Calcineurin-GATA4 pathway is involved in β-adrenergic agonist-responsive endothelin-1 transcription in cardiac myocytes

Tatsuya Morimoto
Koji Hasegawa*
Hiromichi Wada
Tsuyoshi Kakita
Satoshi Kaburagi
Tetsuhiko Yanazume
Shigetake Sasayama

* Address correspondence to:
Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan

TEL: 81-75-751-3190
FAX: 81-75-751-3203
e-mail: koj@kuhp.kyoto-u.ac.jp
Increases in the expression of endothelin-1 (ET-1) in cardiac myocytes play a critical role in the development of heart failure in vivo. While norepinephrine (NE) is a potent inducer of ET-1 expression in cardiac myocytes, the signaling pathways that link NE to inducible cardiac ET-1 expression are unknown. Adrenergic stimulation results in an increase in intracellular calcium levels, which in turn activates calcineurin. Here, we showed that stimulation with NE markedly increased the expression of ET-1 gene in primary cardiac myocytes from neonatal rats. This increase was severely attenuated by a β-adrenergic antagonist, metoprolol, but not by an α-adrenergic antagonist, prazosin. Consistent with these data, the β-adrenergic agonist isoproterenol (ISO) activated the rat ET-1 promoter promoter activity to a similar extent as NE. The ISO-stimulated increase in promoter activity was significantly inhibited by a Ca-antagonist, nifedipine, and an immunosuppressant, cyclosporin A, which blocks calcineurin. Mutation analysis indicated that the GATA4 binding site is required for ISO-responsive ET-1 transcription. Stimulation with ISO enhanced the interaction between NFATc and GATA4 in cardiac myocytes. Consistent with this interaction, overexpression of GATA4 and NFATc synergistically activated the ET-1 promoter. These findings demonstrate that NE-stimulated ET-1 expression in cardiac myocytes is mediated predominantly via a β-adrenergic pathway and that calcium-activated calcineurin-GATA4 plays a role in this process.

Key Words
cardiac myocyte, endothelin-1, calcineurin, GATA4, heart failure, transcription

Abbreviations used in this paper:
NE, norepinephrine
ISO, isoproterenol
ET-1, endothelin-1
NFAT, nuclear factor of activated T lymphocytes
Cys A, cyclosporin A
INTRODUCTION

Endothelin-1 (ET-1) was initially identified as a 21 amino acid vasoconstrictive peptide in porcine vascular endothelial cells (1). Later work showed that it acts not only as a vasoconstrictor but also as a potent growth-promoting peptide. For example, ET-1 can induce myocyte hypertrophy (2,3) through coupling of ET receptors with Gq protein. While ET-1 is mainly produced by endothelial cells in the basal state, a number of cell types can synthesize ET-1 in response to various stimuli (4-7). ET-1 expression in cardiac myocytes is induced by myocardial stretch, angiotensin II and norepinephrine (5-7). Left ventricular levels of ET-1 increase markedly in close association with the deterioration of systolic function following myocardial infarction and pressure overload (8-10). Immunohistochemical studies have demonstrated that ET-1 in the failing heart is localized in cardiac myocytes. ET receptor antagonists bosentan or BQ123 prevent the remodeling of the heart and have been shown to improve survival following myocardial infarction and pressure overload (9,10). These findings demonstrate that upregulated expression of ET-1 in cardiac myocytes plays a critical role in the development of heart failure in vivo. However, the precise mechanisms leading to this upregulation in the failing heart are unclear at present.

Since a number of neurohormonal factors are activated in congestive heart failure (11,12), they may possibly play positive and negative roles in regulating ET-1 expression in cardiac myocytes. Norepinephrine (NE) is one such factor, of which the elevation in plasma closely is correlated with the severity and poor prognosis of heart failure (12). It was shown that NE is a potent inducer of ET-1 expression in cardiac myocytes both in vitro and in vivo (7). NE exerts its effect on cardiac myocytes via both α- and β-adrenergic receptor pathways. However, the signaling pathways that link activation of α- and β-adrenergic receptors to inducible cardiac ET-1 expression are unknown.

