Phosphorylation of GATA-4 Is Involved in \( \alpha_1 \)-Adrenergic Agonist-responsive Transcription of the Endothelin-1 Gene in Cardiac Myocytes*

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The expression of endothelin-1 (ET-1) in cardiac myocytes is markedly induced during the development of heart failure in vivo and by stimulation with the \( \alpha_1 \)-adrenergic agonist phenylephrine in culture. Although recent studies have suggested a role for cardiac-specific zinc finger GATA factors in the transcriptional pathways that modulate cardiac hypertrophy, it is unknown whether these factors are also involved in cardiac ET-1 transcription and if so, how these factors are modulated during this process. Using transient transfection assays in primary cardiac myocytes from neonatal rats, we show here that the GATA element in the rat ET-1 promoter was required for phenylephrine-stimulated ET-1 transcription. Cardiac GATA-4 bound the ET-1 GATA element and activated the ET-1 promoter in a sequence-specific manner. Stimulation by phenylephrine caused serine phosphorylation of GATA-4 and increased its ability to bind the ET-1 GATA element. Inhibition of the extracellularly responsive kinase cascade with PD098059 blocked the phenylephrine-induced increase in the DNA binding ability and the phosphorylation of GATA-4. These findings demonstrate that serine phosphorylation of GATA-4 is involved in \( \alpha_1 \)-adrenergic agonist-responsive transcription of the ET-1 gene in cardiac myocytes and that extracellularly responsive kinase 1/2 activation plays a role upstream of GATA-4.

Endothelin-1 (ET-1)\(^1\) 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 \( G_{\alpha} \) protein. ET-1 signaling is also coupled with \( G_{i} \) protein. Therefore, it is able to decrease intracellular cAMP levels (4). Although 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 (5–8). ET-1 expression in cardiac myocytes is induced by myocardial stretch, angiotensin II, and norepinephrine (6–8). Left ventricular levels of ET-1 increase markedly in close association with the deterioration of systolic function following myocardial infarction and pressure overload (9, 10). Immunohistochemical studies have demonstrated that ET-1 in the failing heart is localized in cardiac myocytes. ET receptor antagonists bosentan and 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 up-regulated expression of ET-1 in cardiac myocytes plays a critical role in the development of heart failure in vivo. However, the molecular mechanisms leading to this up-regulation in the failing heart are unclear at present.

The mechanisms regulating the transcription of the ET-1 gene have been studied in endothelial cells. The 204-bp sequences proximal to the transcription starting site is sufficient to drive high levels of expression in these cells in culture (11). Mutation of a putative GATA element in this sequence diminishes the transcriptional activity of the ET-1 promoter (12–14). One endothelial factor that binds to the ET-1 GATA element has been shown to be GATA-2 (12, 13). Although cardiac myocytes also express the subfamily of zinc finger GATA transcription factors (GATA-4/5/6), it is unknown whether the ET-1 GATA element is functional in this context. We and others have shown that GATA factors are required for transcriptional activation of the genes for \( \beta \)-myosin heavy chain and angiotensin II type 1a receptor during pressure overload-induced hypertrophy in vivo (15, 16). For these reasons, it would be of interest to know whether GATA factors also mediate the up-regulation of the expression of cardiac ET-1 during myocardial cell hypertrophy. In addition, if this were the case, it would be important to examine how GATA factors are regulated during this process. The \( \alpha_1 \)-adrenergic agonist phenylephrine (PE) is a potent inducer of hypertrophy and ET-1 expression in cardiac myocytes (17), providing a useful tool to study the mechanisms for the induction of ET-1 expression in the failing heart. Therefore, in the present study, we investigated the role of GATA factors in PE-stimulated transcription of cardiac ET-1.

