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Local neurohumoral regulation in the transition to isolated diastolic heart failure in hypertensive heart disease: absence of AT1 receptor downregulation and 'overdrive' of the endothelin system

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Abstract

Objective: Although isolated diastolic heart failure with preserved left ventricular (LV) systolic function frequently occurs, regulation of local neurohumoral factors in the transition from diastolic dysfunction without signs of heart failure to diastolic failure, a target for therapeutic strategy, remains to be clarified, partly because of a lack of animal models. Our laboratory recently demonstrated that Dahl–Iwai salt-sensitive (Dahl-S) rats fed on a high-salt diet since 7 weeks of age develop hypertension followed by compensated LV hypertrophy at 13 weeks and transition to isolated diastolic heart failure at 19 weeks. **Methods:** Gene expression of the components of the renin–angiotensin system, endothelin (ET) system and natriuretic peptide system in the left ventricle was investigated in the transition to isolated diastolic heart failure in this model. **Results:** The compensated ventricular hypertrophy was associated with slight increases in angiotensin-converting enzyme (ACE) and angiotensin II type-1a (AT1a) receptor mRNA levels. Although preproET-1 (ppET-1) and ET-converting enzyme-1 (ECE-1) mRNA levels were not increased, mRNA levels of ET type-A (ETA) and ET type-B (ETB) receptors were increased. Atrial natriuretic peptide (ANP) mRNA level increased, but not brain natriuretic peptide (BNP) mRNA level. At the decompensated failing stage (at 19 weeks), ACE mRNA level further increased without downregulation of AT1a receptor mRNA level. The mRNA levels of ppET-1 and ECE-1 increased with persistent upregulation of mRNA levels of ETA and ETB receptors, and immunohistochemical staining for ET-1 was found at endothelial cells and myocytes. BNP mRNA level increased with a further increase in ANP mRNA level. **Conclusions:** The transition to isolated diastolic heart failure in hypertrophied hearts was associated with preserved gene expression of the renin–angiotensin system and 'overdrive' of gene expression of the ET system. BNP gene expression is likely to be activated by the progression of diastolic failure rather than by LV hypertrophy alone. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Angiotensin; Endothelins; Heart failure; Hypertension; Natriuretic peptide

1. Introduction

Clinical and animal studies have suggested that local activation and/or inactivation of neurohumoral systems play key roles in the development of systolic heart failure with left ventricular (LV) enlargement and systolic dysfunction. Previous studies have reported the activation of the renin–angiotensin and endothelin (ET) systems in the heart in the transition to systolic heart failure due to myocardial infarction and hypertensive heart disease [1,2]. Natriuretic peptide system is also activated in the heart in the development of heart failure, and its activation is considered to play protective roles [3,4]. Several studies have reported beneficial effects of pharmacological regulation of these neurohumoral systems [1,5]. However, different pathogenesis of heart failure is associated with different neurohumoral regulation [1,6–10].

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Several clinical studies have reported that 30–50% of patients with congestive heart failure have isolated LV diastolic dysfunction with preserved systolic function, i.e., isolated diastolic heart failure [11,12], which should be discriminated from systolic heart failure. The Framingham study has indicated that hypertension is the most common underlying cardiovascular disease in patients with isolated diastolic heart failure [12], and a recent study conducted in the community population reported poor prognosis of this type of heart failure [13]. In spite of these findings, local activation and/or inactivation of neurohumoral systems in the transition from compensated diastolic dysfunction without signs of heart failure to isolated diastolic heart failure, a target for therapeutic strategy, remains unclear.

Animal models for systolic heart failure or for LV hypertrophy, preserved systolic function and diastolic dysfunction unassociated with overt heart failure are available, but not an animal model with overt isolated diastolic heart failure. Such lack of an appropriate model for isolated diastolic heart failure is largely responsible for the absence of our understanding about the neurohumoral regulation in this type of heart failure. Recently we have demonstrated that Dahl–Iwai salt-sensitive (Dahl-S) rats fed on a high-salt diet since 7 weeks of age develop hypertension followed by compensated LV hypertrophy at 13 weeks and transition to overt heart failure at 19 weeks [14]. At 19 weeks, this model showed signs of overt heart failure such as tachypnea, labored respiration and loss of activity with LV hypertrophy, progressive fibrosis, diastolic dysfunction and increases in LV end-diastolic pressure and lung weight in the absence of an increase in LV end-diastolic diameter or a decrease in fractional shortening. The characteristic of this model mimics that of clinically observed isolated diastolic heart failure and is different from that of the conventional pressure overload model, i.e., aorta-banding model, spontaneously hypertensive rat (SHR) model, etc. where overt heart failure is associated with systolic dysfunction [15,16]. The current study was conducted to investigate the serial gene expression of the components of the renin–angiotensin system, ET system and natriuretic peptide system in the left ventricle during the transition from diastolic dysfunction without signs of heart failure to overt isolated diastolic heart failure in hypertensive heart disease using this model.

2. Methods

This study conforms to the guiding principles of Osaka University Medical School with regard to animal care and to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health.

2.1. Subjects

Laboratory chow containing 0.3% NaCl was fed to weaning male Dahl-S rats (DIS/Eis, Eisai, Tokyo, Japan) until diet was switched to laboratory chow containing 8% NaCl at 7 weeks (high-salt diet group, $n=18$). The male Dahl-S rats continuously fed on laboratory chow containing 0.3% NaCl were used as age-matched control ($n=18$). In nine rats of each group, the harvest of the heart was performed following echocardiographic and hemodynamic studies at 13 weeks. In the other nine rats of each group, the studies were performed at 19 weeks. These schedules were decided according to the results of our previous study in which the time course of the transition from compensated LV hypertrophy to heart failure was studied [14]. The diet and tap water were provided ad libitum throughout the experiment. Systolic blood pressure and heart rate were measured every 2 weeks with a tail cuff system (BP-98A, Softron, Tokyo, Japan).

2.2. Echocardiographic study

Transthoracic echocardiographic recordings were obtained as previously described [14]. Specifically, rats were anesthetized with intraperitoneal administration of ketamine HCl (50 mg/kg) and xylazine HCl (10 mg/kg), and were held in the half left-lateral position. Dose of the anesthetics was reduced to half or one third in the high-salt diet rats at 19 weeks with signs of heart failure. Rats were allowed to breathe spontaneously during the echocardiographic studies. A commercially available echocardiographic machine equipped with a 7.5-MHz transducer (SONOS 2000, Hewlett–Packard, Andover, MA, USA) was used to record the M-mode echocardiogram of the left ventricle at a paper speed of 100 mm/s with simultaneous recording of the electrocardiogram.