Adrenergic stimulation results in an increase in intracellular calcium levels. The calcium-activated phosphatase calcineurin is necessary for the nuclear import of nuclear factor of activated T lymphocytes (NFAT) transcription factors, which mediate changes in gene expression in response to calcium signaling from the T-cell receptor (reviewed in 13). NFAT3, a member of the NFAT family, interacts with high affinity and specificity with the cardiac-restricted zinc finger protein GATA4 (14). While calcineurin-GATA4 is involved in the transcriptional pathways that modulate cardiac hypertrophy (14), a role for this pathway in cardiac ET-1 transcription is unknown. The present study was conducted to determine whether the calcineurin-GATA4 pathway could account for the stimulation of ET-1 expression by NE in cardiac myocytes.
EXPERIMENTAL PROCEDURES

Cell Culture

Primary ventricular cardiac myocytes were prepared as previously described (15-18). Briefly, hearts from 1-2-day-old Sprague-Dawley rats were removed, the ventricles were pooled, and the ventricular cells were dispersed by digestion with pancreatin (Life Technologies, Gaithersburg, MD). The cells were pre-plated for 1 hour to enrich for myocytes (90-95% of the cells after this step). Cells were plated at a density of 250 cells /mm2 onto 60-mm tissue culture dishes (Primaria, Falcon; Becton Dickinson & Co., Lincoln Park, NJ) and cultured in medium consisting of Hanks’ salts plus minimal essential medium (MEM) vitamin stock, MEM amino acids, MEM nonessential amino acids, 2 mM L-glutamine, 0.67 mM glycine, 0.92 mM hypoxanthine, 19.6 mM NaHCO3 (pH 7.1-7.2), penicillin, streptomycin, and 10% (vol/vol) fetal bovine serum (FBS) (all from Gibco BRL, Gaitherburg, MD) at 37°C in 5% CO2 for 24 hours.

RNA analysis

Northern blot analysis of 10 µg total RNA was performed as described (16-18). A cDNA probe consisting of the nucleotides - of rat prepro ET-1 cDNA was generated by PCR as described (10) and radiolabelled by random priming and used to detect prepro ET-1 mRNA. Abundance of mRNAs were quantified by phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA). Values of prepro ET-1 mRNA were normalized to those of GAPDH mRNA.

Plasmid constructs

The plasmid construct pwtET-CAT was the transcription start site-proximal 204-bp wild-type rat ET-1 promoter fused to the bacterial chloramphenicol acetyl transferase (CAT) gene (19). In pmutGATA-ET-CAT, a consensus GATA element located at nucleotides -136 to -131 was mutated in the context of the 204-bp rat ET-1 promoter (19). These plasmids were gifts from Dr. Thomas Quertermous, Stanford University, Palo Alto, CA. A promoter-less CAT plasmid (basic CAT) was purchased from Promega, Madison, WI. pRSVCAT and pRSVLuc contain the CAT and luc genes, respectively, driven by the Rous sarcoma virus (RSV) long terminal repeat sequence (16,20). The murine GATA4 expression plasmid pGATA4 has been described previously (17,18,21). The murine NFATc expression plasmid pNFATc (22) was a generous gift from Dr. Ken-ichi Arai, University of Tokyo, Japan. Plasmids were purified by anion exchange chromatography (QIAGEN, Hilden, Germany), quantified by measurement of OD260, and examined on agarose gels stained with ethidium bromide prior to use.

Transfection and luciferase/CAT assays
Twenty-four hours after plating, cells were washed twice with serum-free medium and then co-transfected with 2 µg of the CAT construct of interest and 0.1 µg of pRSVluc using Lipofectamine Plus (GIBCO BRL, Gaithersburg, MD) according to the manufacturer’s recommendations. After a 2-hour incubation with DNA-lipofectamine complex, the cells were washed twice with serum-free medium and further incubated for 24 hours in serum-free medium in the presence of 10^{-6} M of NE, isoproterenol (ISO) or saline as controls. The cells were then washed twice with ice-cold PBS, lysed with lysis buffer and subjected to assays for luciferase and CAT activities as described previously (15-18,20).

