Expression of endothelin-1, $\text{ET}_A$ and $\text{ET}_B$ receptors, and ECE and distribution of endothelin-1 in failing rat heart

TSUTOMU KOBAYASHI,1 TAKASHI MIYAUCHI,1 SATOSHI SAKAI,1 MASAHIKO KOBAYASHI,3 IWAO YAMAGUCHI,1 KATSUTOSHI GOTO,2 AND YASURO SUGISHITA1
1Cardiovascular Division, Department of Internal Medicine, Institute of Clinical Medicine and 2Department of Pharmacology, Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8575; and 3Tsukuba Research Institute, Banyu Pharmaceutical Company, Tsukuba, Ibaraki 300-2611, Japan

Kobayashi, Tsutomu, Takashi Miyauchi, Satoshi Sakai, Masahiko Kobayashi, Iwao Yamaguchi, Katsutoshi Goto, and Yasuro Sugishita. Expression of endothelin-1, $\text{ET}_A$ and $\text{ET}_B$ receptors, and ECE and distribution of endothelin-1 in failing rat heart. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H1197–H1206, 1999.—Endothelin (ET)-1 has a positive inotropic effect and induces hypertrophy in cardiomyocytes. We previously reported that the peptide level of ET-1 is increased in the failing heart of rats with chronic heart failure (CHF) and that treatment with an $\text{ET}_A$-receptor antagonist greatly improves survival in rats with CHF. However, precise analysis for alteration of the myocardial ET system in the failing heart is not known. In this study, we used rats with CHF due to chronic myocardial infarction. Sham-operated rats served as a control. The results showed that the level of preproendothelin (preproET)-1 mRNA and the peptide level of ET-1 were markedly increased in the heart of rats with CHF, whereas the expression of endothelin-converting enzyme (ECE)-1 mRNA in the heart did not differ between CHF and control rats. The intensity of ET-1 staining (ET-1-like immunoreactivity) in cardiomyocytes was markedly stronger in rats with CHF than in control rats, and the fibrotic tissues of the infarcted area were not stained. The mRNA and protein levels of both $\text{ET}_A$ and $\text{ET}_B$ receptors in the heart were significantly higher in rats with CHF than in control rats. The present study suggests that the increase in ET-1 peptide level in the heart of the rats with CHF originated from upregulation of preproET-1 mRNA, which was not attendant with the alteration of ECE-1 mRNA expression, and that both the $\text{ET}_A$- and $\text{ET}_B$-receptor systems are greatly accelerated in the failing heart.

Heart failure; endothelin-receptor subtypes; endothelin-converting enzyme; angiotensin-converting enzyme

The clinical presentation of chronic heart failure (CHF) is characterized by alterations of various hemodynamic and neurohumoral mechanisms in the circulation. Compensations of the cardiovascular system in CHF may involve factors that act locally at the site of synthesis. Circulating plasma endothelin (ET)-1 is increased in patients with CHF (10, 13, 23, 34) and in animal models of CHF (12, 28). ET-1, initially identified as a potent vasoconstrictor derived from endothelial cells (44), is also produced by cardiomyocytes (37). ET-1 induces cardiac hypertrophy (7, 14, 31, 36) and cellular injury of cardiomyocytes (21, 33) in addition to its potent positive inotropic (5) and chronotropic (6) actions. We previously reported (25, 28) that the tissue level of ET-1 (peptide) is markedly increased in the failing heart of rats with CHF due to myocardial infarction. We also reported that long-term treatment with an $\text{ET}_A$-receptor antagonist greatly improved the survival rate and hemodynamic parameters in rats with CHF (25).

Two subtypes of ET receptors, $\text{ET}_A$ and $\text{ET}_B$ receptors, have been identified and characterized (1, 30). Both of these receptors are actively involved in mediating a variety of biological actions through their G protein-coupled signal transduction pathways (1, 30). Both ET receptors are widely distributed and are found in the heart (15). Previously, it was reported (16, 25) that the administration of an $\text{ET}_A$-receptor antagonist or an $\text{ET}_A/\text{ET}_B$ dual antagonist ameliorated CHF in several animal models. However, the extent of the alteration of the expression of each ET-receptor subtype in the failing heart is unknown. In the present study, we examined the protein levels of both $\text{ET}_A$ and $\text{ET}_B$ receptors in the failing heart. We also examined the mRNA level of each receptor subtype in the failing heart.