Echocardiographic recordings were analyzed with the commercial analysis software supplied with the system to obtain LV inner diameter and wall thickness at end-diastole and at end-systole. LV ejection fraction was calculated as described previously [4]. LV mid-wall fractional shortening was calculated with a Shimizu's model to avoid the overestimation of the systolic function in hypertrophied heart [17], as described previously [14].

2.3. Hemodynamic study

Following the echocardiographic study, a 1.5 F high-fidelity manometer-tipped catheter (SPR-407, Millar Instruments, Houston, TX, USA) was introduced through the right carotid artery retrograde across the aortic valve into the left ventricle after the manometer was calibrated relative to atmospheric pressure. Tracings of aortic pressure, LV pressure and electrocardiogram were digitized at a rate of 2000 samples per second with a commercially available analog to digital converter (MP100WS, Biopac Systems, Goleta, CA, USA) and a personal computer (Power Book 550c, Apple computer, Cupertino, CA, USA) using a dedicated software (ACKNOWLEDGE III, version 3,

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Biopac Systems). The digitized LV pressure recording was used to calculate the time constant of isovolumic LV pressure fall (tau) using a non-zero asymptote method as previously described [14]. LV end-diastolic pressure was determined as the pressure at the point just before the onset of an increase in LV systolic pressure [14].

2.4. Tissue sampling

After the hemodynamic studies, adequate anesthesia was achieved by additional intraperitoneal administration of ketamine HCl (50 mg/kg) and xylazine HCl (10 mg/kg). Blood was sampled from the right carotid artery for measurement of plasma ET-1. Then, an incision was made in the chest and the heart was quickly harvested followed by the removal of the atria, great vessels and the right ventricular free wall. The apical site of the left ventricle below the papillary muscle was removed, weighed, immediately placed in liquid nitrogen and stored at −80°C for the measurement of hydroxyproline content according to the method of Stegemann and Stalder [18]. Results were calculated as hydroxyproline content per wet weight of tissue. Samples of the left ventricle for the measurement of the amount of mRNAs were weighed, immediately placed in liquid nitrogen and stored at −80°C. The other section was weighed and immersed in a cold 4% paraformaldehyde solution for 16 to 24 h. The summed LV weight corrected for body weight was determined as LV mass index.

2.5. Measurement of plasma ET-1

Blood for measurement of ET-1 was placed in a tube containing EDTA, which was immediately placed on ice. After centrifugation at 4°C, 2 ml of plasma acidified with 0.5 ml of 60% acetonitrile/0.09% trifluoroacetic acid (TFA) was applied to a Spe-C8 cartridge (J.T. Baker Chemical, Phillisburg, NJ, USA) prewashed sequentially with methanol, distilled water and 0.09% TFA. The materials adsorbed to the cartridge were eluted with 2 ml of 60% acetonitrile/0.09% TFA and the eluates were evaporated by a centrifugal concentrator. The dried residues were reconstituted in the assay buffer, 0.01 M phosphate buffer, pH 7.4, containing 0.14 M NaCl, 0.01 M EDTA dipotassium salt, 0.02 M glycine, 0.01 M ϵ-aminocaproic acid, 0.001 M sodium azide, 0.1 mg/ml of heat-inactivated human serum albumin and 0.1% Triton X-100, and subjected to radioimmunoassay with the use of antibody to ET-1 (Sumitomo Metal Industry Bioscience, Sagamihara, Japan). The cross-reactivity was <0.1% with ET-2 or ET-3 and 2% with big ET-1.

2.6. Pathological study

The specimen immersed in 4% paraformaldehyde solution was embedded in paraffin, and a 2-μm thick transverse section of the LV free wall was microscopically examined with Azan Mallory stain at ×100 magnification to evaluate the degree of fibrosis by two independent data-blind observers.

2.7. Immunohistochemistry

Two-μm thick transverse sections of paraformaldehyde-fixed, paraffin-embedded tissue were incubated in a 0.3% H_2O_2 solution for 30 min at room temperature to inactivate the endogenous peroxidase activity. Tissue sections were then incubated with blocking solution (10% goat serum, DAKO A/S, Glostrup, Denmark) at room temperature for 10 min followed by 24-h incubation with mouse monoclonal ET-1 antiserum diluted 1:800 (BMA Biomedicals AG, Rheinstrasse Switzerland) at 4°C. The sections were incubated with biotinylated goat anti-mouse antibody (Dako LSAB kit, Dako A/S) for 20 min at room temperature and then with an avidin–biotinylated horseradish peroxidase complex (Dako LSAB kit, Dako A/S) for 20 min at room temperature. The sections were rinsed in 0.05 M Tris–HCl buffer after each incubation step. The peroxidase activity was visualized with 0.05% 3,3-diaminobenzidine. The section was counterstained with methyl-green, dehydrated in ethanol, and then mounted. The degree of the ET-1 immunoreactivity in the section was evaluated by two independent data-blind observers. The specificity of immunoreactivity was determined by an absorption test; the consecutive section was subjected to the same procedure with use of the supernatant of ET-1 antiserum diluted 1:200 preabsorbed with 20 μM of synthetic ET-1 (Biogenesis, Poole, BH17ONF, UK) overnight at 4°C.

2.8. RNA preparation and quantification of mRNA

Total RNAs were extracted from the left ventricle by using ISOGEN, and treated with RNase-free DNaseI (Nippon Gene, Japan). Two-μg total RNA samples were reverse-transcribed with oligo d(T)16 as a reverse primer by using a GeneAmp RNA PCR kit (Perkin–Elmer, Foster, CA, USA). To quantify mRNA levels in total RNA samples, real-time quantitative polymerase chain reactions (PCR) were employed with Prism 7700 Sequence Detector (Perkin–Elmer) [19]. Table 1 summarizes nine measured mRNAs and sequences of all oligo-nucleotides used as forward primers, reverse primers and detection probes. According to the manufacturer's instruction, one fortieth of reverse transcription samples were carried out by PCR by using the TaqMan PCR core kit (Perkin–Elmer) and PCR conditions were; 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, 50 cycles at 95°C for 15 s, 60°C for 1 min. All nucleotide sequences of amplified fragments were confirmed by DNA sequencing. To correct the efficiency of cDNA synthesis, the amounts of each measured mRNA were divided by the amounts of glyceraldehyde-3-phos-