**Immunocytochemistry**

The cardiac myocytes were grown on silicon-coated glass and then stimulated with saline or 10^{-6} M of ISO for 24 hours. The cells were then fixed with 3% formaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature. Immunocytochemical staining for ET-1 was performed using the indirect immunoperoxidase method, as previously described (8,10,15). Immunocytochemical staining for NFATc was performed using the indirect immunofluorescence method. Cells were incubated with anti-NFATc monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:100. Signals of NFATc were detected using anti-mouse FITC-conjugated secondary antibody at a dilution of 1:500 for 45 min.

**Immunoprecipitation / Western Blotting**

Immunoprecipitation followed by Western blotting was performed as previously described (16-18) in nuclear extracts prepared from neonatal rat cardiac myocytes stimulated with ISO or saline as a control for 3 hours. Aliquots of the extracts containing 100 µg of protein were immunoprecipitated using anti-mouse GATA4 polyclonal antiserum (Catalog number SC 1237, Santa Cruz Biotechnology, Santa Cruz, CA) or normal goat IgG in low-stringency buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 10 µg/ml aprotinin and leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride) for 16 h at 4°C and incubated with protein G (Sigma Chemicals, St. Louis, MO) beads for 1 h at 4°C. The precipitate was washed four times in the same buffer, resuspended in 50 µl of SDS-lysis buffer (20 mM Tris [pH 7.5], 50 mM NaCl, 0.5% SDS, 1 mM dithiothreitol), heated to 95°C for 2 min, electrophoresed on an SDS-polyacrylamide gel (6%), transferred to an Immobilon membrane, and reacted with a monoclonal antibody against murine NFATc (Santa Cruz Biotechnology, Santa Cruz, CA) which was subsequently detected using horseradish peroxidase-conjugated anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove PA). Signals were detected using the enhanced chemiluminescence Western blotting detection system (Amersham, Buckinghamshire, UK) according to the manufacturer’s instructions.
To confirm the specificity of Western blotting for GATA4, a five-fold excess of the blocking peptide (Santa Cruz Biotechnology, Santa Cruz, CA) was combined with the primary antibody and incubated for 2 hours in a small volume of PBS. Then, antibody/peptide mixture was diluted and reacted with a blotting membrane in a similar protocol.

**Statistical analysis**

Data are presented as means ± S.E. Statistical comparisons were performed using unpaired two-tailed Student’s t tests or ANOVA with Scheffe’s test when appropriate, with a probability value less than 0.05 taken to indicate significance.

**RESULTS**

**Norepinephrine is a potent inducer of ET-1 gene expression in cardiac myocytes**

To determine whether stimulation of both α- and β-adrenergic receptors by NE would induce the expression of ET-1 gene in cardiac myocytes, primary cardiac myocytes from neonatal rats were stimulated with saline or 10⁻⁶ M NE for 24 hours. Then, we examined the expression of ET-1 gene by northern blot using a cDNA probe for the prepro ET-1 mRNA. We performed these experiments using three independent preparations of cardiac myocytes. As shown in Fig. 1, stimulation with NE markedly increased the expression of ET-1 gene in cardiac myocytes (lane 2) compared with the saline-stimulated state (lane 1). To determine whether the NE-stimulated increase in the ET-1 gene expression is mediated through an α- or β-adrenergic receptor, cardiac myocytes were stimulated with NE in the presence of an α-adrenergic receptor antagonist, prazosin (10⁻⁶ M), or a β-adrenergic receptor antagonist, metoprolol (10⁻⁶ M), for 24 hours. Administration of prazosin in addition to NE only slightly reduced the ET-1 gene expression (lane 3). In contrast, administration of metoprolol in addition to NE severely attenuated the expression (lane 4). NE, prazosin or metoprolol did not alter the expression of the ubiquitously and constitutively expressed GAPDH gene. We confirmed that in our preparation, more than 90% of the NE-stimulated cells were positive for staining with HHF35, which recognizes the α-actin of cardiac muscle cells but not that of fibroblasts. These data indicate that NE, an important neurohormonal factor activated in congestive heart failure, is a potent inducer of ET-1 gene expression in cardiac myocytes, and that NE-responsive ET-1 expression is mediated predominantly through a β-adrenergic receptor-dependent pathway.

