Endothelin-1 induces phosphorylation of GATA-4 transcription factor in the HL-1 atrial-muscle cell line

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The transcription factor GATA-4 plays a central role in the regulation of cardiac-muscle gene transcription. The present study demonstrates that endothelin-1 (ET-1) induces GATA-4 activation and phosphorylation. The treatment of HL-1 adult mouse atrial-muscle cells with ET-1 (30 nM) caused a rapid increase in the DNA binding activity of GATA-4 within 3 min. The activation was associated with an upward mobility shift of the GATA-4 band on native PAGE in an electrophoretic-mobility-shift assay. The upward shift of the GATA-4 band also occurred on SDS/PAGE as monitored by immunoblotting. The *in vitro* treatment of nuclear extracts with λ -protein phosphatase abolished the upward shift, indicating that GATA-4 was

phosphorylated. ET-1 activated the p44/42 mitogen-activated protein kinase (MAPK) and the MAPK kinase (MEK) within 3 min, and PD98059 (a specific inhibitor of MEK) abolished the ET-1-induced GATA-4 phosphorylation. PMA also caused the rapid activation of MAPK and the phosphorylation of GATA-4. In contrast, the activation of MAPK by phenylephrine or H_2O_2 was weak and did not lead to GATA-4 phosphorylation. Thus ET-1 induces a GATA-4 phosphorylation by activating a MEK–MAPK pathway.

Key words: cardiac muscle, heart, mitogen-activated protein kinase.

INTRODUCTION

Endothelin-1 (ET-1), a peptide composed of 21 amino acids, is a potent vasoconstrictor originally identified in vascular endothelial cells [1]. In addition to its actions on smooth muscle, ET-1 has also been shown to elicit cell signalling in the myocardium. ET-1 induces atrial expression of atrial natriuretic factor (ANF) [2,3] and brain natriuretic peptide (BNP) [4], which regulate fluid and electrolyte metabolism and blood pressure homeostasis. ET-1 also induces hypertrophy of isolated ventricular myocytes [5,6] and may be involved in mechanical stretch-induced hypertrophy of isolated ventricular myocytes [7]. The endothelin receptor antagonists have been shown to reduce pressure overload- [8] or norepinephrine- [9] induced cardiac hypertrophy *in vivo*. ET-1 also serves as a preconditioning agent against atrial and ventricular damage during ischaemia–reperfusion injury [10,11]. ET-1 has been shown to exert anti-apoptotic activities in various cell types [12,13]. More recently, anthracycline-induced cardiac myocyte injury was shown to be attenuated by ET-1 [14], suggesting that ET-1 may induce anti-apoptotic signalling in the heart. Therefore it is important to understand the mechanism of ET-1 signal transduction in cardiac myocytes.

GATA-4 is a member of the GATA family of zinc-finger transcription factors which play important roles in cell growth, differentiation and survival. Six GATA family members have been identified and shown to regulate transcription of target genes via binding to the consensus 5′-WGATAR-3′ sequence. The GATA-4/5/6 subfamily is expressed in the heart. The GATA-binding sites have been identified in promoter regions of cardiac genes, including α -myosin heavy chain [15], β -myosin heavy chain [16], myosin light chains [17], Na^+/Ca^{2+} exchanger [18,19], angiotensin II type_{1a} receptor [20], ANF [21] and BNP [22]. Many of these genes are up-regulated during cardiac

hypertrophy and heart failure. The GATA elements are also found in promoters of genes which may be involved in anti-apoptotic activities such as *bcl-x* [23], nitric oxide synthases [24,25] and glutathione peroxidase [26].

The regulatory mechanisms of GATA-4 may involve post-translational modifications. Molkenin et al. [27] proposed a signal-transduction pathway for GATA-4 activation involving calcineurin and nuclear activator of T-cells-3 (NFAT-3). In this pathway, the Ca^{2+} /calmodulin-activated calcineurin induces dephosphorylation and migration of NFAT-3 into the nucleus, which, in turn, interacts with GATA-4. The treatment with the calcineurin inhibitor cyclosporin blocked the development of cardiac hypertrophy induced by pressure overload or genetic manipulations [27,28]. Recently, Morimoto et al. [29] demonstrated that the α -adrenergic agonist phenylephrine (PE) induced the serine phosphorylation of GATA-4, suggesting that GATA-4 is also regulated via the phosphorylation mechanism.