MATERIALS AND METHODS

Cell Culture—Primary ventricular cardiac myocytes were prepared as described previously (17–19). Briefly, hearts from 1–2-day-old Harlan Sprague-Dawley rats were removed, the ventricles were pooled, and the ventricular cells were dispersed by digestion with pancreatic (Life Technologies, Inc.). The cells were preplated for 1 h to enrich for myocytes (90–95% of the cells after this step). Cells were plated at a density of 250 cells/mm\(^2\) onto 60-mm tissue culture dishes (Primaria, Falcon; Becton Dickinson & Co., Lincoln Park, NJ) and cultured in medium consisting of Hanks’ salts plus 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 NaHCO\(_3\) (pH 7.1–7.2), penicillin, streptomycin, and 10% (v/v) fetal bovine serum (all from Life

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1 The abbreviations used are: ET, endothelin; PE, phenylephrine; luc, luciferase; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; ERK, extracellularly responsive kinase; bp, base pair; MEM, minimal essential medium.
Technologies, Inc.) at 37 °C, 5% CO2 for 24 h.

**Phasmid Constructs**—The plasmid construct pwtET-CAT was the transcription start-site-proximal 204-bp wild type rat ET-1 promoter fused to the bacterial CAT gene (14). In pmutGATA-ET-CAT, a consensus GATA element located at sequence −136 to −131 was mutated in the expression construct 204-bp rat ET-1 promoter (14). These plasmids, together with gifts of Drs. Thomas Quertermous (Stanford University, Palo Alto, CA). A promotorless CAT plasmid (basic CAT) was purchased from Promega (Madison, WI). prSVCAT and prSVluc contain the CAT and luc genes, respectively, driven by Rous sarcoma virus long terminal repeat sequences (15, 18). The murine GATA-5 and GATA-6 expression plasmids were generous gifts of Dr. Michael P. Parmacek (University of Pennsylvania, Philadelphia, PA) and were described elsewhere (20, 21). The murine GATA-4 expression plasmid pcDNA4 was subcloned by digesting pMT2-GATA-4 (22) (a generous gift of Dr. David Wilson, Washington University, St. Louis, MO) with EcoRI to isolate the 1.9-kilobase insert and subcloning the resulting cDNA fragment encoding the murine GATA-4 into the EcoRI site of the eukaryotic expression plasmid pcDNA3 (Invitrogen, Carlsbad, CA). Plasmids were purified by anion exchange chromatography (Qiagen, Hilden, Germany), quantified by measurement of A_{260}, and examined on agarose gels stained with ethidium bromide prior to use.

**Transfection and Luciferase/CAT Assays**—24 h after plating, cells were washed twice with serum-free medium and then co-transfected with the 204-bp ET-1 promoter construct of interest and 0.1 pg of pRSVluc using LipofectAMINE Plus (Life Technologies, Inc.) according to the manufacturer's recommendation. After a 2-h incubation with DNA-Lipo-fectAMINE complex, the cells were washed twice with serum-free medium and further incubated for 48 h in serum-free medium in the presence of 1.0 × 10^{-5} M PE or saline as controls. The cells were then washed twice with ice-cold phosphate-buffered saline, lysed with lysis buffer, and subjected to assays for luciferase and CAT activities as described previously (15, 18, 19).

**Electrophoretic Mobility Shift Assays (EMSAs)**—Nuclear extracts were prepared from cultures of primary neonatal rat cardiac myocytes as described (19). Double-stranded oligonucleotides that contained GATA motifs from the ET-1 promoter were designed. The sequences of the 4%-strand-specific oligonucleotides were as follows: ET-GATA, 5'-CTCTTAGGCGGTCTTATCT-CCTCGCGTCAACGTGTC-3', and mutET-GATA, 5'-CTCTTAGGCGGTCTGCA-TCCGGGCTGCAC-GTTGC-3'. We also used a double-stranded oligonucleotide that contained p53-binding site in the p21 promoter as a control probe (19). Oligonucleotides were synthesized by Genencor, Inc. (Tokyo, Japan) and purified by SDS-polyacrylamide gel electrophoresis.