Similarly to other biologically active peptides, the inactive precursor molecule preproendothelin (preproET)-1 is converted into ET-1, its biologically active form, via Big ET-1 by means of two endopeptidases (18, 24, 44). The enzyme that transforms Big ET-1 into ET-1 is designated as endothelin-converting enzyme (ECE-1) and acts in a highly specific manner (43). ECE-1 is a key enzyme in the biosynthesis of ET-1 because the biological activities of Big ET-1 are negligible (8). A previous study (41) showed that the ECE-1 mRNA level in the carotid artery of rats was temporally increased after balloon angioplasty. However, it is not known whether the ECE-1 mRNA level is altered in the failing heart. One of the aims of this study was to examine whether the increase in ET-1 peptide is attendant with the upregulation of ECE-1 in the failing heart of rats with CHF. On the other hand, angiotensin II is another neurohumoral factor that plays an important role in the pathogenesis of heart failure (3). Angiotensin II is converted from angiotensin I by angiotensin-converting enzyme (ACE). Increased expression of ACE has been

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
reported in the failing heart (46). In the present study, we measured the mRNA level of preproET-1 (as the index of precursor level), the peptide level of ET-1, and the mRNA level of ECE-1 in the heart of rats with CHF. Furthermore, we measured the mRNA level of ACE in the heart of rats with CHF to compare the roles of ECE-1 and ACE in the synthesis pathway of each biologically active peptide in the failing heart.

Previous studies (37) demonstrated that cardiomyocytes produce ET-1. In vitro studies (4, 45) showed that cardiac fibroblasts can produce ET-1 under certain conditions. One of the features of the failing heart is structural cardiac remodeling, e.g., hypertrophy of cardiomyocytes and proliferation of fibroblasts. However, it is not known whether the fibroblasts proliferated in the failing heart produce ET-1 in vivo. In the present study, we also performed detailed analysis of the cellular distribution of ET-1 (ET-1 staining) in the cardiomyocytes and fibrotic tissues of failing rat hearts due to myocardial infarction.

**MATERIALS AND METHODS**

Animals. The left coronary artery was ligated in the rats to create a model of CHF. This is a well-characterized model that is pathophysiologically similar to human myocardial infarction with subsequent CHF (19, 25, 28). Male Sprague-Dawley rats were purchased from Charles River Japan (Yokohama, J apan). Left ventricular myocardial infarction was induced in male Sprague-Dawley rats (weighing 170–200 g) according to the method of Pfeffer et al. (19); the details are described in our previous reports (25, 28). Each rat was anesthetized with ether. The heart was rapidly exteriorized, and the left coronary artery was ligated with a 5-0 silk suture. About 60% of the rats with myocardial infarction died within the first 24 h. The surviving rats were maintained on standard rat chow and water ad libitum for 3 wk. As controls, we used rats that underwent the same operation except for coronary ligation. All procedures were approved by the University of Tsukuba and conformed to the "Position of the American Heart Association on Research Animal Use" adopted in November 1984.

Hemodynamic measurement and tissue sampling. On the day of the experiment, the rats were anesthetized with pentobarbital sodium (50 mg/kg body wt ip). A microtip pressure-transducer catheter (model SPC-320; Millar Instruments, Houston, TX) was inserted into the right carotid artery. After arterial blood pressure was measured, the catheter was advanced into the left ventricle for the evaluation of left ventricular pressure. These hemodynamic measurements were recorded by a polygraph system (AP-601G amplifier and WT-687G thermal pen recorder; Nihon Koden, Tokyo, J apan). The peak positive first derivative of left ventricular pressure (LV +dP/dtmax) was derived by active analog differentiation of a pressure signal differentiation amplifier (ED-601G; Nihon Koden). Subsequently, a curved polyethylene catheter was inserted into the right jugular vein to measure central venous pressure and right ventricular pressure. Sham-operated rats were randomly selected as controls. Only rats that underwent ligation and had a left ventricular end-diastolic pressure (LVEDP) 15 mmHg were considered to have CHF.

After hemodynamic measurement, the hearts were excised and the left ventricles were removed and rapidly frozen in liquid nitrogen. The tissue samples were stored at −80°C until use. Some of the rats were used for immunohistochemical study.

Cardiac membrane preparation and binding experiments for ET receptors. The left ventricles, which were stored at −80°C until use, were placed in MOPS buffer containing 20% (wt/vol) sucrose at 4°C, cut into small pieces, and homogenized for 60 s with a Polytron homogenizer (PT10SK/35; Kinematica, Lucerne, Switzerland). The homogenates were centrifuged at 1,000 g for 15 min at 4°C. The supernatants were centrifuged at 10,000 g for 15 min at 4°C. Finally, the resulting supernatants were centrifuged at 105,000 g for 40 min at 4°C. The pellets were suspended in 5 mmol/l HEPES-Tris buffer (pH 7.4) and stored at −80°C until use. The protein concentration was determined by bicinchoninic acid protein assay (42).