The HL-1 cardiac-muscle cell line was established from transgenic mouse atria expressing the simian-virus-40 large T antigen [30]. These immortalized cells maintain the differentiated atrial myocyte phenotype and the ability to contract. We studied the effects of ET-1 on the GATA-4 transcription factor in HL-1 cells and found that ET-1 rapidly phosphorylated and activated GATA-4. The signalling mechanism of ET-1-induced GATA-4 phosphorylation appears to involve a mitogen-activated protein kinase (MAPK)–MAPK kinase (MEK) pathway.

EXPERIMENTAL

Cell culture

HL-1 cells (obtained from Dr. William Claycomb, Department of Biochemistry and Molecular Biology, Louisiana State University, New Orleans, LA, U.S.A.) were maintained in EXCELL 320 medium (JRH Biosciences, Lenexa, KS, U.S.A.) supple-

Abbreviations used: ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; EMSA, electrophoretic-mobility-shift assay; ET-1, endothelin-1; λ PPase, λ -protein phosphatase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; NFAT-3, nuclear factor of activated T-cells-3; PE, phenylephrine.

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mented with 10 % (v/v) fetal-bovine serum (Life Technologies, Grand Island, NY, U.S.A.), 10 μ g/ml insulin (Life Technologies), 20 μ g/ml endothelial-cell growth supplement (Upstate Biotechnology, Lake Placid, NY, U.S.A.), 1 μ M retinoic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.), 100 μ M norepinephrine (Sigma), 1 % non-essential amino acid supplements (Life Technologies), 100 units/ml penicillin, 100 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin B (Sigma) in plastic dishes, coated with 12.5 μ g/ml fibronectin and 0.02 % gelatin, in a 5 % CO₂ atmosphere at 37 °C. The cells were replenished with fresh media every 2–3 days. For experiments, the cells were starved in EXCELL 320 medium supplemented only with non-essential amino acids, penicillin, streptomycin, and amphotericin B for 18 h and treated with ET-1 (Sigma).

Electrophoretic-mobility-shift assay (EMSA)

To prepare nuclear extracts, the cells were washed in PBS and incubated in 10 mM Hepes (pH 7.8)/10 mM KCl/2 mM MgCl₂/0.1 mM EDTA/0.1 mM PMSF/5 μ g/ml leupeptin/10 μ g/ml aprotinin/1 mM sodium fluoride/0.1 mM sodium orthovanadate/1 mM tetrasodium pyrophosphate for 15 min at 4 °C. Triton X-100 (Sigma) was then added at a final concentration of 0.6 % (v/v). The samples were mixed vigorously, and centrifuged. The pelleted nuclei were resuspended in 50 mM Hepes (pH 7.8)/50 mM KCl/300 mM NaCl/0.1 mM EDTA/0.1 mM PMSF/5 μ g/ml leupeptin/10 μ g/ml aprotinin/1 mM sodium fluoride/0.1 mM sodium orthovanadate/1 mM tetrasodium pyrophosphate/1 % (v/v) glycerol, then mixed for 20 min and centrifuged at 15800 g for 5 min. The supernatants were harvested, and protein concentrations determined [31].

To perform EMSA, binding reaction mixtures contained nuclear extract (2 μ g of protein), 0.5–1 μ g of poly(dI-dC)·poly(dI-dC), 1 mM EDTA, 0.5–1 mM dithiothreitol and 0.25 ng (> 20000 c.p.m.) of ³²P-labelled double-stranded oligonucleotide containing consensus GATA sequence (5'-CAC TTG ATA ACA GAA AGT GAT AAC TCT-3') in either 20 mM Tris/HCl (pH 7.5)/100 mM NaCl/10 % (v/v) glycerol [32] or 5 mM Tris/HCl (pH 7.5)/37.5 mM KCl/4 % (w/v) Ficoll 400 [29]. Binding reactions were performed for 20 min at 25 °C. An electrophoresis of samples through a native 6 % (w/v) polyacrylamide gel was followed by autoradiography.