**NE-responsive ET-1 transcription is mediated predominantly through a β-adrenergic receptor-dependent pathway**

To determine if transcriptional activation could account for the stimulation of ET-1 expression by NE in neonatal rat cardiac myocytes, these myocytes were
transfected with a CAT reporter construct driven by the 204-bp rat ET-1 upstream sequence (pwtET-CAT). To control for transfection efficiency, the cells were cotransfected with a small quantity of pRSVLuc. After 24 hours of stimulation with 10^{-6} M NE or saline as a control, cardiomyocytes were harvested for luciferase and CAT assays. The 204-bp ET-1 promoter fragment conferred NE-inducible expression on the CAT reporter gene (Fig.2). In contrast, NE stimulation did not induce the activity of a promoter derived from the ubiquitously expressed β-actin gene (data not shown). The increase in the activity of the ET-1 promoter suggests that NE stimulation of ET-1 expression in cardiac myocytes is mediated, at least in part, through a transcriptional mechanism and that the 204-bp promoter sequence of the ET-1 gene contains important elements that mediate NE-responsive transcription.

NE can activate α- and β-adrenergic receptors in cardiac myocytes. To determine whether the NE-stimulated increase in the ET-1 promoter activity is mediated through these receptors, cardiac myocytes were transfected with pwtET-CAT/pRSVLuc and then stimulated with NE in the presence of an α-adrenergic receptor antagonist, prazosin (10^{-6} M), or a β-adrenergic receptor antagonist, metoprolol (10^{-6} M), for 24 hours. NE-stimulated ET-1 promoter activity was severely attenuated by metoprolol (Fig.2, lane 3) but not by prazosin (Fig. 2, lane 4). Consistent with these data, the β-adrenergic agonist isoproterenol (ISO, 10^{-6} M) activated the ET-1 promoter to an extent similar to the activation by NE (Fig. 3, lane 4). These findings indicate that NE-responsive ET-1 transcription in cardiac myocytes is mediated predominantly through a β-adrenergic receptor-dependent pathway.

Calcium activated calcineurin pathway is involved in β-adrenergic agonist-responsive ET-1 transcription in cardiac myocytes

Stimulation with β-adrenergic agonists results in an increase in intracellular calcium levels. To determine whether the calcium-activated phosphatase calcineurin is involved in ISO-responsive ET-1 transcription in cardiac myocytes, these myocytes were transfected with pwtET-CAT and a small quantity of pRSVLuc and then exposed to saline or ISO in the presence and absence of a Ca-antagonist, nifedipine, or an immunosuppressant, cyclosporin A (Cys A), which blocks the activation of calcineurin. Twenty-four hours later, luciferase and CAT activities were assessed in lysates from these cells. As shown in Fig. 3, the ISO–stimulated increase in the ET-1 promoter activity was significantly inhibited by nifedipine (lane 5) and Cys A (lane 6). However, neither nifedipine nor Cys A affected the basal level of the ET-1 promoter activity (lanes 2 and 3), indicating that these agents specifically blocked the ISO-mediated pathway. These findings demonstrate that the calcium-activated calcineurin pathway is, in part, involved in β-adrenergic agonist-responsive ET-1 transcription in cardiac myocytes.

To examine whether the reduction of ET-1 transcriptional activity results in the
change of ET-1 production in cardiac myocytes, we performed immunocytochemistry for ET-1. ISO stimulation was not sufficient to induce an increase in cell size in our experimental system. However, as shown in Fig. 4, brown positive signals indicating ET-1 were stronger in ISO-stimulated cardiac myocytes (B) than in saline-stimulated cells (A). The specificity of the staining was confirmed by its abolition by the addition of an excess of synthetic ET-1 (C). Treatment with nifedipine (D) and Cys A (E) significantly reduced the staining intensity. These findings demonstrate that the calcium-activated calcineurin pathway is, in part, involved in the β-adrenergic agonist-stimulated increase in ET-1 synthesis in cardiac myocytes as well as ET-1 transcription in these cells.