Experiments regarding the binding of 125I-labeled ET-1 to the membranes of the left ventricle of the rats were performed according to a previously described method (28). The membranes were incubated with 10 pmol/l ET-1 and ET-1- and unlabeled ET-1 at concentrations ranging from 900 fmol/l to 200 µmol/l in triplicate at 25°C in 50 mmol/l Tris-HCl (pH 7.4) containing 0.1 mmol/l phenylmethylsulfonnyl fluoride, 2 µmol/l leupeptin, 1 µmol/l 1.10-phenanthroline, 1 mmol/l EDTA, and 0.1% bovine serum albumin (BSA). After 4 h of incubation, cold 5 mmol/l HEPES-Tris buffer (pH 7.4) containing 0.3% BSA (buffer A) was added to the mixture. Free and bound 125I was separated using a cell harvester (M-24, Brandel, Gaithersburg, MD) by rapid filtration through glass fiber filters (GF/C, Whatman) that had been presoaked in buffer A. After the filters were washed with buffer A, radioactivity was measured in a gamma counter (ARC-1000M; Aloka, Tokyo, J apan). Specific 125I-ET-1 binding was defined as the difference between total binding and nonspecific binding in the presence of 200 nmol/l ET-1. The binding site density (Bmax) and dissociation constant (Kd) values were determined by regression analysis of displacement curves using the LIGAND program (17). Furthermore, to study ET A- and ET B-receptor subtypes in the rat hearts, we performed a competitive displacement experiment of 125I-ET-1 binding to rat cardiac membranes using BQ-123 (an ET A-receptor antagonist).

RT-PCR to evaluate the mRNA levels of ET A-receptor and ET B-receptor mRNA, preproET-1 mRNA, ECE-1 mRNA, and ACE mRNA in left ventricle. The expression of ET A- and ET B-receptor mRNA, preproET-1 mRNA, ECE-1 mRNA, and ACE mRNA was analyzed by RT-PCR. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was also determined as an internal control.

Total tissue RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction with LiSogen (Nippon Gene, Tokyo, J apan) according to the manufacturer’s instructions. The tissue was homogenized in LiSogen (0.2 g tissue per 2 ml LiSogen) with a Polytron tissue homogenizer. Chloroform extraction, isopropanol precipitation, and 75% (vol/vol) ethanol washing of precipitated RNA were subsequently performed. The obtained RNA was resolved in diethyl pyrocarbonate-treated water. For the elimination of the genomic DNA, the RNA was treated with DNase I (TaKaRa, Otsu, Japan) formed at 43°C for 60 min. Whatman filters were washed with buffer A, radioactivity was measured in a gamma counter (ARC-1000M; Aloka, Tokyo, J apan). Specific 125I-ET-1 binding was defined as the difference between total binding and nonspecific binding in the presence of 200 nmol/l ET-1. The binding site density (Bmax) and dissociation constant (Kd) values were determined by regression analysis of displacement curves using the LIGAND program (17). Furthermore, to study ET A- and ET B-receptor subtypes in the rat hearts, we performed a competitive displacement experiment of 125I-ET-1 binding to rat cardiac membranes using BQ-123 (an ET A-receptor antagonist).

RT-PCR to evaluate the mRNA levels of ET A-receptor and ET B-receptor mRNA, preproET-1 mRNA, ECE-1 mRNA, and ACE mRNA in left ventricle. The expression of ET A- and ET B-receptor mRNA, preproET-1 mRNA, ECE-1 mRNA, and ACE mRNA was analyzed by RT-PCR. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was also determined as an internal control.

Total tissue RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction with LiSogen (Nippon Gene, Tokyo, J apan) according to the manufacturer’s instructions. The tissue was homogenized in LiSogen (0.2 g tissue per 2 ml LiSogen) with a Polytron tissue homogenizer. Chloroform extraction, isopropanol precipitation, and 75% (vol/vol) ethanol washing of precipitated RNA were subsequently performed. The obtained RNA was resolved in diethyl pyrocarbonate-treated water. For the elimination of the genomic DNA, the RNA was treated with DNase I (TaKaRa, Otsu, J apan) and was extracted again with LiSogen. The RNA concentration was measured spectrophotometrically at 260 nm.

Total RNA (5 µg) was primed with 0.05 µg oligo-d(T)12–18 and reverse transcribed by avian myeloblastosis virus reverse transcriptase using the First-Strand cDNA Synthesis Kit (Life Sciences, St. Petersburg, FL). The reaction was performed at 43°C for 60 min.