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Table 1
Sequences of all oligonucleotides used as forward primers, reverse primers and detection probes[a]

mRNA		Oligo-nucleotides sequence	Bp of amplification	GenBank reference	Reference
GAPDH	Forward primer	AGTATGACTCCACTCACGGCAA	100	RATGAPDHA	[37]
	Reverse primer	TCTCGCTCCTGGAAGATGGT			
	Probe	AACGGCACAGTCAAGGCCGAGAAT			
ppET1	Forward primer	CTCCTCCTTGATGGACAAGG	370	RATET1	[38]
	Reverse primer	CTTGATGCTGTTGCTGATGG			
	Probe	AGCAGCTGGTGGAGGGAAGAAA			
ETA	Forward primer	TTCCCTCTTCACTTAAGCCGAA	201	RATENDOR	[39]
	Reverse primer	GCAACAGAGGCATGACTGAAAA			
	Probe	CTGCTCAGCTTCTTGCTGCTCATGGATT			
ETB	Forward primer	GCCACCCACTAAGACCTCCT	201	RNETBREC	[40]
	Reverse primer	ATGCCTAGCACGAACACGAG			
	Probe	CTAGAGGTTCCAACTCCAGTCTGATGCGT			
ECE-1	Forward primer	GGACTTCTTCAGCTACGCCTGT	201	RATECE	[41]
	Reverse primer	CTAGTTTCGTTCATACACGCACG			
	Probe	AGCGAGGCAGAGAAAAAGGCGCAAGT			
ACE	Forward primer	CAAGACATTTGACGTGAGCAAC	201	RNU03734	[42]
	Reverse primer	GTCAGATCAGGCTCCAGTGACA			
	Probe	CAGTGCTGCCTCCCAACGAGTTAGAAGA			
AT1a	Forward primer	CTTACGACCAAAGGACCATTCAC	201	RRVT1AIIR	[43]
	Reverse primer	CAGCGAACTGTTTTCCAGACC			
	Probe	TGAAGGAGTAACCAAGCAAAGCCGTCTT			
ANP	Forward primer	AAATCCCGTATACAGTGCGG	105	RATANFC	[44]
	Reverse primer	GGAGGCATGACCTCATCTTC			
	Probe	TCAAGAACCTGCTAGACCACCTGGAGGAGA			
BNP	Forward primer	CCAGAACAATCCACGATGC	162	RATBNP	[45]
	Reverse primer	TCGAAGTCTCTCCTGGATCC			
	Probe	ACAGCTCTCAAAGGACCAAGGCCCTACA			

[a] Abbreviations: ACE=angiotensin-converting enzyme; ANP=atrial natriuretic peptide; AT1a=angiotensin II type-1a receptor; BNP=brain natriuretic peptide; ECE-1=endothelin-converting enzyme-1; ETA=endothelin type A receptor; ETB=endothelin type B receptor; GAPDH=glyceraldehyde-3-phosphate-dehydrogenase; ppET-1=prepro endothelin-1.

phate-dehydrogenase (GAPDH) mRNA used as internal standards. Each RNA preparation was measured by at least three individual experiments.

2.9. Statistics

Statistical analysis of the results were performed by one-way analysis of variance, followed by a multiple comparison test (Scheffe's test). All data were expressed as means±S.E. A level of $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Hemodynamics

Representative echocardiograms were shown in Fig. 1 and all echocardiographic and hemodynamic data were summarized in Table 2. Aortic pressure was significantly higher in the high-salt diet group than in the age-matched control group at 13 and 19 weeks. LV end-diastolic dimension was significantly smaller and posterior wall thickness was significantly greater in the high-salt diet group than in the control group at 13 weeks. At 19 weeks, the high-salt diet group showed signs of overt heart failure such as tachypnea, labored respiration and loss of activity, but not the age-matched control group. LV end-diastolic dimension was not different between the two groups, but posterior wall thickness was significantly greater in the high-salt diet group than in the control group. LV mass index progressively increased in the high-salt diet group and was significantly higher at 13 and 19 weeks than in the control group. Ejection fraction was higher at 13 and 19 weeks in the high-salt diet group than in the control group. LV mid-wall fractional shortening was not different between the two groups at 13 or 19 weeks.

LV end-diastolic pressure was not different at 13 weeks between the two groups, but was significantly higher at 19 weeks in the high-salt diet group than in the control group. A ratio of lung weight to body weight was significantly higher at 19 weeks in the high-salt diet group than in the control group (12.1±1.7 vs. 3.8±0.1 mg/g, $P<0.01$). Tau was significantly longer at 13 and 19 weeks in the high-salt

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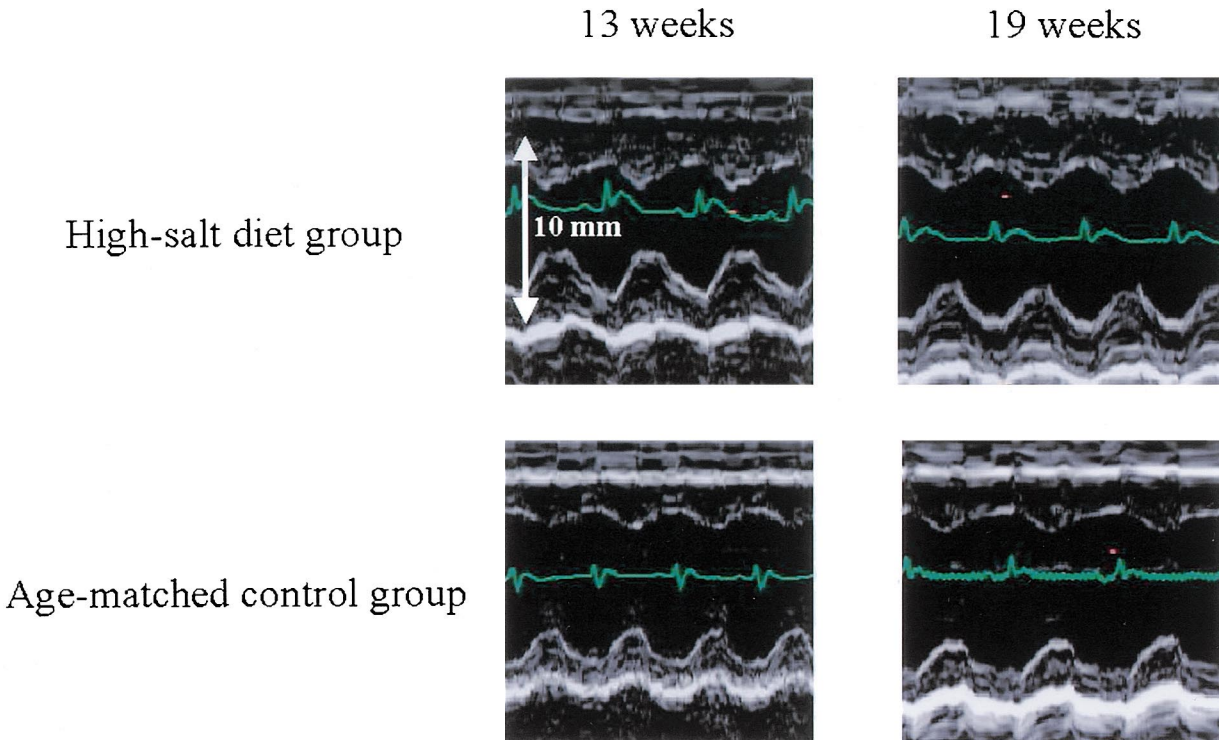