EMSAs were carried out at 4 °C for 20 min in 15-μl reaction mixtures containing 10 μg of nuclear extract, 0.25 ng (≥20,000 cpm) of radiolabeled double-stranded oligonucleotide, 500 ng of poly(dI-dC), 5 μm Tris-HCl (pH 7.5), 1 μm EDTA, 0.5 μm dithiothreitol, 37.5 μm KCl, and 4% Ficoll 400. For cold competition experiments, a 100 molar excess of unlabeled competitor oligonucleotide was included in the binding reaction mixture. Protein-DNA complexes were separated by electrophoresis on 4% polyacrylamide gels. Filters were heated to 95 °C for 2 min, electrophoresed on an SDS-polyacrylamide gel (10%), transferred to Immobilon membranes, and reacted with anti-phospho-specific antibody (New England Biolabs, Beverly, MA), gel (10%), transferred to Immobilon membranes, and reacted with anti-phosphoserine antibody (New England Biolabs, Beverly, MA), and further incubated for 48 h in serum-free medium in the presence of 1.0 × 10^{-5} M PE or saline as controls. The cells were then washed twice with ice-cold phosphate-buffered saline, lysed with lysis buffer, and subjected to assays for luciferase and CAT activities as described previously (15, 18, 19).

**Analysis of the Phosphorylation State of ET-1 Transcription**—To normalize for protein loading after the expression of other genes associated with hypertrophy (15, 16). To clarify the role of the GATA element in PE-induced hypertrophy in the context of the ET-1 promoter, cardiac myocytes were transfected with CAT gene driven by the 204-bp ET-1 promoter containing a mutation in the GATA site (pmut-GATA-ET-CAT), which abolishes the binding of cardiac GATA factors (see Fig. 3). As shown in Fig. 1A, the basal transcriptional activity of the transfected 204-bp ET-1 promoter was modestly decreased by the mutation of the GATA element (53% decrease versus wild type). In contrast, PE-responsive transcription was severely attenuated (2.2-fold for wild type versus 1.3-fold for GATA mutant). Thus, an intact GATA element is required for both basal and PE-responsive ET-1 transcription in cardiac myocytes.

**GATA-4/5 Are Sequence-specific Activators of the ET-1 Promoter**—Among the six members of the GATA transcription factor family, only GATA-4, -5, and -6 are expressed in the heart (19–22). To determine whether expression of GATA-4, -5, and -6 is induced by PE in cardiac myocytes via the GATA sites that mediate PE responsiveness (Fig. 1), we performed co-transfection experiments in NIH3T3 cells, which lack all GATA factors. A CAT reporter driven by the 204-bp ET-1 promoter was transiently transfected with a eukaryotic expression plasmid encoding GATA-4, -5, or -6 or β-galactosidase as a control. Transfection efficiency was monitored by co-transfection with a CAT construct of interest and 0.1 pg of pRSVluc. After 48 h of stimulation with 1.0 × 10^{-5} M of PE or saline as a control, cardiomycocytes were harvested for luciferase and CAT assays. The 204-bp ET-1 promoter fragment conferred PE-inducible expression on the CAT reporter gene (Fig. 1A). In contrast, PE stimulation did not increase the activity of a promoter derived from the ubiquitously expressed β-actin gene (data not shown). PE stimulation did not increase the background activity of a promotorless CAT (basic CAT) transfected into cardiomycocytes (Fig. 1A). To rule out the possibility that the increase by PE of the ET-1 promoter activity occurs in contaminating cells other than cardiac myocytes (less than 10% in our preparation), which mainly consist of fibroblasts, NIH3T3 cells (mouse fibroblasts) were transfected with pwtET-CAT. In these cells, PE stimulation did not increase the activity of the pwtET-CAT (Fig. 1B), indicating the cardiac-specific response of this promoter to PE. These findings demonstrate that the proximal 204-bp ET-1 promoter sequence contains element that are responsive to PE stimulation in cardiac myocytes.

**Results**

**Phenoehrine-responsive ET-1 Transcription Requires an Intact GATA Element**—The α1-adrenergic agonist PE is a potent inducer of hypertrophy and ET-1 expression in cardiac myocytes (17). To determine whether the 204-bp promoter sequence of the ET-1 gene is sufficient to mediate PE-responsive transcription in neonatal rat ventricular cells, these cells 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 co-transfected with a small quantity of prSVluc. After 48 h of stimulation with 1.0 × 10^{-5} M of PE or saline as a control, cardiomycocytes were harvested for luciferase and CAT assays. The 204-bp ET-1 promoter fragment conferred PE-inducible expression on the CAT reporter gene (Fig. 1A). In contrast, PE stimulation did not increase the activity of a promoter derived from the ubiquitously expressed β-actin gene (data not shown). PE stimulation did not increase the background activity of a promotorless CAT (basic CAT) transfected into cardiomycocytes (Fig. 1A). To rule out the possibility that the increase by PE of the ET-1 promoter activity occurs in contaminating cells other than cardiac myocytes (less than 10% in our preparation), which mainly consist of fibroblasts, NIH3T3 cells (mouse fibroblasts) were transfected with pwtET-CAT. In these cells, PE stimulation did not increase the activity of the pwtET-CAT (Fig. 1B), indicating the cardiac-specific response of this promoter to PE. These findings demonstrate that the proximal 204-bp ET-1 promoter sequence contains element that are responsive to PE stimulation in cardiac myocytes.