The obtained cDNA was diluted in a 1:10 ratio, and 1 µl was used for PCR. Each PCR reaction contained 10 mM Tris·HCl
(pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 µM of each dNTP, 0.5 µM of each gene-specific primer, and 0.025 U/µl Taq polymerase (TaKaRa). The gene-specific primers were synthesized according to the published cDNA sequences. The sequences of the oligonucleotides were as follows: ET₄ receptor (sense), 5'-ATCGCTGACATGTGAGAG-3'; ET₅ receptor (antisense), 5'-CCACGATGAAAAATGTACAG-3'; ET₆ receptor (sense), 5'-GAAGAGATCTCCACCTTG-3'; ET₇ receptor (antisense), 5'-ACGAGGACGAGCAGTAC-3'; propreET-1 (sense), 5'-TCTCTCTCTGCTGTTTGTG-3'; prepET-1 (antisense), 5'-TAGTTTTCTCTCCCTCCACC-3'; ECE-1 (sense), 5'-TGGTCTCGGTACGTCTCTC-3'; ECE-1 (antisense), 5'-GGTCATACACGCACGGTAG-3'; ACE (antisense), 5'-CGTTCATACACGCACGGTAG-3'; ACE (sense), 5'-GTGGAGGAGTATGACCG-3'; preproET-1 (sense), 5'-TCTTCTCTGCTGTTTGTG-3'; rat ETA receptor (antisense), 5'-CCACGATGAAAATGGTACAG-3'; rat ETB receptor (antisense), 5'-GAAAAGAGGATTCCCACCTG-3'; 50% formamide, 3% SSPE-0.05% SDS at room temperature for 30 min and in 1× SSPE-0.1% SDS at 60°C for 40 min. The membrane was subjected to autoradiography for a suitable time period. The same membrane was rehybridized with each labeled probe.

Immunohistochemical analysis for ET-1 staining in heart. To study the distribution of ET-1 staining (ET-1-like immunoreactivity) in the heart of rats with CHF due to myocardial infarction and in sham-operated rats, the hearts were subjected to immunohistochemical analysis according to the method described in our previous report (25). In brief, after hemodynamic measurement, the hearts were subjected to perfusion fixation with 0.01 mol/l periodate-0.075 mol/l lysine-2% paraformaldehyde solution at 4°C overnight. The specimens were embedded in paraffin wax (melting point 58°C). The sections were cut into 3-µm thicknesses and stained immunohistochemically using the biotin-streptavidin-horseradish peroxidase method for the detection of ET-1. Rabbit anti-ET-1 polyclonal antibody (Peninsula Laboratories, Belmont, CA) was used as the primary antibody. Deparaffinized sections were incubated with rabbit anti-ET-1 antibody (dilution 1:400) at room temperature for 3 h. After the sections were washed, they were incubated with secondary antibody diluted at 1:100 (biotinylated anti-rabbit IgG; IBL, Fujioka, Japan) for 30 min and then with horseradish peroxidase-conjugated streptavidin diluted at 1:100 for 30 min. Immunoreactive ET-1 products were visualized with 0.05% 3,3'-diaminobenzidine-0.02% hydrogen peroxide. The slides were counterstained with methyl green. The specificity of the immunoreactivity was determined by an absorption test, i.e., the consecutive sections were subjected to the same procedure with the use of the supernatant of anti-ET-1 antibody (dilution 1:100) preabsorbed with synthetic ET-1 (20 µmol/l) at 4°C overnight.

Statistical analysis. All data are means ± SE. Statistical comparisons were performed using the unpaired Student's t-test or Welch's t-test with a commercially available statistical package for the Macintosh personal computer (StatView, v. 4.5; Abacus Concepts, Berkeley, CA). The results were considered statistically significant at P < 0.05.

RESULTS

Figure 1 shows the hemodynamic parameters of control sham-operated rats and rats with coronary artery ligation 3 wk after surgery. Mean arterial blood pressure was significantly lower in rats with CHF than in control rats (Fig. 1A). LV +dP/dt max was significantly lower in rats with CHF than in control rats (Fig. 1B). LVEDP was significantly higher in rats with CHF than in control rats (Fig. 1C). Right ventricular systolic pressure was significantly higher in rats with CHF than in control rats (Fig. 1D). In addition, central venous pressure was significantly higher in rats with CHF than in control rats (Fig. 1E). These results suggest that the rats with coronary artery ligation developed heart failure.

We studied the expression of ET₄ and ET₅ receptors in the failing heart. The protein levels were evaluated by -125I-ET-1 binding assay and a BQ-123 displacement experiment. The mRNA levels were evaluated by RTPCR. The binding assay of rat cardiac membranes revealed that B max for ET-1 was significantly higher in rats with CHF than in control rats [CHF vs. control =
243.0 ± 20.0 vs. 154.8 ± 17.4 (means ± SE) fmol/mg protein, *P* < 0.05, *n* = 5 for both groups), whereas the values of *K*<sub>d</sub> for ET-1 were not different between the two groups (CHF vs. control = 28.7 ± 7.0 vs. 29.8 ± 1.9 pmol/l, *n* = 5 for both groups). To determine the change in each receptor subtype, we performed competitive displacement experiments using BQ-123. The experiment showed that the cardiac membranes of rats with CHF and control rats contained ET<sub>A</sub> and ET<sub>B</sub> receptors in ratios of 86:14 and 91:9 (each *n* = 5), respectively. The protein level of the ET<sub>A</sub> receptors in the heart was significantly higher in rats with CHF than in control rats (Fig. 2A). The protein level of the ET<sub>B</sub> receptor in the heart was significantly higher in rats with CHF than in control rats (Fig. 2B). The expression of ET<sub>A</sub>-receptor mRNA in the failing heart was significantly increased in rats with CHF compared with that in control rats (Fig. 3A). The expression of ET<sub>B</sub>-receptor mRNA in the heart was significantly higher in rats with CHF than in control rats (Fig. 3B).