**β-adrenergic agonist-responsive ET-1 transcription requires GATA4**

Activated calcineurin dephosphorylates NF-AT transcription factors, which then translocate to the nucleus and interact with the cardiac zinc finger protein GATA4 (14). Since the 204-bp rat ET-1 promoter sequence contains a binding site for GATA4, we investigated the role of this site in ISO-responsive ET-1 transcription. Neonatal rat cardiac myocytes were transfected with a CAT gene driven by the 204-bp ET-1 promoter containing a mutation in the GATA site (pmutGATA-ET-CAT), which abolishes the binding of cardiac GATA4 (23). As shown in Fig. 5, the mutation of the GATA element within the 204-bp ET-1 promoter caused a modest decrease in the basal transcriptional activity and abolished ISO-responsive transcription. Thus, GATA4 plays a critical role for ISO-responsive ET-1 transcription in cardiac myocytes.

**β-adrenergic stimulation translocates NFATc into the nucleus and increases NFATc/GATA4 complex**

To examine nuclear translocation of endogenous NFATc in response to ISO treatment in cardiac myocytes, we performed immunofluorescence. As shown in Figure 6, NFATc was detected in cytoplasm of nearly all saline-stimulated cardiac myocytes (A). However, the stimulation of cardiac myocytes with ISO markedly changed this localization and caused the nuclear translocation of NFATc (B). ISO-mediated translocation was reversed by Cys A (C), suggesting that calcineurin activation is required for this translocation.

To determine whether NFATc in cardiac myocytes is dephosphorylated by β-adrenergic stimulation, we performed Western blotting. Neonatal cardiac myocytes were stimulated with ISO or saline as a control for 45 minutes, and then whole-cell lysates were subjected to Western blotting using the anti-NFATc antibody and anti-GATA4 antibody. As shown in Fig. 7, the expression level of NFATc and GATA4 in whole-cell lysates did not differ between saline- and ISO-stimulated cardiac myocytes and was not altered by Cys A. However, NFATc in cardiac myocytes was converted to the high-mobility form following ISO stimulation. This conversion was reversed by
the administration of Cys A, which blocks calcineurin. Since dephosphorylated NFATc migrates faster than phosphorylated NFATc (24), the conversion of NFATc to the high mobility form might indicate dephosphorylation of NFATc by ISO stimulation.

To determine whether the interaction between NFATc and GATA4 in cardiac myocytes is modulated by β-adrenergic stimulation, we performed immunoprecipitation followed by Western blotting. Neonatal cardiac myocytes were stimulated with ISO or saline as a control for 3 hours, and then nuclear extracts derived from these cells were subjected to immunoprecipitation with an anti-NFATc antibody as a positive control (Fig. 8, lane 4), IgG as a negative control (Fig. 8, lane 1) or an anti-GATA4 antibody (Fig. 8, lanes 2 and 3). Western blotting using the anti-NFATc antibody showed that the anti-GATA4 antibody (Fig. 8, lanes 2 and 3), but not IgG (Fig. 8, lane 1) coprecipitated NFATc protein in nuclear extracts from cardiac myocytes stimulated with ISO, even after extensive washing. However, the anti-GATA-4 antibody coprecipitated a smaller amount of NFATc in nuclear extracts from saline-stimulated myocytes (Fig. 8, lane 2) than in extracts from ISO-stimulated cells (Fig. 8, lane 3). The expression level of GATA4 did not differ between saline- and ISO-stimulated cells. Addition of the blocking peptide eliminated the band, confirming the specificity of anti-GATA4 antibody. Thus, stimulation with ISO increased the complex of NFATc/GATA4 in cardiac myocytes.