Fig. 1. Representative tracings of LV M-mode echocardiogram of the high-salt diet group (upper panels) and the age-matched control group (lower panels) at 13 (left panels) and 19 (right panels) weeks. LV contraction was preserved and the LV cavity was not enlarged even at failing stage (19 weeks of age) in the high-salt diet group.

diet group than in the control group. Plasma ET-1 was significantly higher at 19 weeks in the high-salt diet group than in the control group.

These indicate that the high-salt diet rats were compensated for hypertension at 13 weeks but progressed to decompensated heart failure at 19 weeks. The characteris-

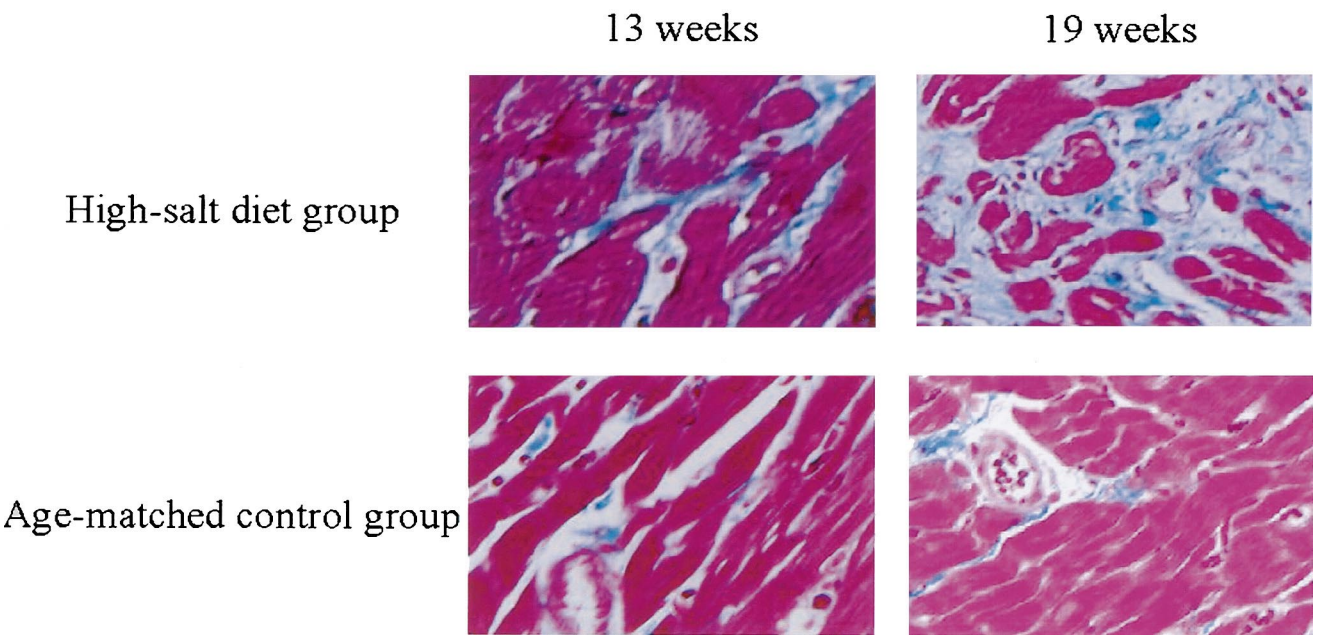

Fig. 2. Photomicrographs of Azan Mallory staining of the left ventricle of the high-salt diet group (upper panels) and the age-matched control group (lower panels) at 13 (left panels) and 19 (right panels) weeks. At failing stage (19 weeks of age) of the high-salt diet group, progressive interstitial and perivascular fibrosis was observed.

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Table 2
Characteristics of the high-salt diet group at compensated (13 weeks) and decompensated (19 weeks) stages and of the age-matched control group[ac]

	13 Weeks		19 Weeks	
	High-salt diet	Age-matched control	High-salt diet	Age-matched control
n	9	9	9	9
LVDd (mm)	7.5±0.3[b]	8.8±0.1	8.5±0.2	8.7±0.2
PWd (mm)	2.0±0.1[b]	1.5±0.1	2.5±0.1[b]	1.5±0.1
Ejection fraction (%)	68±3[b]	56±1	61±1[b]	52±2
Mid-wall FS (%)	18±1	17±1	16±1	14±1
Systolic AoP (mmHg)	191±6[b]	141±5	203±5[b]	146±3
LVEDP (mmHg)	7±1	5±1	19±2[b]	9±1
Tau (ms)	28±2[b]	21±2	28±2[b]	23±2
LV mass index (10^{-3})	2.9±0.1[b]	2.1±0.1	4.1±0.2[b]	2.2±0.1
Pro-OH (μmol/g)	2.4±0.1	2.8±0.1	3.6±0.3[b]	2.4±0.2
Plasma ET-1 (pg/ml)	3.6±0.2	3.3±0.2	7.4±1.1[b]	4.2±0.2

[a] Values are expressed as mean±S.E.
[b] $P<0.05$ versus age-matched control group.
[c] Abbreviations: AoP=aortic pressure; ET-1=endothelin-1; FS=fractional shortening; LVDd=left ventricular end-diastolic dimension; LVEDP=left ventricular end-diastolic pressure; LV mass index=a ratio of left ventricular mass to body weight; Pro-OH=hydroxyproline concentration; PWd=left ventricular posterior wall thickness at end-diastole; Tau=the time constant of isovolumic left ventricular pressure fall.

tic at 19 weeks is compatible with that of clinically observed isolated diastolic heart failure based on hypertensive heart disease.