We evaluated the ET-1 production system in the left ventricle of control rats and rats with CHF. The expression of preproET-1 mRNA in the heart was significantly higher in rats with CHF than in control rats (Fig. 4A).
However, the expression of ECE-1 mRNA in the heart did not differ between the two groups (Fig. 4B). The peptide level of ET-1 in the heart was significantly higher in rats with CHF than in control rats (Fig. 4C).

The expression of ACE mRNA was also evaluated (Fig. 5). The level of ACE mRNA in the heart of rats with CHF was significantly higher than that in the heart of control rats.

To confirm the alteration of gene expressions studied by RT-PCR analysis in the failing heart, we also performed Northern hybridization (Fig. 6). The results of Northern hybridization showed findings similar to those in the RT-PCR analysis. Northern hybridization analysis showed that the expressions of preproET-1 mRNA, ETA- and ETB-receptor mRNA, and ACE mRNA in the heart were higher in rats with CHF than in control rats and that the expression of ECE-1 mRNA in the heart did not differ between the two groups.

The cellular distribution of ET-1 in the hearts failing due to myocardial infarction was evaluated by immunohistochemical analysis. Typical examples of the ET-1 staining (ET-1-like immunoreactivity) in the rat heart are shown in Fig. 7. ET-1 staining was observed as a brown color. This experiment revealed that the inten-
University of ET-1 staining in cardiomyocytes was significantly higher in the noninfarcted area of the left ventricle in rats with CHF than in that of control rats (Fig. 7, A and C). There was no difference in the intensity of staining in endothelial cells of coronary arteries between the two groups (compare Fig. 7, B and D), whereas the intensity of staining in cardiomyocytes was stronger in rats with CHF than in control rats (Fig. 7, B and D). The ET-1 staining in the marginal zone of the infarcted area in rats with CHF is shown in Fig. 7E. Strong intensity of ET-1 staining can be seen in the surviving cardiomyocytes in the marginal zone of the infarcted area (Fig. 7E); however, the fibrotic tissues including fibroblasts in the marginal zone of the infarcted area were not stained (Fig. 7E). Furthermore, ET-1 staining cannot be seen in the fibrous scar tissue of the infarcted area (Fig. 7E).