**GATA4 and NFATc synergistically activated the ET-1 promoter**

The fact that NFATc can bind to GATA4 suggests that these two proteins might co-activate the transcriptional activity of the ET-1 gene. To test this hypothesis, we performed transient transfection assays in COS7 cells, which express neither NFATc nor GATA4. We cotransfected the ET-1 reporter plasmid together with GATA4 or NFATc expression vector alone, or in combination with both vectors. Total amounts of transfected DNA were kept constant by co-transfecting β-gal-expression vector. Then these cells were incubated in a serum-free condition in the presence of ISO. Forty-eight hours later, we measured the ET-1 reporter activity. The transfection of the GATA4-expressing vector or the NFATc-expressing vector alone produced only a minimal stimulation of transcription in these cells (Fig. 9, lanes 2 and 3, respectively). The coexpression of NFATc and GATA4 induced a significant stimulation of the reporter (Fig. 9, lane 4) compared to the level observed with GATA4 or NFATc alone. To check the expression level of NFATc and GATA4 in COS7 cells transfected with expression vectors encoding these proteins, we performed Western blotting in these cells. As shown in Fig. 9, GATA4 and NFATc were abundantly expressed in COS7 cells transfected with each corresponding expression vector, but absent in cells transfected with β-gal expression vector instead of GATA4- or NFATc-expression vector. In addition, co-transfection of both GATA4- and NFATc-expression vectors did not alter the respective levels of expression compared with that after transfection of either expression vector alone. Therefore, the effect of co-expression of NFATc and
GATA4 on enhanced ET-1 transcriptional activity can not be explained by artifactual mechanism. Taken together with the fact that NFATc can bind to GATA4, these results suggest that these two proteins co-operatively work in the transcriptional activation of the ET-1 gene.
DISCUSSION

Although ET-1 was initially identified as an endothelial cell-derived vasoconstrictor, it is now recognized as a growth-promoting peptide produced by a variety of cell types. Expression of ET-1 in cardiac myocytes is markedly increased in failing hearts (8-10). In addition, the administration of ET receptor antagonists prevents remodeling of the heart following myocardial infarction and pressure overload independent of hemodynamic effects (9,10). These findings suggest that the local synthesis of ET-1 is involved in the development of heart failure in vivo. However, the precise mechanisms that mediate the upregulation of ET-1 expression in the failing heart are unknown. NE is an important neurohormonal factor activated in congestive heart failure and a potent inducer of ET-1 expression in cardiac myocytes (7). The major conclusions of the present study are 1) stimulation of ET-1 expression by NE in cardiac myocytes is mediated predominantly via a β-adrenergic pathway and 2) calcium-activated calcineurin-GATA4 plays a role in β-adrenergic agonist-stimulated ET-1 transcription in cardiac myocytes.

NE exerts its effects on cardiac myocytes via both α- and β-adrenergic receptor pathways. Using primary neonatal rat cardiac myocytes, we showed that NE markedly stimulated the rat ET-1 promoter activity and that this stimulation was severely attenuated by the β-adrenergic receptor blocker metoprolol, but not by the α-adrenergic receptor blocker prazosin. In addition, the ET-1 promoter activity was activated by the β-adrenergic agonist ISO to an extent similar to the activation by NE. These findings indicate that NE-inducible expression of ET-1 in cardiac myocytes is controlled, at least in part, at the level of transcription and that NE stimulation of ET-1 transcription is mediated predominantly via a β-adrenergic pathway. β-adrenergic stimulation results in an increase in the intracellular calcium level. The present study demonstrated that β-adrenergic agonist-stimulated ET-1 transcription was significantly inhibited by the calcium antagonist nifedipine. This finding suggests that calcium signaling may be involved in this process. However, the inhibition by nifedipine was not complete. Nifedipine blocks the entry of extracellular calcium into the cytosol but does not block the release of calcium from the sarcoplasmic reticulum. Therefore, the partial effect of nifedipine on β-adrenergic agonist-stimulated ET-1 transcription might be attributable to incomplete blockage of the increase in the intracellular calcium level. To clarify the precise role of calcium signaling, measurement of the intracellular calcium level and complete blockage of the increase of the calcium level are needed.

A sufficient increase of the calcium level activates calcineurin, a ubiquitously expressed serine/threonine phosphatase, by binding the 19-kDa regulatory subunit of calcineurin (25). Calcineurin dephosphorylates NFAT transcription factors, which enables them to translocate to the nucleus (13,14). Cys A binds the immunophilin cyclophilin and forms a complex that binds the calcineurin catalytic subunit, thereby
inhibiting calcineurin’s ability to activate NFAT transcription factors (26,27). The present study demonstrated that Cys A significantly but not completely inhibited β-adrenergic agonist-stimulated ET-1 transcription in cardiac myocytes. We used a physiological concentration of Cys A, which has been shown to specifically block calcineurin activation. Use of a higher dose of Cys A did not augment its ability to inhibit ET-1 transcription (data not shown). These results demonstrate that calcineurin activation is, in part, involved in β-adrenergic-stimulated ET-1 transcription, while the results also suggest the existence of alternative pathways.