3.2. Histological study

Fibrosis was not developed at 13 weeks in the high-salt diet group, but both perivascular and interstitial fibrosis progressed at 19 weeks without massive regional necrosis or broad fibrosis in replacement of the myocardium (Fig. 2). The hydroxyproline content in the left ventricle was not different at 13 weeks between the two groups but was significantly greater at 19 weeks in the high-salt diet group than in the control group (Table 2).

3.3. The mRNA levels in the left ventricle

To measure the amount of nine mRNAs in the left ventricle (see Table 1), we employed real-time quantitative RCR with a Prism 7700 Sequence Detector. First, we evaluated whether real-time quantitative RCR is usable for quantifying the mRNA amount. A cDNA sample was diluted serially and the amount ET type B (ETB) receptor mRNA measured. As shown in Fig. 3, each threshold cycle was calculated from collected fluorescent emission data, and the plotted standard curve was linear, indicating that the amount of mRNA was correctly evaluated in a wide range in this method.

We examined mRNA levels of angiotensin-converting enzyme (ACE) and angiotensin II type-1a (AT1a) receptor to get insights into the activation of renin–angiotensin system (Fig. 4). The ACE mRNA level was slightly but significantly higher at 13 weeks in the high-salt diet group than in the age-matched control group, and further increased 4.1-fold relative to the age-matched control group at 19 weeks. The AT1a receptor mRNA level was higher at 13 weeks in the high-salt diet group than in the age-matched control group, but was not different at 19 weeks. Thus, ACE mRNA level increased with the progression of LV hypertrophy and diastolic heart failure in the absence of down-regulation of the AT1a receptor mRNA level.

We next examined mRNA levels of the ET system (Fig. 4). The preproET-1 (ppET-1) mRNA and ET converting enzyme-1 (ECE-1) mRNA levels were not different between the two groups at 13 weeks, but significantly elevated in the high-salt diet group to 7.6 and 1.9-fold relative to the age-matched control group at 19 weeks. The ET type A (ETA) and ETB receptor mRNA levels were significantly higher at 13 and 19 weeks in the high-salt diet group than in the age-matched control group. Thus, ppET-1 and ECE-1 mRNA levels increased from the compensated stage to the decompensated heart failure stage with consistent upregulation of ETA and ETB receptor mRNA levels.

Finally, mRNA levels of natriuretic peptide system were examined (Fig. 5). The atrial natriuretic peptide (ANP) mRNA level progressively increased in the high-salt diet group to 30.8 and 50.5-fold of the age-matched control group at 13 and 19 weeks, respectively. Brain natriuretic peptide (BNP) mRNA level was not different at 13 weeks between the two groups, but was significantly elevated in the high-salt diet group to 4.3-fold of the control group at 19 weeks. Thus, ANP mRNA level progressively increased with the progression of LV hypertrophy. In contrast, BNP mRNA expression was not enhanced with LV hypertrophy

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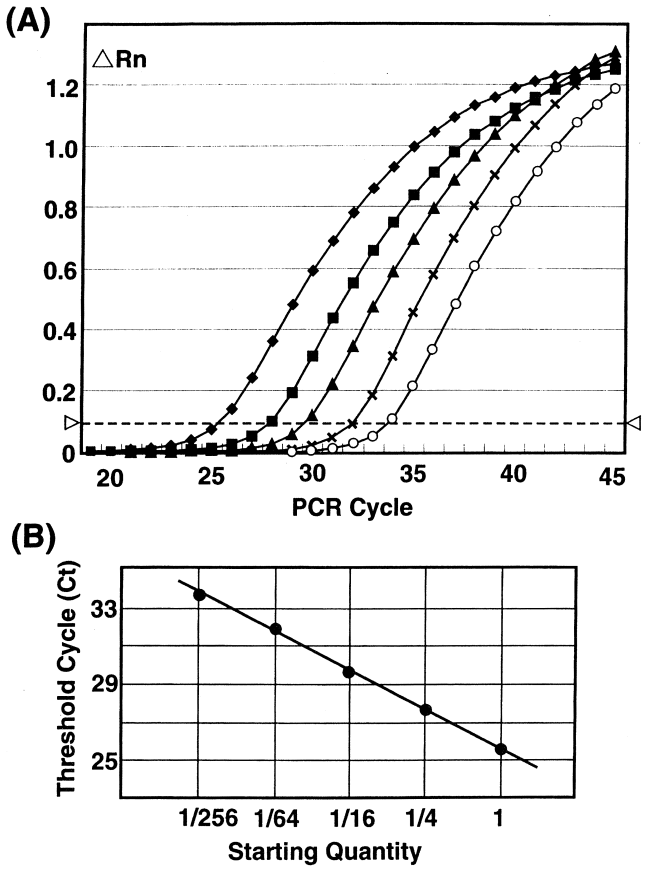

Fig. 3. Quantification of ETB receptor mRNA level by real-time quantitative PCR. Upper panel is the representative amplification plots of ETB receptor mRNA levels measured by real-time quantitative PCR. A cDNA sample diluted at 1 (closed diamond), 1:4 (closed square), 1:16 (closed triangle), 1:64 (cross), 1:256 (open circle) was amplified using the ETB receptor mRNA detection set. For each dilution the ΔCRn (fluorescent emission) was plotted against cycle numbers. As the larger amounts of the standard samples were applied, the significant emission of fluorescence was detected at the earlier phase of the threshold cycle in the amplification plot (dotted line). Lower panel is a standard curve for ETB receptor mRNA levels measured by real-time quantitative PCR. The amounts of standard samples were plotted versus threshold cycle number.

alone and was enhanced in association with the development of heart failure.

3.4. Immunohistochemistry

Immunohistochemical staining for ET-1 was found at the endothelial cells of the intramyocardial coronary arteries at 13 and 19 weeks in the control group. In the high-salt diet group, the staining for ET-1 was similar at 13 weeks to the control group. However, only in the failing hearts at 19 weeks of the high-salt diet group, did ET-1 become detectable in the cardiomyocytes in addition to the endothelial expression (Fig. 6). The specificity of immunostaining was confirmed when the signals were completely abolished by preincubation of the primary antibody with the excess of synthetic ET-1.