Supershift experiments were performed by incubating 2 μ g of anti-GATA-4, anti-GATA-6 or anti-NFAT-3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) in the binding reaction mixture for 1 h at 4 °C before the addition of the ³²P-labelled oligonucleotide to start the binding reaction.

A densitometry analysis of the bands was performed using the Sigma gel software. All the experiments were repeated at least three times.

Western-blot analysis

To prepare lysates, the cells were washed in PBS and solubilized with 50 mM Hepes solution, pH 7.4, containing 1 % (v/v) Triton X-100, 4 mM EDTA, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1 mM tetrasodium pyrophosphate, 2 mM PMSF, 10 μ g/ml leupeptin and 10 μ g/ml aprotinin. Following centrifugation in a Microfuge, protein concentrations in the supernatant were determined [31]. Cell lysates (10 μ g of protein) were electrophoresed through a reducing SDS/10 % (w/v) polyacrylamide gel and electroblotted on to a nitrocellulose membrane. After the transfer, the gel was stained with Coomassie Blue to check for transfer efficiency. The membrane was blocked and incubated with the polyclonal IgG for phospho-specific p44/42 MAPK (New England Biolabs, Beverly, MA, U.S.A.),

p44/42 MAPK (Santa Cruz Biotechnology), phospho-specific MEK1/2 (New England Biolabs) or MEK1 (Zymed Laboratories, South San Francisco, CA, U.S.A.). The levels of proteins and phosphoproteins were detected with horseradish peroxidase-linked secondary antibodies and the ECL® (enhanced chemiluminescence) System (Amersham Life Science, Arlington Heights, IL, U.S.A.). The membranes were stained with Coomassie Blue to confirm the equal loading and transfer.

SDS/PAGE (10 % gel) was used to separate phosphorylated from non-phosphorylated GATA-4, and the gel contained acrylamide and bisacrylamide in the ratio 30:0.165. For a Western-blot analysis, the gel was electroblotted on to a PVDF membrane, and the rabbit polyclonal IgG for GATA-4 (H-112) (Santa Cruz Biotechnology) was used at a concentration of 1 μ g/ml. In these experiments, 20 μ g of protein of nuclear extracts were loaded on to a gel. To determine the effects of γ -protein phosphatase (λ PPase), nuclear extracts were isolated as described above, except that the phosphatase inhibitors (sodium fluoride, sodium orthovanadate and tetrasodium pyrophosphate) were omitted. Nuclear extracts (20 μ g) were incubated with 2500 units/ml λ PPase (New England Biolabs) at 30 °C for 30 min before subjecting them to SDS/PAGE.

RESULTS

EMSA revealed that the nuclear extracts from HL-1 cells had some constitutive binding activity towards the consensus GATA sequence. The supershift experiments using the GATA-4 antibody revealed that this GATA binding protein was GATA-4 (Figure 1A). Neither the anti-GATA-6 antibody nor a control IgG caused a supershift of the GATA band (results not shown). The GATA-4 binding activity was enhanced by the treatment of cells with ET-1 (30 nM) for 10 min. Furthermore, the complex between DNA and ET-1-activated GATA-4 migrated slightly more slowly than the complex with unstimulated GATA-4 (Figure 1A). Activation of the DNA-binding activity and the reduced mobility of GATA-4 induced by ET-1 was also observed when we used an EMSA protocol similar to that employed by Morimoto et al. [29] (Figure 1B). This protocol decreased smearing, and thus the reduced migration was more clearly visible. The ET-1-mediated increase in GATA-4 DNA binding activity and the shift of DNA–GATA-4 complex were transient, occurring as early as 3 min (Figure 1C).

