Upregulated Expression of Cardiac Endothelin-1 Participates in Myocardial Cell Growth in Bio14.6 Syrian Cardiomyopathic Hamsters

Tsukasa Inada, MD,* Hisayoshi Fujiwara, MD,* Koji Hasegawa, MD,* Makoto Araki, MD,* Rikako Yamauchi-Kohno, MS,† Hideo Yabana, Ph.D,† Takako Fujiwara, MD,‡ Masaru Tanaka, MD,* Shigetake Sasayama, MD, FACC*

Kyoto, Japan

OBJECTIVES The purpose of this study is to investigate the role of endogenous endothelin-1 (ET-1) in myocardial growth in Bio14.6 Syrian cardiomyopathic hamsters (Bio).

BACKGROUND While ET-1, as a growth-promoting peptide, has been implicated in the development of secondary cardiac hypertrophy, the role of endogenous ET-1 in cardiac growth in primary myocardial disease is unknown.

METHODS We measured left ventricular ET-1 levels by a specific sandwich enzyme-linked immunosorbent assay. Furthermore, we examined the chronic effect of T0201, an ET type A receptor-specific antagonist.

RESULTS The ET-1 levels in the left ventricles were 1.8-fold higher (p < 0.0005) at 20 weeks and 6.4-fold higher (p < 0.0001) at 35 weeks in the Bio compared to age-matched control F1B hamsters (F1B). The Bio ET-1 levels in the lungs exhibited an only 1.3-fold elevation at 35 weeks. Immunohistochemistry demonstrated the localization of ET-1 mainly in the cardiac myocytes. The treatment with T0201 significantly reduced the heart weight/body weight ratio in the Bio, but did not affect the heart weight/body weight ratio in the F1B. Histologically, T0201 reduced the myocyte diameter of Bio to a level similar with that of F1B. However, T0201 did not affect the extent of fibrosis in Bio or F1B.

CONCLUSIONS The ET-1 level in the heart of cardiomyopathic hamsters increases in stage-dependent and organ-specific manners. Though myocyte degeneration and subsequent replacement fibrosis do not require an ET-1 pathway, the accelerated synthesis of ET-1 in the heart may contribute to the pathological growth of remaining myocytes in this animal model. (J Am Coll Cardiol 1999;33:565–71) © 1999 by the American College of Cardiology

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide originally isolated from the supernatants of cultured endothelial cells (1). It exerts its effect through the stimulation of specific receptors (ET_A and ET_B receptors) widely distributed in the cardiovascular system (2,3). The plasma levels of ET-1 are elevated in various pathophysiological states including congestive heart failure (4,5). The elevated level of ET-1 in heart failure is closely associated with the extent of pulmonary hypertension (5). In this state, an acute administration of endothelin receptor blocker reduced vascular resistance and improved an overall left ventricular performance (6,7). These findings suggest that ET-1 has a pathophysiological role in heart failure as a circulating hormone. In addition to its action as a systemic hormone, ET-1 has a number of actions as a local factor (8). Endothelin-1 is sufficient to induce the myocardial cell hypertrophy associated with the reactivation of the fetal gene program; i.e., the induction of atrial natriuretic factor and β-myosin heavy chain expression (9,10). Endothelin-1 mediates load-induced hypertrophy in cultured neonatal cardiac myocytes as an autocrine factor (11). Previous studies demonstrated that the expression of ET-1 in the heart is accelerated following pressure overload and myocardial infarction, and that the chronic administration of an ET receptor antagonist blocks the development of hypertrophy in animal models (12,13). These findings demonstrate that ET-1 is involved in the development of cardiac hypertrophy secondary to hemodynamic overload. Little is known regarding a role of ET-1 in primary myocardial disease, however.
We previously reported that in hypertrophic cardiomyopathy patients with normal pulmonary arterial pressure, plasma ET-1 levels are significantly elevated (14). The elevated ET-1 levels closely correlated with the myocardial cell diameter in endomyocardial biopsy specimens. These findings raise the possibility that ET-1 has a role in primary myocardial disease. However, it is not known whether the elevated plasma levels of ET-1 are derived from hearts with hypertrophic cardiomyopathy, or whether endogenous ET-1 directly contributes to the development of hypertrophy in primary myocardial disease. To identify the role of endogenous ET-1 in the development of hypertrophy in primary myocardial disease, we investigated the cardiac synthesis of ET-1 in Bio14.6 Syrian cardiomyopathic hamsters (Bio) and examined the effect of an oral ET\(_A\) receptor antagonist on the pathogenesis of this animal model.

**METHODS**

**Experimental animals.** Male cardiomyopathic hamsters of the Bio 14.6 Syrian strain (Bio) and control F1B hamsters (F1B) aged 5 weeks old were purchased from Charles River Japan (Atsugi, Japan). Hamsters were individually housed in our animal room maintained at a temperature of 24 ± 1°C and with a 12-hour light/dark cycle (0600-1800 hours). The animals received powdered diet (CE-7, Nihon Clea, Tokyo, Japan) and tap water ad libitum. The following experiments were conducted for 35 weeks. These animals were sacrificed at the age of 20 weeks or 35 weeks.

**Tissue preparation.** After the heart was excised and weighed, the atria and the right ventricles were trimmed off, and the left ventricle was rinsed in cold physiological saline. The basal one-third of the left ventricle was cut into two slices, one of which was embedded in OCT compound (Miles Laboratories, Elkhart, Indiana), frozen in dry ice acetone and stored at −70°C until use for immunohistochemistry. The other slice was fixed with 10% formalin for histological examination with hematoxylin–eosin and Masson-trichrome staining. For the measurement of cardiac ET-1 levels, the apical two-thirds of the left ventricle was immediately homogenized with a Polytron homogenizer for 30 s in 9 volumes of 1 mol/L acetic acid containing 0.1% Triton-X, boiled for 7 min and centrifuged at 20,000g for 30 min at 4°C. Following the measurement of the protein concentrations (pg/g), the supernatant was stored at −80°C until use.

**Measurement of myocardial ET-1 levels.** ET-1 was extracted from the supernatant of homogenized ventricular tissues according to the method of Kitamura et al. (15). Briefly, 1 milliliter of the supernatant was acidified with 3 ml of 4% acetic acid and applied to a Sep-Pak C18 cartridge (Waters, Milford, Massachusetts). The absorbed peptides were eluted with 86% ethanol containing 4% acetic acid, dried under nitrogen gas and reconstituted with 250 μl of assay buffer. The ET-1 levels were measured by means of a sandwich enzyme-linked immunosorbent assay (EIA) kit (Wako Pure Chemical, Osaka, Japan) as previously described (14). This sandwich EIA utilized two antibodies that recognize different portions of ET-1: a mouse monoclonal antibody against the NH\(_2\)-terminal portion of rat ET-1 immobilized on a 96-well plate and a horseradish peroxidase-labeled rabbit antibody against the COOH-terminal portion of rat ET-1. After color development with o-phenylenediamine as a substrate, the absorbance of each well was measured at 492 nm. This EIA for ET-1 could detect as little as 0.5 pg/ml of ET-1. The cross-reactivity with ET-3 or big ET-1 was less than 0.1%. The values by EIA presented as (pg/ml) were standardized by the protein concentrations of the supernatant (g/ml) and expressed as pg/g.

**Immunohistochemical staining for ET-1.** Sections (4 μm) were cut on a cryostat at −20°C and mounted on glass slides. After rinsing in PBS, the hydrated sections were fixed in 10% phosphate-buffered formalin solution for 10 min. Then they were immunostained using an indirect immunoperoxidase method as described previously (16). Briefly, the sections were incubated with 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase activity, followed by further incubation with normal goat serum for 30 min to block nonspecific bindings. They were then incubated with ET-1 antiserum (Peptide Institute, Osaka, Japan) at a final dilution of 1:40 for 16 hr at 4°C. In the second step, they were treated with a 1:200 dilution of peroxidase-conjugated goat anti-rabbit IgG (Jackson Immuno-Research Laboratories, West Grove, Pennsylvania) for 45 min. Peroxidase activity was visualized by use of diaminobenzidine and hydrogen peroxide. The sections were counterstained with hematoxylin, dehydrated through alcohol, and cleared in xylene before coverslipping. The slides were evaluated microscopically. As a control to check the specificity, the primary antibody was preincubated with 1 μg ET-1 peptide (Peptide Institute, Osaka, Japan) for 3 h before application to the slides.

**Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio</td>
<td>Bio 14.6 Syrian cardiomyopathic hamsters</td>
</tr>
<tr>
<td>ET(_A)</td>
<td>endothelin-1 receptor antagonist</td>
</tr>
<tr>
<td>EIA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ET-1</td>
<td>endothelin-1</td>
</tr>
<tr>
<td>F1B</td>
<td>F1B hamsters</td>
</tr>
</tbody>
</table>

Inada et al.  
Endothelin-1 in Cardiomyopathy  
February 1999:565–71
Histological assessment. Myocyte diameters were measured separately in the inner, middle and outer thirds of the left ventricular free wall. Two investigators (M.A., T.I.), who were unaware of the treatment protocol, independently reviewed the sections and randomly selected a total of 100 myocardial fibers from each area. The myocyte diameters were measured semiautomatically with the aid of an image analyzer (LUZEX 3U, Nikon, Tokyo) in sections stained with hematoxylin-eosin (17–19). The shortest diameters of transversely cut fibers were measured at the level of the nucleus. Myocardial fibers were excluded if their boundaries were not clear, if their nuclei were not located centrally or if degenerative changes were prominent. The myocardial cell diameter of an animal was expressed by averaging the data determined by these two investigators.

The investigator (M.A.) who was unaware of the treatment protocol measured all of the fibrotic areas in the sections. The areas of fibrosis stained blue were quantified semiautomatically using the LUZEX 3U image analyzer in sections stained with Masson’s trichrome (19–21). The ratio of the fibrotic area to the total tissue area was calculated as the percent area of fibrosis.

The left ventricular inner and outer cavity areas were measured using the LUZEX 3U image analyzer. The myocardial area was expressed as the outer cavity area subtracted by the inner cavity area.

Statistical analysis. The results are expressed as mean value ± SEM. Statistical comparisons of ET-1 levels at specific time points between Bio and F1B were assessed using unpaired Student t tests. Otherwise, multiple comparisons were performed using ANOVA with Scheffe’s test. In all tests, a p value <0.05 was considered significant.

RESULTS

The cardiac expression of ET-1 in Bio is markedly upregulated in state-dependent and organ-specific manners. To investigate the possible contribution of cardiac ET-1 synthesis to the elevation of plasma ET-1 levels in cardiomyopathy, we determined the left ventricular ET-1 levels in Bio and F1B using a specific sandwich EIA. At 5 weeks of age the left ventricular ET-1 levels of the Bio did not differ from those of the F1B. At the age of 20 weeks, the heart weight/body weight ratio was 25.5% higher in the Bio compared with the age-matched F1B. The left ventricular inner cavity area of Bio (12.6 ± 0.20 mm²) did not differ significantly from that of F1B (13.8 ± 0.21 mm²). However, the myocardial area of Bio (48.3 ± 0.70 mm²) was significantly (p < 0.01) larger than that of F1B (38.9 ± 0.69 mm²). The histological examination revealed focal myocyte degeneration with fibrosis and inhomogenous zones of hypertrophy. In these cardiomyopathic hamsters, the ventricular ET-1 levels were mildly (1.8-fold) elevated (Fig. 1A). At the age of 35 weeks, the left ventricular inner cavity area of Bio (30.4 ± 0.33 mm²) was much larger (p < 0.0001) than that of F1B (16.3 ± 0.29 mm²) while the myocardial area of Bio (42.1 ± 0.47 mm²) was similar to that of F1B (44.5 ± 0.58 mm²). The histological examination revealed the replacement of degenerative myocytes with fibrosis. The heart weight/body weight ratio increase (22.9%) was similar compared to that at the age of 20 weeks. At this stage, the ventricular ET-1 levels were markedly elevated (6.4-fold) in the Bio compared with the F1B (Fig. 1A). Thus, the left ventricular ET-1 levels increased in the Bio in a stage-dependent manner. We also determined the
ET-1 levels in the lungs (Fig. 1B). The pulmonary ET-1 levels did not differ between the F1B and Bio at the ages of 5 weeks and 20 weeks. At 35 weeks, the levels were 1.3-fold higher in the Bio than in the F1B. Thus, the elevation of ET-1 levels in the Bio compared with the F1B was much more prominent in the heart than in the lung, suggesting the organ-specific activation of ET-1 synthesis.

Immunohistochemical detection of ET-1 in the left ventricular myocardium. In addition to endothelial cells, various cell types including cardiac myocytes have the ability to synthesize ET-1 (11,22). To determine which cell type is responsible for the elevated levels of ET-1 in the Bio heart, we performed immunohistochemical staining. Strong positive brown signals for ET-1 were widely distributed within the left ventricular myocardium of the Bio (Fig. 2A). The specificity of the immunostaining was confirmed by the finding that the signals were completely abolished by preincubating the primary antibody with an excess of synthetic ET-1 (Fig. 2B). The ET-1 expression was homogenous, suggesting that systemic hormonal factors are involved in the ET-1 expression in cardiac myocytes.

The effect of T0201 on organ weights and histological changes. To determine whether the accelerated synthesis of ET-1 in the heart participates in the pathogenesis of cardiomyopathy, we randomly assigned five-week-old Bio and F1B hamsters to treatment with vehicle or an oral ET type A receptor-specific antagonist, T0201. No deaths occurred in the vehicle- or T0201-treated Bio and F1B until sacrifice at the ages of 20 weeks and 35 weeks. Organ weights were compared between the T0201- and vehicle-treated groups. As shown in Figure 3, T0201 did not affect the heart weight/body weight ratio in the F1B. However, in the Bio, the heart weight/body weight ratio was significantly lower in the T0201-treated group than in the vehicle-treated group at both 20 weeks and 35 weeks. T0201 did not affect the lung weight/body weight ratio or the kidney weight/body weight ratio in the F1B or Bio (data not shown).

The hearts of cardiomyopathic hamsters spontaneously develop focal necrosis followed by fibrotic replacement and secondary hypertrophy (23). We examined the effect of T0201 on these histological changes in Bio hamsters as shown in Figure 4. The extent of fibrosis in the Bio did not differ between the T0201-treated group and the vehicle-treated group (Fig. 4A). However, the cardiac myocyte diameter in the Bio was significantly lower in the T0201-treated group than in the vehicle-treated group at 20 weeks and 35 weeks (Fig. 4B). Administration of T0201 reduced the cell diameter of Bio to a level similar to that of F1B. By contrast, T0201 did not affect the myocyte diameter in F1B. Thus, T0201 did not inhibit replacement fibrosis but suppressed the growth of remaining myocytes in the Bio hamsters.

DISCUSSION

To clarify the role of endogenous ET-1 in cardiac growth in primary myocardial disease, we have studied Syrian cardiomyopathic hamsters of the Bio14.6 strain. The results of the present study demonstrated that cardiac ET-1 levels in these animals increased in stage-dependent and organ-specific manners. The elevated levels of ET-1 were localized mainly in cardiac myocytes. In addition, a chronic ET\textsubscript{A} receptor blockade with T0201 inhibited myocardial cell growth in the Bio14.6 hamsters but not in the control F1B.
animals. These findings suggest that the accelerated cardiac synthesis of ET-1 contributes to the pathological myocardial growth in this animal model.

Cardiac expression of ET-1 in the Bio14.6 cardiomyopathic hamsters. Plasma ET-1 levels are reported to be high in various kinds of cardiovascular diseases. In patients with chronic heart failure, the increase of circulating ET-1 levels correlates with functional class and alterations in hemodynamics (4,5). Although plasma ET-1 levels are increased in patients with hypertrophic cardiomyopathy (14), the increase in these patients is only 2- to 3-fold. The present study demonstrated that the myocardial ET-1 levels in cardiomyopathic hamsters increased in a stage-specific manner. At 35 weeks, the ET-1 levels in the left ventricles were markedly (6.4-fold) increased in the cardiomyopathic hamsters compared with the controls. The prominent elevation of ET-1 levels in the heart is compatible with the idea that ET-1 plays a pathophysiological role as a local factor rather than a systemic hormone (8,24). In contrast, the increase of ET-1 levels in the lungs of the Bio hamsters was only 1.3-fold. Although these data do not rule out the possible contribution of other organs to the increased levels of ET-1 in plasma, these findings suggest that the heart is one of the main sources of plasma ET-1 in cardiomyopathy.

ET-1 is primarily identified in the supernatant of cultured endothelial cells (1). However, the expression of ET-1 is not confined to these cells but is also observed in various cell types including cardiac myocytes (11,22). Stimulation with angiotensin II or stretch induces the expression of ET-1 in cultured neonatal cardiac myocytes, and an ETA receptor antagonist can block the hypertrophy evoked by these stimuli (11,22). Using an immunohistochemical technique, the present study demonstrated that the elevated level of ET-1 in the heart of cardiomyopathic hamsters is localized mainly in cardiac myocytes. In addition, the chronic administration of an ETA receptor antagonist reduced the myocardial cell diameter of Bio to a level similar to that of control F1B. Since vasodilative agents such as hydralazine do not improve the pathology of cardiomyopathic hamsters (25), the complete blockade of myocardial cell size by an ETA receptor in cardiomyopathy may not be explained by hemodynamic unloading. In fact, the dosage of T0201 used in the present study was not sufficient to decrease the blood pressure in the animals (data not shown). Our findings are compatible with in vitro data (11,22) and suggest that ET-1 plays a role as an autocrine factor for hypertrophy in cardiomyopathy. The triggers that stimulate the cardiac synthesis of ET-1 in these animals are unclear at present. It has been reported that sympathetic nervous systems are activated in these cardiomyopathic hamsters (26). Since an \( \alpha_1 \)-adrenergic agonist is a potent inducer of ET-1 expression in cultured neonatal cardiac myocytes (27), such neurohormonal factors might contribute to the accelerated synthesis of cardiac ET-1 in this animal model. However, further studies are needed to clarify the precise mechanisms that induce ET-1 expression in cardiac myocytes.

The effect of ETA receptor blocker in the cardiomyopathic hamsters. The Bio 14.6 Syrian hamster has been pharmacologically well-studied as an experimental model of cardiomyopathy. In this model, focal myocyte degeneration occurs at an early stage and is followed by replacement with fibrosis (23). The number of calcium channels is increased early in the course of the cardiomyopathy in these hamsters (28). Calcium-channel blockers such as verapamil have been shown to reduce the extent of fibrosis and collagen content in the hearts of these hamsters (25,28). These findings demonstrate that voltage-dependent calcium uptake and subsequent calcium accumulation play a significant role in the development of myocardial cell injury in this animal model. ET-1 has been shown to increase cytosolic calcium levels in cardiac myocytes (29). However, while focal myocardial degeneration starts as early as 5 weeks of age in Bio hamsters, the cardiac ET-1 levels were not elevated at this stage. In addition, the present findings demonstrate that the
chronic ETA receptor blockade with T0201 reduced the myocardiad cell diameter in the Bio but not the extent of fibrosis in cardiomyopathy. These findings suggest that genetically determined myocyte degeneration occurs through ET-1-independent pathways while myocardial ET-1 plays a role in the secondary hypertrophy of the remaining myocytes. As pharmacological intervention to prevent the hypertrophic process (for example, angiotensin-converting enzyme inhibitor treatment) has been shown to be beneficial in this hamster model of cardiomyopathy (30), blockade of hypertrophy by T0201 would be beneficial in this animal model.

Hypertrophic cardiomyopathy is a cardiac disease characterized by a hypertrophied, nondilated left ventricle in the absence of other cardiac disease. Some patients have a left ventricular outflow obstruction with a relatively high incidence of sudden death, and others display dilatation of the left ventricle and heart failure in the later stage. Although missense mutations in the cardiac β-myosin heavy chain gene have been identified in patients with familial hypertrophic cardiomyopathy (31), the precise mechanisms that lead to cardiac hypertrophy are still unclear. The present findings demonstrate that an ET-1 receptor blocker can inhibit the development of myocellular hypertrophy in the Syrian hamster model of cardiomyopathy. This model has focal zones of necrosis and scarring, while hypertrophic cardiomyopathy in humans shows diffuse abnormalities. Therefore, we cannot apply the data of the present study to cardiomyopathy in humans. Nevertheless, taken together with our previous report that plasma ET-1 levels are elevated in patients with hypertrophic cardiomyopathy, it would be of particular interest to investigate whether ET receptor blockers are useful as therapeutic agents for hypertrophic cardiomyopathy in humans.

Acknowledgments

We greatly appreciate Ms. T. Hoshino and Dr. S. Murata for their scientific help. This work was supported in part by grants from the Kanae Foundation of Research for New Medicine, the Japanese Heart Foundation, the Japan Cardiovascular Research Foundation and the Ministry of Education, Science and Culture of Japan.

Reprint requests and correspondence: Koji Hasegawa, MD, Department of Cardiovascular Medicine, Kyoto University, Graduate School of Medicine, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: koj@kuhp.kyoto-u.ac.jp.

REFERENCES