intracellular cAMP levels [1,3] and that CGRP stimulated growth hormone secretion in rats and humans [30], Taylor et al. revealed that AM2/IMD inhibited growth hormone release in Sprague–Dawley rats [29]. There were no reports about the existence of such unidentified AM2/IMD unique receptors in peripheral tissues. Further studies are therefore required to clarify whether the cardio-protective effects of AM2/IMD are mediated by CRLR/RAMPs complexes or by unidentified AM2/IMD unique receptors.

Production and secretion of certain vasoactive peptides and cytokines, such as atrial natriuretic peptide, brain natriuretic peptide, AM, angiotensin II, tumour necrosis factor-α and urotensin II, are known to be increased in the failing heart [16]. The present study has shown that expression of AM2/IMD was also elevated in the heart of CHF rats. These findings have raised the possibility that AM2/IMD plays a certain pathophysiological role in myocardial infarction and the heart failure syndrome.

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