GATA4 is a cardiac-restricted zinc finger protein which plays an important role in cardiac-specific transcription (28-31). In addition, GATA4 is required for transcriptional activation of the genes for β-myosin heavy chain and angiotensin II type 1α receptor during hemodynamic overload-induced cardiac hypertrophy in vivo (20,32). The present study demonstrated that mutating the GATA4 binding site abolished β-adrenergic agonist-responsive ET-1 transcription, suggesting a critical role for GATA4 in this process. Downstream of calcineurin, GATA4 interacts with high affinity and specificity with NFAT transcription factors (14). Consistent with this interaction, we showed that NFATc and GATA4 co-activated the ET-1 promoter. In addition, β-adrenergic stimulation increased NFATc/GATA4 complex in cardiac myocytes. All of these data suggest that a calcineurin-GATA4 pathway controls transcription of the ET-1 gene in cardiac myocytes and is involved in β-adrenergic agonist-responsive ET-1 transcription in these cells.

Although ET-1 was initially identified as an endothelial cell-derived vasoconstrictor, it is now recognized as a growth-promoting peptide produced by a variety of cell types. A recent study suggested a role of ET-1 produced by cardiac fibroblasts in myocardial cell hypertrophy (33). In our preparation, more than 90% of cells consist of cardiac myocytes as identified by immunostaining with HHF35, which recognizes α-actin of cardiac myocytes but not that of other cell types. Although GATA4 is specifically expressed in the heart and endodermal derivatives (21,28), it should be further investigated whether a calcineurin-GATA4 pathway represents a common or cell type-specific mechanism for the induction of ET-1 expression.

Calcineurin-GATA4 is involved in the transcriptional pathways that modulate cardiac hypertrophy (14). Inhibition of calcineurin by Cys A is sufficient to block
cardiac hypertrophy in a transgenic animal model of hypertrophic cardiomyopathy (34). A calcineurin pathway appears to be involved in the development of heart failure in some animal models, while it is still being debated whether calcineurin plays a critical role in this process (35). The present study demonstrated that calcineurin-GATA4 is involved in the activation of ET-1 transcription in cardiac myocytes. Since ET-1 is one of the local factors that play important roles in the development of heart failure, our findings provide further insights into the role of calcineurin in heart failure in vivo. However, several limitations should be taken into account when the data of this study are applied to the in vivo setting in the adult. First, as myocardial development is not complete at birth, differences may exist between neonatal and adult cardiac myocytes. Second, the biological properties of disassociated myocytes in culture and myocytes in the organized heart in vivo may differ. Therefore, it would be of particular interest to clarify the role of calcineurin in the activation of the myocardial ET-1 pathway during the development of heart failure in vivo.

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**Figure Legends**

**Figure 1.** NE stimulation increased the expression of ET-1 gene in cardiac myocytes. Neonatal rat ventricular myocytes were incubated in the presence of $10^{-6}$ M NE, prazosin ($10^{-6}$ M), metoprolol ($10^{-6}$ M) as indicated for 24 hours. Blots containing total RNA (10 µg) from these myocytes were sequentially hybridized with a rat prepro ET-1 cDNA and with a rat GAPDH cDNA.

**Figure 2.** Metoprolol inhibits norepinephrine-mediated activation of the ET-1 promoter. Two µg of pwtET-CAT and 0.1 µg of pRSVluc were co-transfected into primary cultures of neonatal rat cardiac myocytes which were subsequently stimulated with saline or $10^{-6}$ M NE in the presence or absence of metoprolol ($10^{-6}$ M) or prazosin ($10^{-6}$ M) as indicated for 24 hours. The relative CAT activity (CAT/luc) of pwtET-CAT in the saline-stimulated cells was set at 1.0 in each experiment. Values are means ± S.E. of two independent experiments, each carried out in duplicate.