A two-hybrid screen with a GATA-4 bait showed that GATA-4 can form a complex with NFAT-3 [27]. Thus the upward EMSA shift (Figure 1) could result from the formation of such a complex. However, an anti-NFAT-3 antibody did not supershift the GATA-4 band (results not shown). The absence of interactions of GATA-4 with NFAT-3 in the mechanism of ET-1-mediated upward shift of the GATA-4 band was further supported by our observation that the upward shift can also be detected by reducing-SDS/PAGE. As shown in Figure 2(A), two GATA-4 bands were separated at $\approx$ 50 kDa in an immunoblot analysis using the anti-GATA-4 antibody. In untreated cells the lower band was slightly denser than the band with reduced mobility (see leftmost lane, Figure 2A). The treatment of cells with ET-1 resulted in an upward shift of the lower band. By 10 min of treatment, all of the GATA-4 protein molecules existed as a single species with reduced mobility (lane 2 from the left, Figure 2A). To test the hypothesis that GATA-4 may be phosphorylated and thus give slightly reduced mobility, we incubated nuclear extracts with λ PPase in *in vitro*. The treatment of nuclear extracts from ET-1-treated cells with λ PPase completely abolished the higher band (lane 4 from the left, Figure 2A). Similar results were obtained in EMSA exper-

Figure 1 Activation of GATA-4 by ET-1

(A) HL-1 cells were treated with ET-1 (30 nM) for 10 min. Nuclear extracts were prepared and subjected to an EMSA with the ^{32} P-labelled oligonucleotide containing GATA-binding sequence in the reaction mixtures with 20 mM Tris/HCl, 100 mM NaCl and 10% (v/v) glycerol as described by Suzuki et al. [32]. For the supershift experiments, nuclear extracts were subjected to an EMSA in the presence of the antibody (Ab) for GATA-4 (2 μ g). (B) Nuclear extracts were subjected to an EMSA with the ^{32} P-labelled oligonucleotide containing the GATA-binding sequence in reaction mixtures with 5 mM Tris/HCl, 37.5 mM KCl and 4% (w/v) Ficoll 400 as described by Morimoto et al. [29]. (C) HL-1 cells were treated with ET-1 (30 nM) for 3, 10 or 30 min. Nuclear extracts were prepared and subjected to an EMSA [29] with the ^{32} P-labelled oligonucleotide containing the GATA-binding sequence. The histogram (lower right) indicates fold increases in the intensity of GATA bands after treating cells with ET-1, as determined by densitometry. The values represent means $\pm$ S.E.M. ($n = 4-6$).

Figure 2 Effects of λ PPase on ET-1-induced GATA-4 activation

Nuclear extracts from HL-1 cells treated with ET-1 (30 nM) for 10 min were incubated *in vitro* at 30 $^{\circ}$ C without or with λ PPase (2500 units/ml). These samples were subjected to (A) SDS/PAGE and Western-blot analysis using the antibody for GATA-4 or (B) an EMSA using the protocol described by Suzuki et al. [32].

Figure 3 ET-1 activates MEK and p44/42 MAPK

HL-1 cells were treated with ET-1 (30 nM) for the times indicated. Cell lysates were prepared and subjected to a Western-blot analysis using the antibodies for (A) phospho-specific p44 and p42 MAPK or (C) phospho-specific MEK. (B) The ratio of phosphorylated p44/42 MAPK and MAPK proteins as determined by a densitometric analysis. The values represent means $\pm$ S.E.M. An asterisk (*) denotes the values that were significantly different from the value without ET-1 treatment at $P < 0.05$.

iments (Figure 2B). These results demonstrate that ET-1 induced the phosphorylation of GATA-4.