**Analysis of the Phosphorylation State of ET-1**—We used a double-stranded oligonucleotide that contained p53-binding site in the p21 promoter as a control probe (19). Oligonucleotides were synthesized by Genencor, Inc. (Tokyo, Japan) and purified by SDS-polyacrylamide gel electrophoresis.

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transferring a small quantity of pRSVluc. As shown in Fig. 2, expression of GATA-4 (25-fold), -5 (91-fold), or -6 (13-fold) resulted in significant activation of the 204-bp ET-1 promoter. Moreover, co-transfection experiments employing the ET-1 promoter containing the same GATA site mutation as in Fig. 1 illustrated that only GATA-4 and -5 transactivate the 204-bp ET-1 promoter in a GATA sequence-specific manner (Fig. 2). These data suggest that GATA-4/5 transactivate the ET-1 promoter directly via the GATA site.

GATA-4 in PE-stimulated Cardiac Myocytes Binds to the ET-1 GATA Element—To identify cardiac nuclear factors that bind the ET-1 GATA site, EMSAs were performed with nuclear extracts from PE-stimulated neonatal cardiac myocytes. Nuclear extracts were probed with a radiolabeled ET-1 GATA double-stranded oligonucleotide in the presence or absence of competitor oligonucleotides (Fig. 3A). Competition EMSAs revealed that one retarded band, indicated by an arrow (first lane), represented a GATA sequence-specific complex, as evidenced by the fact that it was competed by an unlabeled ET-1 GATA double-stranded oligonucleotide (second lane) but not by an excess of the ET-1 GATA site containing mutations that abolish PE responsiveness (third lane). This result suggests the presence of factor(s) that specifically bind to the ET-1 GATA sequence in extracts from PE-stimulated rat cardiac myocytes.

Previous studies demonstrated that in vitro translated GATA factors can bind to the ET-1-GATA site (11–14). In addition, our results described above demonstrate that GATA-4/5 can transactivate the ET-1 promoter in a sequence-specific manner. Therefore, we examined by supershift experiments whether the factor(s) that specifically interact with the ET-1-GATA site in cardiac nuclear extracts are GATA-4 and/or GATA-5 (Fig. 3B). Addition of anti-GATA-4 antibody resulted in nearly complete disappearance of the original complex formed by the interaction of the ET-1-GATA site with cardiac nuclear factors (second lane). In addition, it produced a new complex that migrated much more slowly than the original one. In contrast, addition of anti-GATA-5 antibody resulted in only slight diminishment of the original band and in the formation of a slower migrating complex which was much fainter than that formed upon the addition of anti-GATA-4 antibody (lane 3). These findings demonstrate that GATA-4 is the major cardiac nuclear factor that binds the ET-1-GATA site and that GATA-5 binds this site to a much lesser degree.