**Figure 3.** Nifedipine and cyclosporin A inhibit isoproterenol-mediated activation of the ET-1 promoter. Two µg of pwtET-CAT and 0.1 µg of pRSVluc were co-transfected into primary cultures of neonatal rat cardiac myocytes, which were subsequently stimulated with saline or $10^{-6}$ M isoproterenol (ISO) in the presence or absence of nifedipine ($10^{-6}$ M) or cyclosporin A (Cys A, 0.5 µg/ml) as indicated for 24 hours. The relative CAT activity (CAT/luc) of pwtET-CAT in the saline-stimulated state was set at 1.0 in each experiment. Values are means ± S.E. of two independent experiments, each carried out in duplicate.

**Figure 4.** Immunocytochemical staining of cultured rat cardiac myocytes labeled with antibody to ET-1. Primary cultures of neonatal rat cardiac myocytes were stimulated with saline or $10^{-6}$ M isoproterenol (ISO) in the presence or absence of nifedipine ($10^{-6}$ M) or cyclosporin A (Cys A, 0.5 µg/ml) as indicated for 24 hours. The primary antibody to ET-1 was stained with the secondary antibody conjugated with peroxidase (brown signals). The nuclei were counterstained with hematoxylin. The addition of an excess of synthetic ET-1 abolished the signals (C).

**Figure 5.** The GATA4 binding site in the rat ET-1 promoter is required for isoproterenol-responsive transcription in cardiac myocytes. Two µg of pwtET-CAT (wild type) or pmutGATA-ET-CAT (mutation of GATA element) and 0.1 µg of pRSVluc were co-transfected into primary cultures of neonatal rat cardiac myocytes and subsequently stimulated with saline (SS) or $10^{-6}$ M ISO for 24 hours. The relative CAT activity (CAT/luc) of pwtET-CAT in the saline-stimulated state was set at 1.0 in each experiment. Data are presented as the mean ± SEM of 4 independent experiments.
**Figure 6.** Isoproterenol stimulation causes translocation of NFATc into the nucleus in a calcineurin-dependent manner. Cultured cardiac myocytes were treated with $10^{-6}$ M ISO (B), ISO plus cyclosporin A (0.5 µg/ml, C) or saline as a control (A) for 3 hours in serum-free conditions, and subjected to immunofluorescence using anti-NFATc antibody.

**Figure 7.** Isoproterenol stimulation converts NFATc into high mobility forms. Neonatal cardiac myocytes were stimulated with saline or $10^{-6}$ M ISO in the presence or absence of cyclosporin A (Cys A, 0.5 µg/ml) as indicated for 45 minutes. Whole cell lysates derived from these cells were subjected to Western blotting with anti-NFATc or anti-GATA4 antibody.

**Figure 8.** NFATc interacts with GATA4. Neonatal cardiac myocytes were stimulated with saline or $10^{-6}$ M ISO for 3 hours. One hundred µg of nuclear extracts derived from these cells were immunoprecipitated with anti-NFATc antibody (lane 4), IgG (lane 1) or anti-GATA4 antibody (lanes 2 and 3). After electrophoresis and electroblotting, the membranes containing immobilized immuno-complexes were subjected to Western blotting with anti-NFATc antibody.

**Figure 9.** GATA4 and NFATc synergistically activate the ET-1 promoter. COS7 cells were transfected with 1.0 µg of pwtET-CAT, 0.1 µg of pRSVluc and 1.0 µg of GATA4 and/or NFATc expression vector(s) as indicated, and incubated in serum-free condition in the presence of $10^{-6}$ M ISO. Total amounts of transfected DNA were kept constant by co-transfecting β-gal-expression vector. The results, expressed as fold induction of reporter constructs, are the mean ± SEM of 2 independent experiments, each carried out in duplicate. Lysates from these cells were also subjected to Western blotting with anti-NFATc or anti-GATA4 antibody.
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pmutGATA-ET-CAT

GATA element

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Fold activation

p<0.05
Calcineurin-GATA4 pathway is involved in b-adrenergic agonist-responsive endothelin-1 transcription in cardiac myocytes
Tatsuya Morimoto, Koji Hasegawa, Hiromichi Wada, Tsuyoshi Kakita, Satoshi Kaburagi,
Tetsuhiko Yanazume and Shigetake Sasayama

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