Towatari et al. [33] reported that interleukin 3 activated GATA-2 through the p44/42 MAPK pathway. GATA-4 also contains putative MAPK phosphorylation sites [34], and Morimoto et al. [29] demonstrated that PE induced phosphorylation of GATA-4. Therefore we hypothesized that ET-1 phosphorylated GATA-4 through the MAPK pathway. It has been shown that ET-1 can activate MAPK in neonatal rat ventricular myocytes [35,36]. Consistently, the treatment of HL-1 cells with ET-1 caused a rapid phosphorylation of p44/42 MAPK (Figure 3A) and MEK (Figure 3C) within 3 min of treatment. The protein levels of MAPK or MEK were unchanged in response to ET-1 treatment (results not shown). Figure 3(B) shows the ratio of phosphorylated MAPK and MAPK proteins as determined by densitometry analysis.

To determine the role of the MAPK pathway in ET-1-signalling for GATA-4 phosphorylation, we used a specific inhibitor of MEK activation, PD98059 [37]. The treatment of HL-1 cells with PD98059 (30 μ M) inhibited phosphorylation of p44/42 MAPK induced by ET-1 (results not shown), suggesting that ET-1 activated MAPK via MEK. As shown in Figure 4(A), the pre-treatment of cells with PD98059 effectively blocked the upward shift (phosphorylation) of the GATA-4 band induced by ET-1. Further, EMSA experiments showed that PD98059 abolished the upward shift induced by ET-1 (Figure 4B). Densitometry analysis revealed that ET-1-induced increase in the GATA DNA-binding activity was also blocked by PD98059 (Figure 4C).

Recently, Morimoto et al. [29] reported that PE induced the MAPK-dependent phosphorylation of GATA-4 in neonatal rat

Figure 4 Role of a MEK-MAPK pathway in GATA-4 phosphorylation

(A) HL-1 cells were pretreated with 30 μ M PD98059 (PD) for 30 min then treated with ET-1 for 10 min. Nuclear extracts were prepared and subjected to SDS/PAGE and Western-blot analysis using the antibody for GATA-4. (B) Nuclear extracts were subjected to an EMSA using the protocol described by Morimoto et al. [29]. (C) The bar graph indicates means $\pm$ S.E.M. of the intensity of the GATA-4 bands as determined by densitometry. The asterisk (*) denotes the values that were significantly different from each other at $P < 0.05$. PD98059 was dissolved in DMSO and an equal volume of DMSO (0.2% final concn.) alone had no effect.

ventricular myocytes. To determine if PE also phosphorylates GATA-4 in HL-1 cells, cells were treated with ET-1 (30 nM) or PE (10 or 100 μ M). In contrast with the ET-1 treatment, which caused a rapid phosphorylation of GATA-4, the treatment of HL-1 cells with PE for 3, 10 or 30 min failed to elicit GATA-4 phosphorylation (Figure 5A). Results of these EMSA experiments were confirmed by Western-blot analysis, as neither 10 μ M PE (Figure 5B) nor 100 μ M PE (results not shown) caused the upward shift of GATA-4 band. Since both ET-1 and PE are known activators of MAPK in neonatal ventricular myocytes [35], these results raised the question of whether MAPK activation alone may not be sufficient to elicit an early phase GATA-4 phosphorylation. To address this issue, the HL-1 cells were treated with PMA, another known activator of MAPK in neonatal ventricular myocytes [35] and in HL-1 cells [38]. An EMSA (Figure 6A) and Western-blot analysis (Figure 6B) showed that, similarly to ET-1, PMA caused the rapid phosphorylation of GATA-4 in HL-1 cells. By examining the abilities of ET-1, PE and PMA to activate p44/42 MAPK, we found that ET-1 and PMA were much stronger stimuli compared with PE (Figure 7A). Furthermore, H_2O_2 , which only weakly activated MAPK (Figure 7A), did not cause a GATA-4 phosphorylation (Figure 7B). These results suggest that, in

Figure 5 Effect of PE on GATA-4 phosphorylation

HL-1 cells were treated with ET-1 (30 nM) or PE (10 or 100 μ M) for the times indicated. Nuclear extracts were prepared and subjected to (A) an EMSA [29] and (B) Western-blot analysis.