PE Stimulation Causes Serine Phosphorylation of Cardiac GATA-4—To clarify the regulatory mechanisms for GATA-4 activation during PE-stimulated hypertrophy in neonatal rat cardiac myocytes, we examined the expression of GATA-4 following PE stimulation. In neonatal rat ventricular cells incubated with PE for 5 min - 48 h, the total GATA-4 expression levels were not altered by PE (Fig. 4). We next examined the effect of PE on GATA-4 phosphorylation. The cell lysates were immunoprecipitated with anti-GATA-4 antibody, followed by Western blot analysis with anti-phosphoserine antibody. As shown in Fig. 4, PE treatment resulted in a marked increase in the level of the phosphorylated form of GATA-4. The level was maximal at 3 h after PE treatment and decreased thereafter but continued to be high at 48 h after the treatment. To correct for differences in protein loading after immunoprecipitation, the same membrane was reblotted with anti-GATA-4 antibody. As shown in Fig. 4, total GATA-4 immunoreactivity did not change following PE stimulation. Therefore, the ratio of the phosphorylated form of GATA-4 to the total GATA-4 in cardiac myocytes was markedly increased by PE. Similar results were obtained with reciprocal experiments, i.e. immunoprecipitation with anti-phosphoserine antibody and Western blotting with anti-GATA-4 antibody. We could not detect a PE-stimulated...
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**Fig. 3.** Analysis of interactions between cardiac GATA factors and the ET-1 GATA site. Nuclear extracts (10 μg of protein) from PE-stimulated cardiac myocytes were probed with a radiolabeled double-stranded oligonucleotide containing the ET-1 GATA site. The thick arrow indicates the complex formed by the sequence-specific interaction between cardiac GATA-4 and the ET-1 GATA site. F, free probe. A, unlabeled competitor DNAs were present at a 100-fold molar excess as indicated: wild type ET-1 GATA (Wild type) in the second lane; ET-1 GATA with a mutation which abolishes the PE responsiveness of transcription (mut-GATA) in the third lane. B, the thin arrow indicates a supershifted band produced by the addition of anti-GATA-4 antibody (lane 2) or anti-GATA-5 antibody (lane 3).

**Fig. 4.** Phosphorylation of cardiac GATA-4 following PE stimulation. Nuclear extracts (100 μg of protein) from cardiac myocytes stimulated with PE for various periods were immunoprecipitated with anti-GATA-4 antibody. Immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis, transferred to Immobilon membranes, and sequentially probed with anti-phosphoserine antibody and with anti-GATA-4 antibody.

Increasing threonine phosphorylation of cardiac GATA-4. These findings indicate that PE stimulation causes serine phosphorylation of GATA-4, which might be involved in PE-responsive ET-1 transcription in cardiac myocytes.

**Phosphorylation of GATA-4 Increases Its DNA Binding Activity**—We tested the possibility that phosphorylation of GATA-4 results in functional consequences such as an increase in the DNA binding activity. COS7 cells were transfected with an expression plasmid encoding GATA-4 (pcDNAG4) and incubated in medium containing 10% serum. 48 h later, nuclear extracts were prepared by lysis of the transfected cells in lysis buffer in the presence or absence of phosphatase inhibitors (50 mM NaF and 1 mM Na3VO4). These extracts were immunoprecipitated with anti-GATA-4 antibody, and the immunoprecipitates were subjected to Western blot analysis with anti-phosphoserine antibody. As shown in the upper panel of Fig. 5A, the phosphorylation of GATA-4 was evident in the nuclear extract collected with the phosphatase inhibitors but not in that without the inhibitors. However, the amount of GATA-4 (total of phosphorylated and unphosphorylated forms) was similar in these two extracts (Fig. 5A, lower panel). Then we determined using EMSA whether the DNA binding activity of GATA-4 differed in the nuclear extracts used for the experiments shown in Fig. 5A. By competition EMSAs using a radiolabeled ET-1 GATA double-stranded oligonucleotide as a probe (Fig. 5B, second through sixth lanes), one retarded band (indicated by an arrow, second lane) was found to be a GATA sequence-specific complex, as evidenced by the fact that it was competed by an ET-1 GATA oligonucleotide (third lane) but not by an oligonucleotide with a GATA site mutations (fourth lane). In addition, this retarded band was clearly supershifted by anti-GATA-4 antibody (sixth lane) but not by IgG (fifth lane), indicating this band represents a complex of the ET-1 GATA oligonucleotide and GATA-4. Notably, the intensity of this band was much stronger in the nuclear extract prepared with the phosphatase inhibitors (second lane) than in that prepared without the inhibitors (first lane). These results suggest that phosphorylation of GATA-4 increases its DNA binding activity.