4. Discussion

LV hypertrophy and fibrosis progressed following hypertension in Dahl-S rats fed on a high-salt diet since 7 weeks of age. Overt heart failure without systolic dysfunction occurred at 19 weeks through the compensated LV hypertrophic stage at 13 weeks. The compensated hypertrophy was associated with increases in mRNA levels of ACE, AT1a receptor, ETA receptor, ETB receptor and ANP in the left ventricle. The decompensated hypertrophy was associated with increases in mRNA levels of ppET-1, ECE-1 and BNP in the left ventricle with consistent upregulation of ETA and ETB receptor mRNA levels. The mRNA levels of ACE and ANP were further increased without downregulation of AT1a receptor mRNA level.

4.1. Renin–angiotensin system in the left ventricle

The expression of ACE and AT1a receptor is shown to be enhanced in the presence of pressure-overload-induced LV hypertrophy at the compensated stage followed by systolic heart failure [20,21], which is compatible with the current results at the compensated stage. ACE gene expression was enhanced in association with the further progression of LV hypertrophy and the development of isolated diastolic heart failure in this study. AT1a receptor gene expression was not down-regulated and was still preserved at the normal level at the decompensated stage.

The previous reports demonstrated that decompensated heart failure with systolic dysfunction and LV enlargement is associated with downregulation of AT1 receptor, which is considered similar to downregulation of β-adrenoceptor in this type of heart failure [8]. Lopez et al. reported the downregulation of AT1 receptor even at the compensated hypertrophic stage in a pressure-overload hypertrophy model due to aortic banding that develops systolic heart failure [22]. Thus, downregulation of AT1 receptor precedes systolic heart failure, and the absence of the downregulation of AT1 receptor gene expression at the failing stage in the current model may be taken characteristic to isolated diastolic heart failure. Previous in vitro studies demonstrated that angiotensin II plays crucial roles in mechanical stretch-induced myocyte hypertrophy [23] and collagen synthesis in fibroblast [24], and that such effects are mediated through AT1 receptor [23,24]. Ventricular hypertrophy and fibrosis are likely to contribute to the progression of heart failure, and thus, AT1 receptor blocker has been considered to be effective for the treatment of chronic heart failure. Several human and animal studies reported beneficial effects of AT1 receptor blocker in systolic heart failure [5,25]. However, Spinale,

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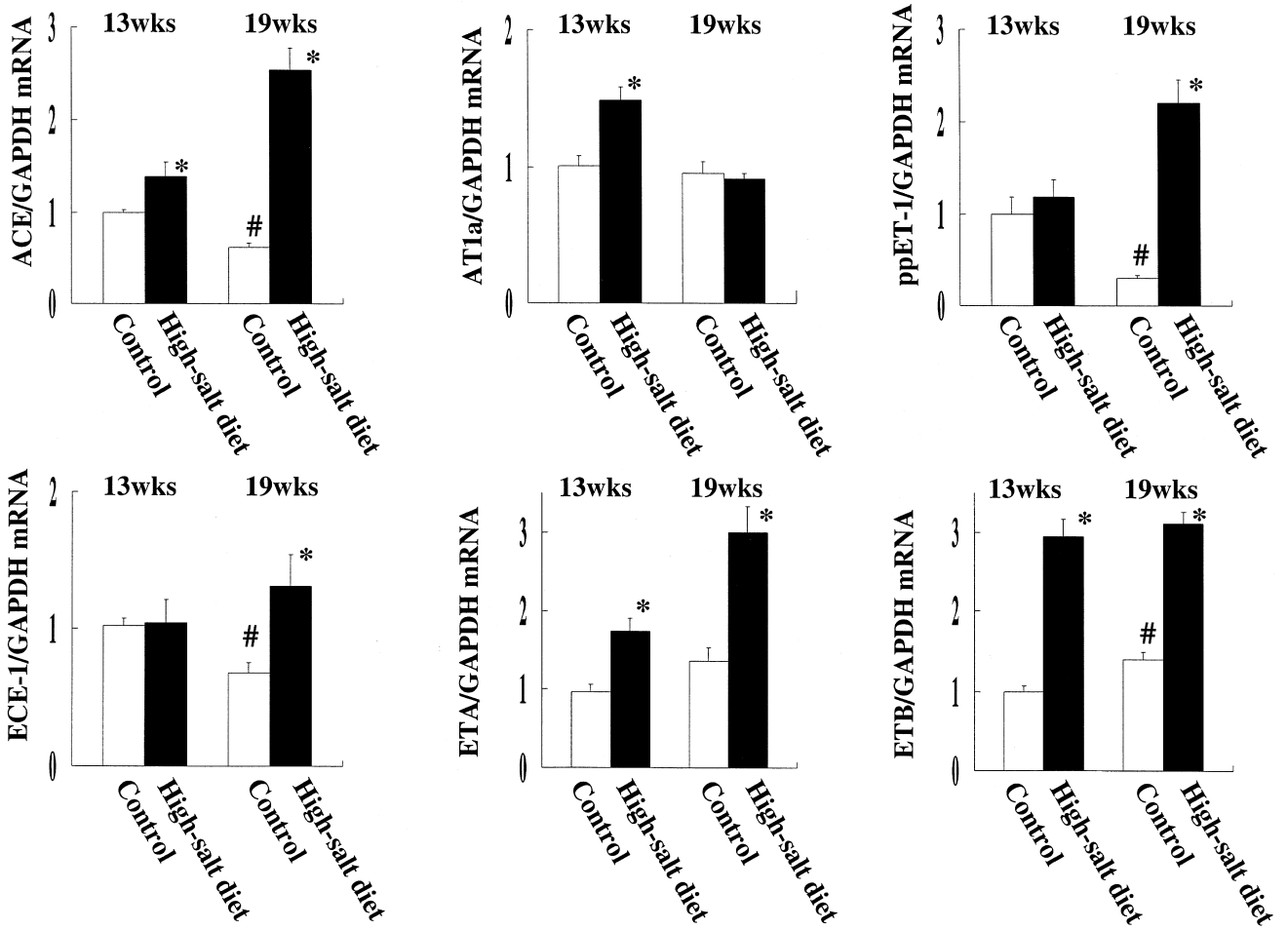

Fig. 4. The mRNA levels of ACE, AT1a receptor, ppET-1, ECE-1, ETA receptor, and ETB receptor of the age-matched control group (control) and the high-salt diet group (high-salt diet) at 13 and 19 weeks. Each mRNA level was corrected for a mRNA level of GAPDH. The mean value of the age-matched control group at 13 weeks is represented as 1.0, and the remaining values were adjusted accordingly. * $P<0.05$ vs. age-matched control, # $P<0.05$ vs. age-matched control at 13 weeks.