Figure 6 Effects of PMA on GATA-4 phosphorylation

HL-1 cells were treated with PMA (10 nM) for 10 min. Nuclear extracts were prepared and subjected to (A) an EMSA [32] and (B) Western-blot analysis.

HL-1 cells, ET-1 and PMA activated MAPK strongly enough to phosphorylate GATA-4.

DISCUSSION

We describe here a signalling pathway in cardiac muscle in which ET-1 rapidly phosphorylates GATA-4. ET-1 also activated MEK and p44/42 MAPK, and the ET-1-mediated phosphorylation of GATA-4 was blocked by a MEK inhibitor, indicating that ET-1 activated the MEK-MAPK-GATA-4 pathway.

Important GATA-binding sites have been identified in multiple cardiac-specific transcriptional regulatory regions, and the over-expression of GATA-4 has been shown to transactivate these elements. GATA-4 regulates various genes involved in cardiac hypertrophy, blood-pressure regulation and cell survival. Since

Figure 7 Effects of H₂O₂ on MAPK activation and GATA-4 phosphorylation

(A) HL-1 cells were treated with PE (100 μM) for the time indicated or with ET-1 (30 nM), PMA (10 nM) or H₂O₂ (100 μM) for 5 min. Cell lysates were prepared and the phosphorylation of p44/42 MAPK was monitored by Western-blot analysis using the phospho-specific p44 and p42 MAPK antibody. The membrane was stripped by incubating in 100 mM glycine, pH 2.5, for 1 h and re-blotted with the antibody for p44/42 MAPK proteins. (B) HL-1 cells were treated with ET-1 (30 nM) or H₂O₂ (100 μM) for the times indicated. Nuclear extracts were prepared and subjected to an EMSA [29].

ET-1 has also been shown to regulate these processes, ET-1 signalling for GATA-4 activation may play important roles in cardiac biology.

GATA-1 and GATA-2 have been shown to be phosphorylated in non-cardiac muscle cells [33,39], and GATA-4 contains putative phosphorylation sites [34]. Morimoto et al. [29] recently showed that PE induced a serine phosphorylation of GATA-4. Consistent with these findings, our experiments using λPPase showed that ET-1 induced phosphorylation of GATA-4. Since λPPase is capable of dephosphorylating serine, threonine and tyrosine residues, these experiments did not determine which residues were phosphorylated by ET-1. The treatment of cells with ET-1 induced the activation of MEK and p44/42 MAPK with the time course of activation similar to the phosphorylation of GATA-4. The ET-1-mediated GATA-4 phosphorylation was effectively abolished by a specific inhibitor of MEK, PD98059, indicating a signal-transduction pathway from MEK–MAPK to GATA-4. The GATA-4 molecule contains a number of putative MAPK phosphorylation sites, including Ser¹⁰⁵, which is within the preferential Pro-Xaa-Ser/Thr-Pro MAPK phosphorylation sequence. We are presently performing mutagenesis studies to identify the site(s) which is (are) phosphorylated in response to ET-1.

Morimoto et al. [29] recently reported that PE activated serine phosphorylation of GATA-4 via MAPK pathway in neonatal rat ventricular myocytes. In contrast with the rapid phosphorylation by ET-1 shown in the present study, a PE-induced phosphorylation occurred after 3 h of treatment. Consistent with the findings by Morimoto et al. [29], PE did not elicit a rapid phosphorylation of GATA-4 in HL-1 cells. The mechanism of the different actions on GATA-4 between ET-1 and PE may involve different extents of MAPK activation by these agents. As shown in Figure 7, the PE-induced activation of MAPK was weaker than that elicited by ET-1. PMA, which caused activation of MAPK similar to that elicited by ET-1, also induced the phosphorylation of GATA-4. In contrast, H₂O₂ which, like

PE, weakly activated MAPK, did not elicit rapid GATA-4 phosphorylation.