**ERK1/2 Activation Is Required for PE-induced Increase in Phosphorylation and DNA Binding Activity of Cardiac GATA-4**—To determine the upstream factors involved in PE-induced phosphorylation of GATA-4, we examined the effect of PD098059, a MEK-1-specific inhibitor, on GATA-4 phosphorylation, as well as on activation of ERK1/2, targets of MEK-1. Neonatal rat ventricular myocytes were preincubated with or without 20 μM PD098059 for 1 h; then PE was added, and the myocytes were further incubated at 37 °C. Activation of ERK1/2 was estimated by Western blot analysis using an antibody that specifically recognizes the phosphorylated, active form of these enzymes. ERK1/2 were markedly activated after 15 min of PE stimulation (Fig. 5A, second lane) compared with saline stimulation (Fig. 5A, first lane). 20 μM PD098059 completely blocked the activation (Fig. 5A, third lane). GATA-4 phosphorylation was examined in cardiac myocytes collected 3 h after the PE stimulation, when the phosphorylation was maximal. Lysates of these cells were subjected to immunoprecipitation with anti-GATA-4 antibody followed by Western blotting with anti-phosphoserine antibody as above. As shown in Fig. 5B, 20 μM PD098059 almost completely inhibited PE-induced GATA-4 phosphorylation. However, GATA-4 phosphorylation was not blocked by a phosphatidylinositol 3-kinase inhibitor (wortmannin) or p38 mitogen-activated protein kinase inhibitor (SB 203580) (data not shown). These results suggest that ERK activation is required for PE-induced phosphorylation of cardiac GATA-4.

Last, to determine whether the ERK pathway is also involved in the DNA binding activity of cardiac GATA-4, EMSAs were performed with nuclear extracts from neonatal cardiac myocytes stimulated with saline and PE in the presence or absence of PD098059. These extracts were probed with a radiolabeled double-stranded oligonucleotide containing the ET-1 GATA site or that containing p53-binding site in the p21 promoter, which is a control. As shown in Fig. 6 (top two panels), the intensity of the specific band indicating GATA-4 binding was increased in nuclear extracts from PE-stimulated myocytes (second lane) compared with those from saline-stimulated cells (first lane). The PE-stimulated increase in the DNA binding activity of cardiac GATA-4 was almost completely blocked by PD098059 (third lane). In contrast, p53 binding activities were altered by PE nor PE plus PD098059 (Fig. 6, bottom two panels, first through third lanes). These experiments were repeated three times using independent preparations of cells and found to be reproducible. Taken together with the above results, these findings indicate that ERK activation and subsequent GATA-4 phosphorylation might be involved in the increased DNA binding activity of cardiac GATA-4 (Fig. 7).
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 (9, 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. The α1-adrenergic agonist PE is a potent inducer of hypertrophy and ET-1 expression in cardiac myocytes (17). The present results demonstrate that PE-inducible expression of cardiac ET-1 is mediated, at least in part, at the level of transcription and that phosphorylation of GATA-4 plays a role in this process.

FIG. 5. Phosphorylation of GATA-4 increases its DNA binding activity. COS7 cells were transfected with pcDNA4. Nuclear extracts were prepared from these cells in lysis buffer in the presence or absence of phosphatase inhibitors (50 mM NaF and 1 mM Na3VO4) as indicated. A, GATA-4 phosphorylation was examined as described in the legend for Fig. 4. B, nuclear extracts were probed with a radiolabeled double-stranded oligonucleotide containing the ET-1 GATA site.

FIG. 6. ERK activation is required for PE-induced phosphorylation of GATA-4 in cardiac myocytes. Cardiac myocytes were preincubated with (third lane) or without (first and second lanes) 20 μM PD098059 (PD) for 1 h and subsequently stimulated with saline (SS, first lane) or PE (second and third lanes) for 15 min for ERK activation and for 3 h for GATA-4 phosphorylation. Activation of ERK was estimated by Western blotting as described under “Materials and Methods,” and GATA-4 phosphorylation was examined as described in the legend for Fig. 4.

FIG. 7. PD098059 inhibits PE-stimulated increase in the cardiac ET-1 GATA binding activity. Cardiac myocytes were preincubated with (third lane) or without (first and second lanes) 20 μM PD098059 (PD) for 1 h and subsequently stimulated with saline (SS, first lane) or PE (second and third lanes) for 3 h. Nuclear extracts (10 μg of protein) from these cells were probed with a radiolabeled double-stranded oligonucleotide containing the ET-1 GATA site in A and with that containing the p53-binding site in B.