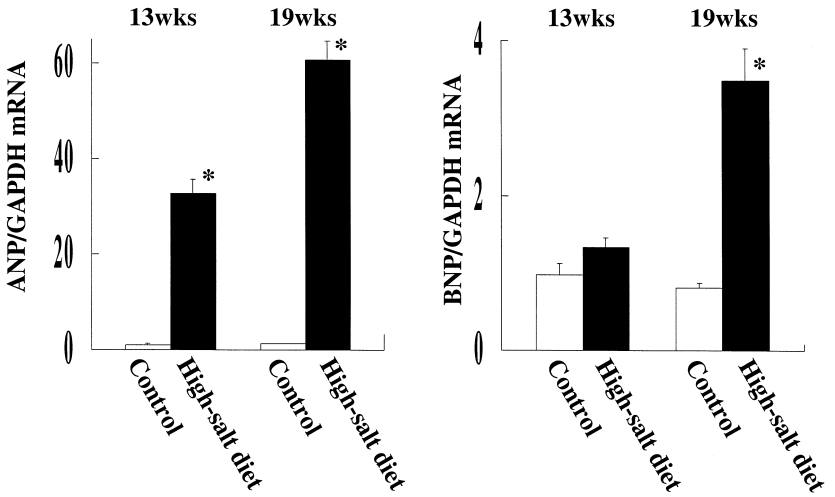

Fig. 5. The mRNA levels of ANP and BNP of the age-matched control group (control) and the high-salt diet group (high-salt diet) at 13 and 19 weeks. Each mRNA level was corrected for a mRNA level of GAPDH. The mean value of the age-matched control group at 13 weeks is represented as 1.0, and the remaining values were adjusted accordingly. * $P<0.05$ vs. age-matched control.

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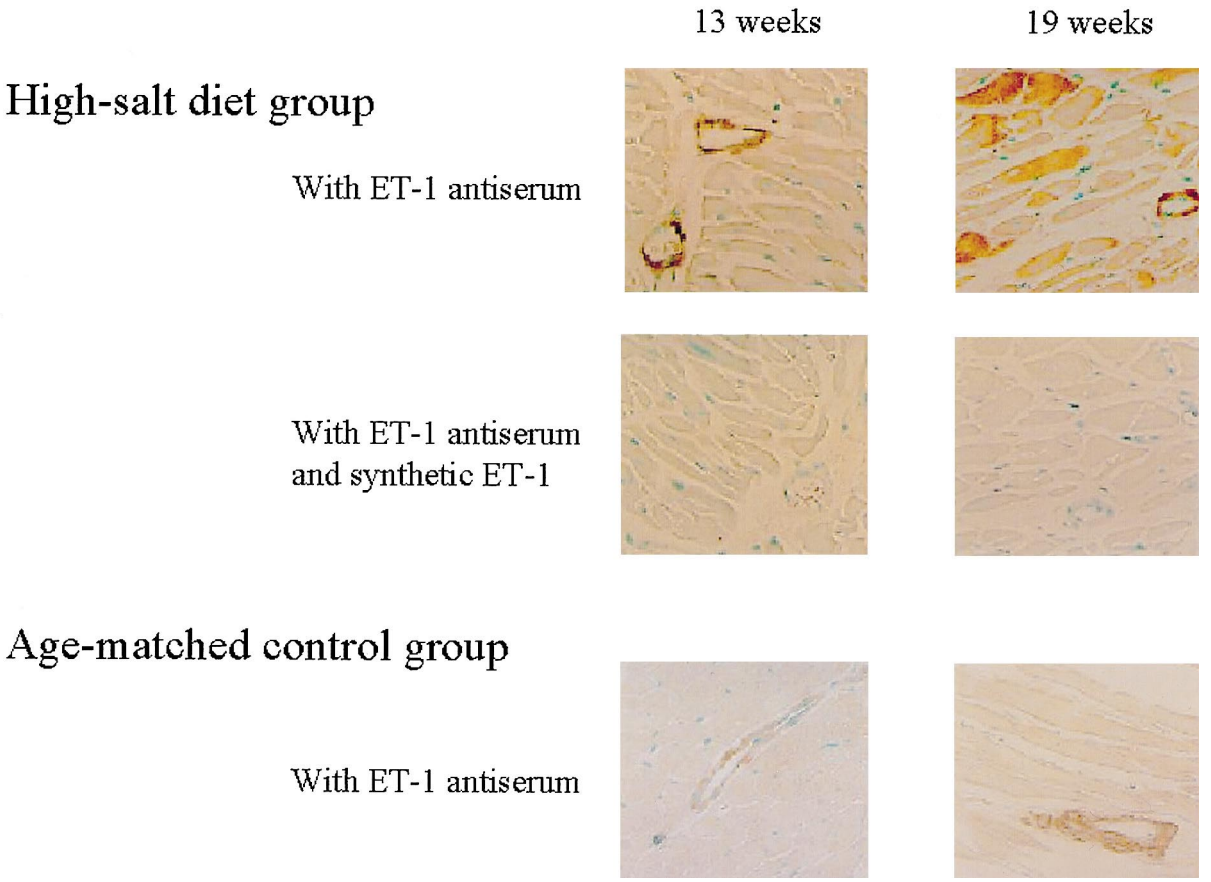

Fig. 6. Representative immunohistochemical staining for ET-1 in the left ventricle of the high-salt diet group (upper panels) and the age-matched control group (lower panels) at 13 (left panels) and 19 (right panels) weeks. Preincubation of the primary antibody with the excess of synthetic ET-1 resulted in no staining in the left ventricle of the high-salt diet group (middle panels).

et al. reported a lack of effects of AT1 receptor blocker on LV remodeling in an animal systolic heart failure model [26]. The RESOLVD trial was interrupted due to the concerns about increased mortality and morbidity events with candesartan monotherapy and the candesartan/enalapril combination vs. the enalapril monotherapy in patients with systolic failure [27]. Such controversy about the issue of the beneficial effects of AT1 receptor blocker on systolic heart failure may be partly explained by variation in the degree of the down-regulation of AT1 receptor among study subjects. The lack of downregulation of AT1 receptor gene expression in the diastolic heart failure model of this study suggests that AT1 receptor blocker is effective in this type of heart failure. Our preliminary study demonstrated that AT1 receptor blocker prevented the transition to diastolic heart failure in this model with inhibition of the progression of LV hypertrophy and fibrosis following the compensated hypertrophy [28].