**DISCUSSION**

In this study, we revealed the details of the changes in the myocardial ET system in rats with CHF due to myocardial infarction. Protein levels of both ETA and ETB receptors in the heart of the rats with CHF were increased in comparison with those in the heart of control rats. These increases were accompanied by an increase in the expression of ETA- and ETB-receptor mRNA. The peptide and mRNA levels of ET-1 were also increased in the heart of rats with CHF. However, the expression of ECE-1 mRNA was not altered in the heart of rats with CHF. On the other hand, the expression of ACE mRNA was increased in rats with CHF. The difference in expression between ECE-1 mRNA and ACE mRNA suggests that the contribution of the converting enzyme of the ET system to the regulation of mature peptide production may differ at this stage from that of the converting enzyme of the angiotensin II system in the failing heart of rats with CHF due to myocardial infarction. The intensity of ET-1 staining in the cardiomyocytes was markedly stronger in rats with CHF, whereas fibrotic tissues in the marginal zone of the infarcted area were not stained. These findings suggest that both the ETA- and ETB-receptor systems are greatly accelerated in the failing heart of rats with CHF and that the increase in the mature peptide level of ET-1 in the heart of rats with CHF originates from upregulation of preproET-1 mRNA, which was not accompanied by alteration of the expression of ECE-1 mRNA. Furthermore, the present study also suggests that the cardiomyocytes, but not the fibrotic tissues, chiefly contribute to the increase in the production of ET-1 in CHF due to myocardial infarction. We evaluated the expression of both ETA and ETB receptors in the heart of control rats and rats with CHF. The present binding study demonstrated that left ventricular sarcolemmal ET-receptor density was primarily of the ETA-receptor subtype in both control rats and rats with CHF in a ratio of 9:1 (ETA receptor:ETB receptor). The calculated Bmax value of ETA receptors in the heart was significantly higher in rats with CHF than in control rats. The level of ETA-receptor mRNA increased in the heart of rats with CHF. However, the expression of ECE-1 mRNA was not altered in the heart of rats with CHF. On the other hand, the expression of ACE mRNA was increased in rats with CHF. The difference in expression between ECE-1 mRNA and ACE mRNA suggests that the contribution of the converting enzyme of the ET system to the regulation of mature peptide production may differ at this stage from that of the converting enzyme of the angiotensin II system in the failing heart of rats with CHF due to myocardial infarction. The intensity of ET-1 staining in the cardiomyocytes was markedly stronger in rats with CHF, whereas fibrotic tissues in the marginal zone of the infarcted area were not stained. These findings suggest that both the ETA- and ETB-receptor systems are greatly accelerated in the failing heart of rats with CHF and that the increase in the mature peptide level of ET-1 in the heart of rats with CHF originates from upregulation of preproET-1 mRNA, which was not accompanied by alteration of the expression of ECE-1 mRNA. Furthermore, the present study also suggests that the cardiomyocytes, but not the fibrotic tissues, chiefly contribute to the increase in the production of ET-1 in CHF due to myocardial infarction. We evaluated the expression of both ETA and ETB receptors in the heart of control rats and rats with CHF. The present binding study demonstrated that left ventricular sarcolemmal ET-receptor density was primarily of the ETA-receptor subtype in both control rats and rats with CHF in a ratio of 9:1 (ETA receptor:ETB receptor). The calculated Bmax value of ETA receptors in the heart was significantly higher in rats with CHF than in control rats. The level of ETA-receptor mRNA increased in the heart of rats with CHF. However, the expression of ECE-1 mRNA was not altered in the heart of rats with CHF. On the other hand, the expression of ACE mRNA was increased in rats with CHF. The difference in expression between ECE-1 mRNA and ACE mRNA suggests that the contribution of the converting enzyme of the ET system to the regulation of mature peptide production may differ at this stage from that of the converting enzyme of the angiotensin II system in the failing heart of rats with CHF due to myocardial infarction. The intensity of ET-1 staining in the cardiomyocytes was markedly stronger in rats with CHF, whereas fibrotic tissues in the marginal zone of the infarcted area were not stained. These findings suggest that both the ETA- and ETB-receptor systems are greatly accelerated in the failing heart of rats with CHF and that the increase in the mature peptide level of ET-1 in the heart of rats with CHF originates from upregulation of preproET-1 mRNA, which was not accompanied by alteration of the expression of ECE-1 mRNA. Furthermore, the present study also suggests that the cardiomyocytes, but not the fibrotic tissues, chiefly contribute to the increase in the production of ET-1 in CHF due to myocardial infarction. We evaluated the expression of both ETA and ETB receptors in the heart of control rats and rats with CHF. The present binding study demonstrated that left ventricular sarcolemmal ET-receptor density was primarily of the ETA-receptor subtype in both control rats and rats with CHF in a ratio of 9:1 (ETA receptor:ETB receptor). The calculated Bmax value of ETA receptors in the heart was significantly higher in rats with CHF than in control rats. The level of ETA-receptor mRNA increased in the heart of rats with CHF. However, the expression of ECE-1 mRNA was not altered in the heart of rats with CHF. On the other hand, the expression of ACE mRNA was increased in rats with CHF. The difference in expression between ECE-1 mRNA and ACE mRNA suggests that the contribution of the converting enzyme of the ET system to the regulation of mature peptide production may differ at this stage from that of the converting enzyme of the angiotensin II system in the failing heart of rats with CHF due to myocardial infarction. The intensity of ET-1 staining in the cardiomyocytes was markedly stronger in rats with CHF, whereas fibrotic tissues in the marginal zone of the infarcted area were not stained. These findings suggest that both the ETA- and ETB-receptor systems are greatly accelerated in the failing heart of rats with CHF and that the increase in the mature peptide level of ET-1 in the heart of rats with CHF originates from upregulation of preproET-1 mRNA, which was not accompanied by alteration of the expression of ECE-1 mRNA. Furthermore, the present study also suggests that the cardiomyocytes, but not the fibrotic tissues, chiefly contribute to the increase in the production of ET-1 in CHF due to myocardial infarction. We evaluated the expression of both ETA and ETB receptors in the heart of control rats and rats with CHF. The present binding study demonstrated that left ventricular sarcolemmal ET-receptor density was primarily of the ETA-receptor subtype in both control rats and rats with CHF in a ratio of 9:1 (ETA receptor:ETB receptor). The calculated Bmax value of ETA receptors in the heart was significantly higher in rats with CHF than in control rats. The level of ETA-receptor mRNA increased in the heart of rats with CHF. However, the expression of ECE-1 mRNA was not altered in the heart of rats with CHF. On the other hand, the expression of ACE mRNA was increased in rats with CHF. The difference in expression between ECE-1 mRNA and ACE mRNA suggests that the contribution of the converting enzyme of the ET system to the regulation of mature peptide production may differ at this stage from that of the converting enzyme of the angiotensin II system in the failing heart of rats with CHF due to myocardial infarction. The intensity of ET-1 staining in the cardiomyocytes was markedly stronger in rats with CHF, whereas fibrotic tissues in the marginal zone of the infarcted area were not stained. These findings suggest that both the ETA- and ETB-receptor systems are greatly accelerated in the failing heart of rats with CHF and that the increase in the mature peptide level of ET-1 in the heart of rats with CHF originates from upregulation of preproET-1 mRNA, which was not accompanied by alteration of the expression of ECE-1 mRNA. Furthermore, the present study also suggests that the cardiomyocytes, but not the fibrotic tissues, chiefly contribute to the increase in the production of ET-1 in CHF due to myocardial infarction. We evaluated the expression of both ETA and ETB receptors in the heart of control rats and rats with CHF. The present binding study demonstrated that left ventricular sarcolemmal ET-receptor density was primarily of the ETA-receptor subtype in both control rats and rats with CHF in a ratio of 9:1 (ETA receptor:ETB receptor). The calculated Bmax value of ETA receptors in the heart was significantly higher in rats with CHF than in control rats. The level of ETA-receptor mRNA
was also higher in rats with CHF than in control rats. The density of ET<sub>B</sub> receptors in the heart of the rats with CHF was significantly higher than that in control rats. ET<sub>B</sub>-receptor mRNA in the failing heart was markedly increased, similarly to the protein level. These data demonstrate that ETA and ET<sub>B</sub> receptors are increased in the heart of rats with CHF and that the increase in the ET receptors was caused by upregulation of ET-receptor mRNA levels. Previously, we reported that acute intravenous infusion of BQ-123, an ETA-receptor antagonist, decreased both myocardial contractility and heart rate in rats with CHF, whereas the infusion of BQ-123 had no apparent effect on these hemodynamic parameters in normal rats (12). This suggests that, in this pathological setting of CHF, ET-1 contributes to modulating myocardial function.