In summary, the present study demonstrates that ET-1 rapidly induces the phosphorylation of GATA-4 via the MEK–MAPK pathway in an HL-1 adult atrial-muscle cell line. The present results, along with those from a previous study on neonatal rat ventricular myocytes [29], indicate that the phosphorylation of GATA-4 may regulate the GATA-dependent transcription of various genes involved in cardiac-muscle pathophysiology.

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REFERENCES

- Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988) A novel potent vasoconstrictor peptide producing vascular endothelial cells. *Nature (London)* **332**, 411–415.
- Fukuda, Y., Hirata, Y., Taketani, S., Kojima, T., Oikawa, S., Nakazato, H. and Kobayashi, Y. (1989) Endothelin stimulates accumulations of cellular atrial natriuretic peptide and its messenger RNA in rat cardiocytes. *Biochem. Biophys. Res. Commun.* **164**, 1431–1436.
- Suzuki, E., Hirata, Y., Kohmoto, O., Sugimoto, T., Hayakawa, H., Matsuoka, H., Sugimoto, T., Kojima, M., Kangawa, K., Minamino, N. and Matsuo, H. (1992) Cellular mechanisms for synthesis and secretion of atrial natriuretic peptide and brain natriuretic peptide in cultured rat atrial cells. *Circ. Res.* **71**, 1039–1048.
- Bruneau, B. G., Piazza, L. A. and De Bold, A. J. (1997) BNP gene expression is specifically modulated by stretch and ET-1 in a new model of isolated rat atria. *Am. J. Physiol.* **273**, H2678–H2686.
- Shubel, H. E., McDonough, P. M., Harris, A. N., Knowlton, K. U., Glembocki, C. C., Brown, J. H. and Chien, K. R. (1990) Endothelin induction of inositol phospholipid hydrolysis, sarcomere assembly, and cardiac gene expression in ventricular myocytes. A paracrine mechanism for myocardial cell hypertrophy. *J. Biol. Chem.* **265**, 20555–20562.
- Ito, H., Hirata, Y., Hiroe, M., Tsujino, M., Adachi, S., Takamoto, T., Nitta, M., Taniguchi, K. and Marumo, F. (1991) Endothelin-1 induces hypertrophy with enhanced expression of muscle-specific genes in cultured neonatal rat cardiomyocytes. *Circ. Res.* **69**, 209–215.
- Yamazaki, T., Komuro, I., Kudoh, S., Zou, Y., Shiojima, I., Hiroi, Y., Mizuno, T., Maemura, K., Kurihara, H., Aikawa, R., Takano, H. and Yazaki, Y. (1996) Endothelin-1 is involved in mechanical stress-induced cardiomyocyte hypertrophy. *J. Biol. Chem.* **271**, 3221–3228.
- Ito, H., Hiroe, M., Hirata, Y., Fujisaki, H., Adachi, S., Akimoto, H., Ohta, Y. and Marumo, F. (1994) Endothelin ETA receptor antagonist blocks cardiac hypertrophy provoked by hemodynamic overload. *Circulation* **89**, 2198–2203.
- Kaddoura, S., Firth, J. D., Boheler, K. R., Sugden, P. H. and Poole-Wilson, P. A. (1996) Endothelin-1 is involved in norepinephrine-induced ventricular hypertrophy in vivo. Acute effects of bosentan, an orally active, mixed endothelin ETA and ETB receptor antagonist. *Circulation* **93**, 2068–2079.
- Wang, P., Gallagher, K. P., Downey, J. M. and Cohen, M. V. (1996) Pretreatment with endothelin-1 mimics ischemic preconditioning against infarction in isolated rabbit heart. *J. Mol. Cell. Cardiol.* **28**, 579–588.
- Bugge, E. and Ytrehus, K. (1996) Endothelin-1 can reduce infarct size through protein kinase C and KATP channels in the isolated rat heart. *Cardiovasc. Res.* **32**, 920–929.
- Shichiri, M., Yokodura, M., Marumo, F. and Hirata, Y. (2000) Endothelin-1 inhibits apoptosis of vascular smooth muscle cells induced by nitric oxide and serum deprivation via MAP kinase pathway. *Arterioscler. