The Role of Endothelin-Converting Enzyme-1 in the Development of \( \alpha_1 \)-Adrenergic-Stimulated Hypertrophy in Cultured Neonatal Rat Cardiac Myocytes

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Background—Accumulating evidence suggests that the local synthesis of endothelin-1 (ET-1) plays a role in the development of heart failure in vivo. We investigated the role of endothelin-converting enzyme-1 (ECE-1), which mediates the conversion of big ET-1 to mature ET-1, in the development of \( \alpha_1 \)-adrenergic-stimulated hypertrophy in cultured neonatal rat cardiac myocytes.

Methods and Results—Phenylephrine (PE) induced the expression of ET-1 in rat cardiac myocytes and accelerated the conversion of big ET-1 to ET-1. The ECE-1 mRNA levels were markedly increased 3 hours after PE stimulation (3.6-fold compared with saline stimulation, \( P < 0.005 \)). A specific ECE-1 antagonist, FR901533, inhibited the PE-stimulated increase in protein synthesis rate by 45% (\( P < 0.05 \)). As genetic markers for the hypertrophic response, FR901533 inhibited the PE-stimulated transcriptional activities of the 3.5-kb \( \beta \)-myosin heavy chain promoter by 79% (\( P < 0.01 \)) but did not affect that of the 3.4-kb atrial natriuretic factor (ANF) promoter. In Bio14.6 Syrian cardiomyopathic hamsters, ventricular ET-1 and ANF mRNA levels did not correlate at 2 different stages.

Conclusions—ET-1-independent pathways may mediate activation of the ANF gene program in ventricular myocytes both in vitro and in vivo. These results also indicate that the conversion of big ET-1 to ET-1 in rat cardiac myocytes is required for the development of \( \alpha_1 \)-adrenergic-stimulated hypertrophy and \( \beta \)-myosin heavy chain gene transcription.

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Key Words: endothelin \( \cdot \) cardiac hypertrophy \( \cdot \) gene expression

Endothelin-1 (ET-1), a potent vasoconstrictor peptide, is synthesized from intermediate big ET-1 by endothelin-converting enzyme-1 (ECE-1). Although ET-1 is mainly produced by endothelial cells at the basal state, a number of cell types can synthesize ET-1 in response to various kinds of stimuli. ET-1 expression in cardiac myocytes is induced by the stimulation of myocardial stretch and by angiotensin II and catecholamine. In addition, ET-1 acts not only as a vasoconstrictor but also as a potent growth-promoting peptide. ET-1 is sufficient to induce the myocardial cell hypertrophy associated with the reactivation of the fetal gene program such as the induction of atrial natriuretic factor (ANF) and \( \beta \)-myosin heavy chain (MHC) expression. The following observations suggest that ET-1 is involved in the development of cardiac hypertrophy and failure as a local factor in vivo. The elevation of left ventricular ET-1 in failing hearts is more marked than that of circulating ET-1. Immunochemistry demonstrated that ET-1 in the failing heart is localized in cardiac myocytes. In addition, the administration of an ET type-A receptor antagonist, BQ123, blocks the development of heart failure by myocardial infarction. However, it is unclear whether this blockade is mediated by the local antagonism of ET-1 action in cardiac myocytes or by a reduction of systemic arterial resistance. Since big ET-1 has little biological action by itself, ECE-1 may play a critical role in the cardiac biosynthesis of ET-1. Thus, the present study investigated the role of ECE-1 in the development of \( \alpha_1 \)-adrenergic-stimulated hypertrophy in cultured neonatal cardiac myocytes.

Methods

Cell Culture
Primary ventricular cardiac myocytes were prepared from 1- to 2-day-old Sprague-Dawley rats (Japan SLC, Inc, Shizuoka, Japan) as previously described. Twenty-four hours after they were plated, cells were washed twice with serum-free media and then stimulated with saline or \( 1.0 \times 10^{-4} \) mol/L of phenylephrine (PE) in serum-free media for 48 hours.

Immunocytochemistry
The cells were fixed with 3% formaldehyde in PBS for 15 minutes at room temperature. Immunocytochemical staining for ET-1 was
performed by use of the indirect immunoperoxidase method, as previously described.11 As the primary antibody, we used anti-ET-1 polyclonal antibody (Peptide Institute) at a dilution of 1:20. The cross-reactivity of this antibody with big ET-1 was <1%. To examine whether ET-1-expressing cells are derived from myocytes, double staining was performed by use of an anti-ET-1 antibody as described above and a monoclonal antibody against muscle-specific α-actin (HHF35) at a dilution of 1:100. Signals of α-actin were detected by the use of an alkaline phosphatase-conjugated Fab fragment of the secondary antibody (a dilution of 1:600, Jackson ImmunoResearch Laboratories) and nitroblue tetrazolium dye as the substrate.