4.2. Endothelin system in the left ventricle

The density of ETA receptor was not altered in human end-stage congestive heart failure due to dilated or ischemic cardiomyopathy [9]. In an animal model with volume overload, ET-1 and ET receptor levels in the left ventricle were not altered [6]. In an animal model with aortic valvular regurgitation and stenosis, tissue ET-1 level and ET receptor density were decreased [10]. In myocardial infarction model, ET-1 production was enhanced in the left ventricle, and ET receptors were upregulated in large myocardial infarction but not in small or moderate infarction [1,29]. In rats with myocardial infarction, ETA receptor blockade improved the survival with amelioration of LV dysfunction and prevention of progressive LV hypertrophy and LV enlargement [1]. Thus, the regulation of the ET system during the progression of heart failure is likely to be diverse according to the pathogenesis of heart failure, and an upregulated ET system may play important roles in the development of heart failure.

The current study demonstrated that upregulation of the gene expression of ET receptors at the compensated stage is followed by the development of isolated diastolic heart failure with the ‘overdrive’ of the gene expression of the ET system in hypertensive heart disease. This was indicated by the consistent and inexplicable upregulation of gene expression of ET receptors in spite of overexpression of ET-1 and ECE-1 mRNAs. Both myocytes and endothelial cells contributed to the production of ET-1. As in

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heart failure due to myocardial infarction, an upregulated ET system may play important roles in the development of isolated diastolic heart failure.

In the previous studies with pressure-overload models, LV hypertrophy was associated with increases in ET-1 and ET receptors in the left ventricle [30] or no alteration [31]. Previous reports about sources of ET-1 production and secretion are not consistent [32,33]. The current study showed that the gene expression of the ET system and the sources of ET-1 production and secretion are altered according to stages of heart failure. The previous studies focused on the relation between ventricular or myocyte hypertrophy and the ET system, but not on that between the development of heart failure and the ET system. Thus, the severity of heart failure was not shown in the previous studies, and the difference in the severity of heart failure may be responsible for the different results among the previous studies. Hypertensive heart disease can result in either systolic heart failure or isolated diastolic heart failure. Animal models used in the previous studies are known to develop systolic heart failure, and such a difference in types of heart failure may be also responsible for the discrepancy between our and previous results. Iwanaga et al. [32] recently demonstrated that the ET-1 production in the left ventricle was enhanced in myocytes at systolic heart failure stage but not at the compensated hypertrophic stage in hypertensive rat models. They showed the beneficial effects of an ET receptor antagonist. Although Iwanaga et al. also used Dahl-S rats, the rats were placed on high-salt diet at 6 weeks of age and progressed to systolic heart failure without development of ventricular fibrosis [34], which is different from our model [14]. As suggested by the lack of downregulation of AT1 receptor gene expression in the current diastolic heart failure model, different types of heart failure may have different neurohumoral regulation. The current study further expanded Iwanaga's study by demonstrating upregulation of the ET system in diastolic heart failure as well as systolic failure in hypertensive hearts, indicated by the enhanced gene expression of ET-1 and ECE-1 and the upregulation of the ETA and ETB receptors' gene expression at diastolic heart failure stage.

4.3. Natriuretic peptide system in the left ventricle

The ANP mRNA level increased with progression of LV hypertrophy in this model. BNP mRNA level increased in the presence of both LV hypertrophy and heart failure, but not in the presence of LV hypertrophy alone. Thus, our results suggest that gene expression of ANP and BNP is differently regulated in the left ventricle. A close relation between the ANP gene expression and LV hypertrophic process is compatible with a recent concept that ANP gene expression is a marker for myocyte hypertrophy. Nakagawa et al. reported that BNP gene expression was enhanced earlier than ANP gene expression following hypertrophic stimulation to myocytes [35]. However, our results suggest that BNP gene expression is not activated by myocyte hypertrophic stimulation alone even if ANP gene expression is enhanced. BNP gene expression is likely to be enhanced by other stimulations related to the development of heart failure. Nakagawa et al. used ET-1, phenylephrine and phorbol 12-myristate 13-acetate (PMA) as a hypertrophic stimulator [35]. Although angiotensin II is known to induce myocyte hypertrophy through the ET system in vitro [33], pressure overload can induce LV hypertrophy in AT1a receptor knockout mice [36]. Thus, an hypertrophic signaling pathway proven in vitro is not necessarily involved in an early stage of ventricular hypertrophy in vivo, which is likely to explain the discrepancy between our and previous results.

4.4. Study limitation

In the current study, mRNA levels were measured, but not protein levels. As regards the ET-1 protein level, we showed the enhanced immunohistochemical staining for ET-1 at 19 weeks in the high-salt diet rats, which qualitatively indicated an increase in ET-1 protein level suggested by the increase in ppET-1 mRNA level.

Conclusions

The neurohumoral characteristics of the current model of isolated diastolic heart failure were the enhanced gene expression of ACE without downregulation of AT1 receptor and the coordinated upregulation of gene expression of ET-1, ECE-1 and ET receptors. These characteristics are partly different from those of systolic heart failure due to dilated cardiomyopathy, pressure overload, myocardial infarction, etc. Modulation of local neurohumoral factors is considered to be a target for the treatment of heart failure patients. Therapeutic strategy for isolated diastolic heart failure has been under discussion as well as clinical detection of diastolic dysfunction. Thus, further investigation of activation and/or inactivation of local neurohumoral factors in isolated diastolic heart failure should be promoted in parallel with establishment of assessment of diastolic function.

The current study also indicated that gene expression of ANP and BNP in the left ventricle is differently regulated in pressure-overloaded hearts. ANP gene expression was progressively enhanced with the development of LV hypertrophy; however, BNP gene expression was not enhanced by LV hypertrophy alone. The transition to heart failure may be mandatory for the enhancement of BNP production.

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