GATA factors are important for cardiac-specific transcription of many genes, including α-MHC, B-type natriuretic peptide, myosin light chain 1/3, and cardiac troponin C (23–26). Likewise, we showed here that mutation of the GATA element in the 204-bp ET-1 promoter moderately decreased the basal transcriptional activity in cardiac myocytes. In addition, recent studies, including ours, demonstrated that GATA transcription factors are also required for transcriptional activation of the genes for β-myosin heavy chain and angiotensin II type 1a receptor during hemodynamic overload-induced cardiac hypertrophy in vivo (15, 16). The results of the present study provide further evidence that GATA factors are involved in the hypertrophic process, because mutating the GATA element abolished α1-adrenergic-responsive ET-1 transcription. Thus, in the context of the ET-1 promoter, the GATA element plays an important role in both basal and PE-responsive transcription in cardiac myocytes.

To date, six related zinc-finger-containing proteins have been described that recognize and bind the GATA motif (19–22). The proteins fall into two subgroups: one consisting of GATA-1, -2,
and -3 and one consisting of GATA-4, -5, and -6. The subgroups are defined both by sequence homology and expression pattern, with GATA-1, -2, and -3 predominating in blood and endodermal derivatives and GATA-4, -5, and -6 predominating in heart and endodermal derivatives. Interestingly, the genes encoding GATA-4 and -6 are expressed in the heart throughout embryonic and postnatal development, whereas the murine GATA-5 gene is normally expressed in a temporally and spatially restricted pattern within the embryonic heart (20, 21). The present study demonstrated that both GATA-4 and -5 activated the 204-bp ET-1 promoter in a sequence-specific manner. However, GATA-4 is the major cardiac nuclear factor that binds to the ET-1 GATA element, and GATA-5 binds to a much lesser degree. Despite this fact, our data do not rule out a potential contribution of GATA-5 to the PE-responsive ET-1 transcription because the expression of GATA-5 in cardiac myocytes increases during hypertrophy (19). The relative contributions of GATA-4 and GATA-5 to PE-responsive transcription should be further investigated.

A large number of transcription factors have been shown to exist within cells as phosphoproteins. The functional consequences of phosphorylation vary but include regulation of DNA binding and transcriptional activation. It has been shown that other members of the GATA family of transcription factors, namely GATA-1 and -2, exist as phosphoproteins in erythroid or hematopoietic progenitor cells (27, 28). These proteins are phosphorylated exclusively on serine residues. Notably, stimulation with growth factors results in their enhanced phosphorylation. Systematic mutations of serine residues in GATA-1 do not appear to alter its transactivation function as judged by reporter gene assays conducted in COS cells, nor do they alter the DNA binding ability of GATA-1 proteins expressed in COS cells. Our data that stimulation by PE caused serine phosphorylation of GATA-4 in cardiac myocytes are compatible with these previous findings. In contrast to the previous reports, we observed that PE stimulation increased the DNA binding activity of GATA-1 proteins expressed in COS cells. Our data that stimulation by PE caused serine phosphorylation of GATA-4 in cardiac myocytes are compatible with these previous findings. In contrast to the previous report, stimulation by PE caused serine phosphorylation of GATA-4 in cardiac myocytes. The reason for this discrepancy is unclear at present, but further studies on precise mapping of phosphorylation sites are needed to clarify this discrepancy.

Cardiac myocyte hypertrophy is a central feature of all types of heart muscle failure. Hypertrophic stimuli reach the nucleus via multiple signaling pathways within cardiac myocytes and elicit changes in cardiac gene expression. ET-1 is one of the local factors that play important roles in the development of heart failure. Our present findings add ET-1 to the increasing list of factors whose transcriptional activation during cardiac hypertrophy is mediated by GATA factors. In addition, the present study provides the first evidence that post-translational modification of GATA-4 is involved in this process. Further elucidation of the precise mechanisms by which this central pathway modulates the hypertrophic response may provide novel therapeutic approaches to human heart failure.

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Phosphorylation of GATA-4 Is Involved in α1-Adrenergic Agonist-responsive Transcription of the Endothelin-1 Gene in Cardiac Myocytes

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