**Measurement of ET-1 and Big ET-1 Levels in Culture Media**

We measured the immunoreactive ET-1 level in the culture media with an ELISA (Wako Chemical Co) as previously described.12 This ELISA is a 2-step sandwich method by use of a monoclonal antibody that recognizes the N-terminal of ET-1 and a peroxidase-conjugated polyclonal antibody that recognizes the C-terminal of ET-1. In this system, the cross-reactivity with ET-3 or big ET-1 is <0.4%.

The immunoreactive big ET-1 level in the culture media was measured by an ELISA (Iwai Chemical Co), according to the manufacturer’s instructions as described.13 This ELISA is a 2-step sandwich method by use of a polyclonal antibody that recognizes the C-terminal of big ET-1 and a peroxidase-conjugated polyclonal antibody against ET-1. In this system, the cross-reactivity with ET-1 is <0.1%.

**Quantitative Reverse Transcriptase-Polymerase Chain Reaction**

Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform procedure. A quantitative reverse transcription-polymerase chain reaction (RT-PCR) was carried out as described previously.14 The PCR primers were designed on the basis of published rat cDNA sequences for ECE-12,15 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)15 as follows; sense for ECE-1: CGTACCAGATGCTTTAGCAC, antisense for ECE-1: GTGCCACACAAAACCTACAG, sense for GAPDH: TTGCACTCATGGAGCTGGTG GCC, antisense for GAPDH: TTGTCTAGATGACCTTGGCC. To define the optimal amplification conditions, a series of pilot studies were performed by the use of various amounts of RT products from 10 to 550 ng RNA and 20 to 45 cycles of PCR amplification in the presence of α-dCTP as described previously.15 A set of the representative data showing the amplification of ECE-1 and GAPDH is illustrated in Figure 1. On the basis of these initial experiments, the linear portion of the amplification was determined for both genes. The following conditions were therefore chosen as standard for the PCR reactions in a volume of 50 μL: RT products from 300 ng RNA for ECE-1 or 150 ng RNA for GAPDH, 2.5 U TaqAmpli polymerase (Perkin-Elmer Cetus), 35 cycles of PCR, denaturation, 94°C for 45 seconds; annealing, 54°C for 45 seconds; and extension, 72°C for 90 seconds. The PCR products (10 ng) were analyzed with a Bioimaging analyzer (BAS 2000, FUJIX).

**Measurement of Protein Synthesis Rate**

The cells were incubated with 5 μCi/mL of [3H]phenylalanine (120 Ci/mmol) and unlabeled phenylalanine (0.36 mmol/L) in the serum-free medium and incubated for 48 hours. The cells were washed twice with PBS, and 10% trichloroacetic acid was added at 4°C for 60 minutes to precipitate protein. The precipitate was washed 3 times with 95% ethanol and then resuspended in 0.15N NaOH. Aliquots were counted by a scintillation counter.

**Plasmid Constructs**

The plasmid constructs p-3542-MHCluc16 and p-3412ANFluc16 composed of the most proximal 3542 bp of the rat β-MHC or 3412 bp of the rat ANF gene 5′-flanking region, respectively, were inserted into the promoterless firefly luciferase reporter plasmid pXP2. pRSVCAT, containing Rous sarcoma virus (RSV) long-
terminal repeat sequences spliced to chloramphenicol acetyltransferase (CAT), has been described previously.\textsuperscript{10,16}

Transfection and Luciferase/CAT Assays
The cells were cotransfected with 4 \textmu g of the luciferase construct of interest and 1 \textmu g of pRSVCAT with lipofectamine (GIBCO BRL) according to the manufacturer’s recommendation. After a 2-hour incubation with DNA-lipofectamine complex, the cells were washed twice with serum-free media and further incubated for 48 hour in serum-free media. The cells were then washed twice with ice-cold PBS and lysed with lysis buffer.\textsuperscript{10,16} The luciferase and CAT activities were determined in the same cell lysate as previously described.\textsuperscript{10,16}

Experimental Animals and Tissue Preparation
Male cardiomyopathic hamsters of the Bio 14.6 strain and control F1B hamsters aged 20 or 35 weeks old were purchased from Charles River, Japan. 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. For measurement of cardiac ET-1 levels, the basal half of the left ventricle was immediately homogenized with a Polytron homogenizer for 30 seconds in 9 vol of 1 mol/L acetic acid containing 0.1% Triton-X, boiled for 7 minutes, and centrifuged at 20 000 \texttimes\ g for 30 minutes at 4°C. The supernatant was stored at -80°C until the measurement of ET-1 levels by ELISA as described above. For mRNA analysis, total RNA was isolated from the apical half of the left ventricles by the use of the acid guanidinium isothiocyanate-phenol-chloroform method, as previously described.\textsuperscript{16}

RNA Analysis
Northern blotting analysis of aliquots of 10 \textmu g of total RNA was performed as previously described.\textsuperscript{16} mRNA abundance was quantified by phosphorimaging analysis (Molecular Dynamics). Values of ANF mRNA were normalized relative to those of GAPDH mRNA.

Statistical Analysis
Data are presented as mean±SE. Statistical comparisons were performed by use of unpaired 2-tailed Student’s t tests or ANOVA with the Scheffé test when appropriate; P<0.05 was considered statistically significant.

Results

Phenylephrine Accelerates the Conversion of Big ET-1 to Mature ET-1
To determine whether stimulation by an \alpha\textsubscript{1}-adrenergic agonist phenylephrine (PE) would induce the expression of ET-1 in cardiac myocytes, cells were stimulated with saline or 1.0\times\textsuperscript{10}^{-4} \text{ mol/L} of PE for 48 hours and then stained with anti-ET-1 antibody. The CPK levels in the media were very low and did not differ between the media of the saline- (0.63±0.49 IU/L, n=8) and PE-stimulated cells (0.88±0.57 IU/L, n=8). As shown in Figure 2, brown positive signals were observed in almost all of the PE-stimulated cells but in none of the saline-stimulated cells. The specificity of the staining was confirmed by its abolition by the addition of an excess of synthetic ET-1 (Figure 2C). To check whether the ET-1-producing cells are derived from cardiac myocytes, we performed double staining by use of an anti-ET-1 antibody and HHF35, which recognizes the \alpha\textsubscript{actin of cardiac muscle cells but not that of fibroblasts. As shown in Figure 2D, most of the PE-stimulated cells (>95%) were positive for both ET-1 (brown signals) and \alpha\textsubscript{actin (purple signals). A small population of cells (<5%) was negative for HHF35 (data not shown).}

The specific sandwich ELISA revealed that the ET-1 levels were significantly higher in the media of the PE-stimulated

![Figure 2](http://circ.ahajournals.org/)

![Figure 3](http://circ.ahajournals.org/)
cells than in that of the saline-stimulated cells (Figure 3A). In contrast, the big ET-1 levels did not differ between these 2 states (Figure 3B). Since the primary production of big ET-1 may be represented as (mature ET-1 big ET-1), we defined the conversion rate (%) as follows: % conversion rate = (mature ET-1)/(mature ET-1 big ET-1) × 100. Thus, the percent conversion rate was significantly higher in the PE-stimulated state (60.6±0.9%) than in the saline-stimulated state (49.2±3.7%); P < 0.05. The primary production of big ET-1 (mature ET-1 big ET-1) was also higher in the PE-stimulated state (30.9±2.9 pmol/L) than in the saline-stimulated state (24.6±3.1 pmol/L), although this difference was not significant.

ECE-1 Gene Transcript is Increased After PE Stimulation
We examined whether PE stimulation induced the expression of ECE-1 gene in these cells. Representative autoradiograms of the quantitative RT-PCR experiment to detect the expression of ECE-1 mRNA in the cultured neonatal cardiac myocytes are shown in Figure 4A, and the corresponding quantitative data after normalizing to the constitutive control, GAPDH, are illustrated in Figure 4B. As shown, the level of

ECE-1 transcript was markedly increased at 3 hours after PE stimulation (3.6-fold increase of the mean value compared with saline-stimulated control, P < 0.005), and maintained an elevated level up to 12 hours in PE-stimulated cardiac myocytes. The increased ECE-1 transcription at 12 hours after PE stimulation was associated with increased ECE-1 activity, since the percent conversion rate was significantly higher in the PE-stimulated state (63.1±3.8%) than in the saline-stimulated state (51.8±4.5%); P < 0.001. Forty-eight hours after the PE stimulation, the ECE-1 mRNA levels did not significantly differ between saline- and PE-stimulated cells. Other stimuli, such as angiotensin II (10−6 mol/L) and isoproterenol (10−4 mol/L), did not induce the expression of ECE-1 gene in cardiac myocytes.

The Effect of a Specific ECE-1 Inhibitor on PE-Induced Hypertrophic Response
FR901533 (Fujisawa Pharmaceutical Co), isolated from Streptosporangium roseum No. 79089, is a potent and specific inhibitor of ECE-1. 17 FR901533 markedly inhibited the ECE-1 activity, with an IC50 value of 1.4×10−7 mol/L, although it did not inhibit collagenase and NEP activities <4.9×10−5 mol/L. Thus, the inhibitory activity of FR901533 is highly selective for ECE-1. By contrast, phosphoramidon is not a selective ECE-1 inhibitor because this agent is about 50 times more active against NEP than against ECE-1. One hundred mg/mL of this agent markedly reduced the conversion rate from 55.2±3.6% to 26.2±4.6% in cultured neonatal cardiac myocytes (P < 0.001). Thus, we used FR901533 to further clarify the role of ECE-1 in PE-induced hypertrophy in cultured neonatal cardiac myocytes. As shown in Figure 5, this concentration of FR901533 inhibited the PE-stimulated increase in the protein synthesis rate in the cultured neonatal rat cardiac myocytes by 45%. We also examined
the effect of FR901533 on the transcriptional activities of the β-MHC and ANF genes. Both 3.5-kb β-MHC and 3.4-kb ANF promoter sequences have been shown to confer the PE-responsive expression of luciferase gene in cultured neonatal cardiocytes.18,19 Compatible with these previous findings, PE increased the relative luciferase activity of p-3542β-MHCluc and p-3412ANFluc by 2.0-fold and 4.5-fold, respectively, in the present study.

Figure 6. FR901533 (FR) differentially affected the PE-stimulated transcription of β-MHC (A) and ANF (B) genes in cardiac myocytes. Neonatal ventricular cardiac myocytes were co-transfected with 4 mg of β-myosin heavy chain (MHC) or ANF luciferase reporters and 1 μg pRSVCAT, and then stimulated with saline or PE (1.0×10^-4 mol/L). The activities of luciferase and CAT were determined 48 hours later. Relative luciferase activity (mean±SEM) represents the luciferase activity corrected for CAT activity and is expressed as a percentage relative to that in saline-stimulated cardiocytes. Data are mean±SEM of 3 independent preparations of cells, each performed in duplicate.

Figure 7. ANF expression (A) and ET-1 levels (B) in left ventricles of Bio14.6 cardiomyopathic and control F1B hamsters. A, Representative Northern blot showing steady-state levels of ANF mRNA and GAPDH mRNA in left ventricles of Bio14.6 cardiomyopathic and control F1B hamsters. B, ET-1 levels were measured by a sandwich ELISA as described in Methods. Values are mean±SEM. n=4 per group.

ET-1 Levels and ANF Expression in Ventricular Myocardium in Syrian Cardiomyopathic Hamsters at 2 Different Stages
To clarify the relationship between the response to ET-1 and ANF expression in vivo, we examined ventricular ET-1 and ANF mRNA levels in Syrian cardiomyopathic hamsters of the Bio14.6 strain. At the age of 20 weeks (left ventricular hypertrophy with compensation), ventricular ANF mRNA levels normalized with GAPDH mRNA levels were markedly increased (16-fold) compared with those of age-matched control F1B (Figure 7A). However, ventricular ET-1 levels were only mildly elevated (1.7-fold) (Figure 7B). As shown in Figure 7A, the increase of ventricular ANF mRNA levels at 35 weeks old (failing phase) was marked (15-fold) and similar to that in 20-week-old animals. In contrast to the results at 20 weeks, ventricular ET-1 levels were markedly elevated (5.8-fold) at this stage (Figure 7B).

Discussion
The major findings of this study include (1) α1-adrenergic stimulation accelerated the conversion of big ET-1 to bioac-
tive, mature ET-1 in rat cardiac myocytes and induced the expression of ECE-I, which mediates this conversion and (2) a specific ECE-1 antagonist inhibited α1-adrenergic stimu-
lated increase of the protein synthesis rate and β-MHC transcription. We observed that α1-adrenergic stimulation induced the expression of ET-1 in cardiac myocytes and accelerated the secretion of ET-1 into the culture media. It has been reported that the left ventricular content of ET-1 is markedly elevated in an animal model of heart failure.6,8 Immunohistochemistry demonstrated that ET-1 immunoreac-
tivity in failing hearts is localized in cardiac myocytes. Although these data do not rule out a possible role of nonmyocytes for the increased synthesis of ET-1 in the failing heart, the data demonstrate that cardiac myocytes are 1 of the main sources of ET-1. Compatible with the cardiac expression of ET-1, the rat ET-1 promoter contains a GATA element required for its full transcriptional activities.20 Our recent study26 demonstrated a role of GATA transcription factors in the regulation of cardiac gene expression during pressure overload hypertrophy in vivo. It is of interest to as-
to whether the GATA element in the ET-1 promoter plays a role in the upregulated expression of ET-1 in myocardial cell hypertrophy.

We found that the ECE-1 mRNA levels were markedly increased 3 hours after PE stimulation. Wang et al14 reported that the ECE-1 mRNA levels increased immediately (within 6 hours) after rat carotid artery balloon angioplasty. The augmented expression of the ECE-I gene may thus be 1 of the earliest responses to hormonal and mechanical stimuli. In agreement with the PE-inducible expression of the ECE-1 gene, PE accelerated the conversion of big ET-1 to ET-1. It has also been shown that the prepro-ET-1 mRNA levels are transiently increased after PE stimulation.8 These findings suggest that the PE-stimulated synthesis of ET-1 is regulated at both pre- and posttranslational levels.

We examined whether the myocardial ET-1 pathway is involved in the transcriptional activation of β-MHC and ANF genes by α1-adrenergic stimulation. The specific ECE-1 inhibitor FR901533 abolished the PE-stimulated increase of the transfected 3.5-kb β-MHC promoter activities, suggesting that the PE-stimulated β-MHC gene transcription requires myocardial ET-1 pathway. However, FR901533 did not affect the PE-stimulated 3.4-kb ANF promoter activities. Our data do not rule out the possibility that the ET-1-dependent pathway is involved in the activation of ANF gene transcription in the redundant networks. Nevertheless, the data suggest that the α1-adrenergic–stimulated transcription of β-MHC and ANF genes are mediated, at least in part, through differential pathways. cis-acting elements that mediate the PE-inducible expression include the GAG motif in the rat ANF promoter29 and the M-CAT element in the rat β-MHC promoter.14 To date, no conserved PE-responsive elements in different cardiac genes have been identified. Thus, α1-adrenergic stimulation may activate divergent signaling path-
ways. Further studies are needed to determine the target cis element of each pathway in the α1-adrenergic stimulated transcription of β-MHC and ANF genes.

The treatment with FR901533 inhibited the PE-stimulated increase in the protein synthesis rate but did not inhibit that of ANF gene expression. These findings suggest that cardiac hypertrophy and ANF expression are mediated, at least in part, through different pathways. In accordance with this idea, Sadoshima et al31 found that a 70-kDa S6 kinase inhibitor, rapamycin, inhibited the angiotensin II-stimulated increase in protein synthesis but did not affect the angiotensin II-induced activation of fetal genes, including those encoding ANF. It would be of particular interest to determine each intracellular signaling pathway that mediates cardiac hypertrophy and its specific gene expression.

In terms of the relationship between ET-1 level and ANF expression, it is interesting that ventricular ET-1 and ANF mRNA levels did not correlate at 2 different stages of Syrian cardiomyopathic hamster. These findings suggest an involve-
ment of ET-1–independent pathways for activation of the ANF gene program in vivo. In addition, the elevation of the left ventricular ET-1 levels is only slight at the hypertrophic stage but marked at the failing stage, which suggests that the accelerated production of cardiac ET-1 is involved in the transition from hypertrophy to failure in this animal model.

An application of our data in cultured neonatal cardiac myocytes to the in vivo setting in adults must be undertaken carefully because these 2 situations differ significantly. A previous report37 demonstrated that the intravenous adminis-
tration of FR901533 at the concentration 1 mg/kg significa-
tantly inhibited the big ET-1–induced pressor response in rats. Thus, the inhibitory effect of this agent is not confined to in vitro assays, but is also observed in the in vivo context. An ET type A receptor antagonist has been shown to be benefi-
cial in animal models of heart failure.8 However, it was reported that the expression of the ET type A receptor was downregulated in hypertrophied hearts of spontaneously hy-
pertensive rats32 and that ET-1 synthesis increased after the administration of the nonselective ET receptor antagonist bosentan.23 Bird et al38 demonstrated that the ECE-1 inhibitor phosphoramidon provided more pronounced beneficial effects on renal function and structure in ischemic renal failure than did the ET type A receptor antagonist. Thus, a compar-
ison of ECE-1 inhibitors and ET receptor antagonists as therapeutic agents for heart failure in vivo would be of particular interest.

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References


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