We evaluated the level of preproET-1 mRNA and the level of ET-1 peptide in the failing heart. The finding of increased levels of preproET-1 mRNA and ET-1 peptide in the failing heart was in agreement with previous reports (25, 28, 39). In the failing heart of rats with CHF, the expression of ETA receptors, ET<sub>B</sub> receptors, and ET-1 was upregulated. These data suggest that the stimulation of ET-1 on each ET-receptor subtype (ETA receptor and ET<sub>B</sub> receptor) may be elevated in the failing heart of rats with CHF. Furthermore, this is in accord with a report by Qi et al. (22) that the myocardial contractile effects mediated through ETA receptors as well as ET<sub>B</sub> receptors by exogenous ET-1 are increased in the failing rat heart due to myocardial infarction (22). We previously demonstrated that long-term administration of an ETA-receptor antagonist ameliorated CHF in rats (25). Another study demonstrated that the administration of an ETA/ET<sub>B</sub> dual antagonist ameliorated CHF in rats (16). In the case of the chronic treatment of CHF with ET-receptor antagonists on CHF, we consider that the antagonizing effect of ET antagonists on the cardiac ET receptors of the failing heart is one of the mechanisms for improving CHF. It is also considered that the accelerated ET system (increase in ET-1 and ET receptors) in the heart partly contributes to the development of CHF.
We also studied the expression of ECE-1 mRNA to determine the regulation of ET-1 production in the failing heart. The level of ECE-1 mRNA did not alter in the heart of the rats with CHF. This finding is of interest in comparison with the actions of the renin-angiotensin system in failing hearts. Angiotensin II is a vasoactive peptide that induces cardiac hypertrophy similarly to ET-1. ACE converts the biologically inactive precursor, angiotensin I, to angiotensin II as an active form. ACE mRNA is expressed in hearts and is upregulated in failing hearts. Previous studies showed that the level of angiotensin II peptide in the failing heart is increased (20) and that the increase in angiotensin II is accompanied by the upregulation of ACE mRNA and ACE activity in failing hearts (46). It is considered that the upregulation of ACE plays an important role in the increase in cardiac tissue angiotensin II. In this study, we also demonstrated that the mRNA level of ACE was higher in the failing heart than in the control heart. However, the level of ECE-1 mRNA in the heart of rats with CHF was similar to that in the heart of control rats, despite a marked increase in the peptide level of ET-1 in the heart of rats with CHF. We also demonstrated that the expression of preproET-1 mRNA was increased in the failing heart of rats with CHF. The increase in ET-1 peptide in the failing heart may have originated from the upregulation of preproET-1 mRNA, which was not accompanied by an alteration in the expression of ECE-1. A possible explanation for this finding is that the expression of ECE-1, which is translated from ECE-1 mRNA, in the rat heart may be sufficient to synthesize ET-1 in vivo even when the production of preproET-1 is markedly increased. In this study, we found an increase in ACE mRNA in the failing heart. The difference in expression between ECE-1 mRNA and ACE mRNA suggests that the contribution of the converting enzyme of the ET system to the regulation of mature peptide production at this chronic stage may differ from that of the converting enzyme of the angiotensin II system in the failing heart of rats with CHF due to myocardial infarction. Our findings suggest that ECE-1 is not a rate-limiting factor in the increase in mature ET-1 production in the failing heart of rats with CHF.

Our previous studies showed that production of ET-1 is increased in the heart failing due to myocardial infarction (25, 28). One of the features of failing hearts is structural remodeling, e.g., hypertrophy of cardiomyocytes and fibrosis with proliferation of fibroblasts. ET-1 induces cardiac cell hypertrophy and has a mitogenic effect on fibroblasts (2, 38). In vitro studies demonstrated that both cardiomyocytes (37) and fibroblasts (4, 45) can produce ET-1. It was not known which cells mainly produce ET-1 in the failing heart in vivo. To answer this question, we performed immunohistochemical ET-1 staining of cardiac tissue from the failing heart. Cardiomyocytes were stained, whereas fibroblasts were not stained. The ET-1 staining in the failing heart was shown to be increased in the myocytes in noninfarcted areas and in surviving myocytes around infarcted areas. This result suggests that the increase in ET-1 in the heart of rats with CHF due to myocardial infarction is mainly produced by cardiomyocytes at this stage of CHF. Previously, we reported that the administration of the ETA-receptor antagonist BQ-123 in rats with CHF inhibited cardiac remodeling (25). We considered that ET-1 produced by cardiomyocytes in the failing heart promotes hypertrophy of cardiomyocytes in an autocrine/paracrine fashion and that ET-1 from cardiomyocytes promotes proliferation of fibroblasts in a paracrine fashion in the failing heart. It has been reported by others (4, 37, 45) using the culture system that ET-1 is expressed in cardiac fibroblasts as well as cardiomyocytes (4, 37, 45). The present study showed that the intensity of ET-1 staining in the cardiomyocytes was markedly stronger in rats with CHF, whereas the fibrotic tissues of the infarcted area were not stained. Therefore, the present finding that ET-1 is expressed only in cardiomyocytes in the failing heart is interesting.

We reported that long-term (12 wk) treatment with an ET-receptor antagonist greatly improves the survival rate of rats with CHF (25). This beneficial effect was accompanied by significant amelioration of left ventricular dysfunction and prevention of unfavorable ventricular remodeling (an increase in the ventricular mass of the surviving myocardium and cavity enlargement of the ventricle) (25). We recently reported that chronic treatment with an ET-receptor antagonist effectively ameliorated the altered expression of various cardiac genes in the failing heart (26, 27). The switching of cardiac myosin heavy chain (MHC) isoforms from α-MHC to β-MHC was observed in the failing heart of rats with CHF. This switching was significantly ameliorated by chronic treatment with an ET-receptor antagonist (26, 27). The expression of atrial natriuretic peptide mRNA in the heart was markedly increased in rats with CHF, and this increase was greatly inhibited by chronic treatment with an ET-receptor antagonist (26). The expression of the mRNA for cardiac sarcoplasmic reticulum Ca2+-ATPase, which is essential for myocardial function, was decreased in rats with CHF, and this change was normalized by chronic treatment with an ET-receptor antagonist (26). These findings suggest that chronic treatment with an ET-receptor antagonist ameliorates the failing heart at the molecular level.

In summary, we determined the details of the ET system in the failing heart of rats with CHF due to myocardial infarction. In the failing heart of rats with CHF, the mRNA level of preproET-1 and the peptide level of ET-1 were markedly higher than in the heart of control rats, whereas the level of ECE-1 mRNA was equivalent in the two groups. We also showed the upregulation of ACE mRNA in the failing heart. It was considered that each of the converting enzymes in the ET synthesis pathway and the angiotensin II synthesis pathway acts in a different manner in increasing mature peptide levels. A prominent increase in ET-1 staining was shown in the surviving cardiomyocytes in both the infarcted and noninfarcted areas of the failing heart but not in the fibrotic tissues. We demonstrated that the protein levels of both ETA and ETB receptors...
were upregulated in the failing heart. We also demonstrated that the mRNA levels both of ET_A and ET_B receptors were increased in the failing heart. The significant increase in ET-1 and ET receptors suggests that both the ET_A- and ET_B-receptor systems are greatly accelerated in the failing heart of rats with CHF.

We thank Dr. Masaru Nishikibe for useful discussions concerning the study.

This study was supported by a grant from the Study Group of Molecular Cardiology, by a grant from the Miyaiuchi Project of the Center for Tsukuba Advanced Research Alliance at the University of Tsukuba, and by grants-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan (8670757, 9770473). Address for correspondence and reprint requests: T. Miyau, Cardiovascular Div., Dept. of Internal Medicine, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan (E-mail: t-miyau@md.tsukuba.ac.jp).

Received 11 June 1998; accepted in final form 8 December 1998.

REFERENCES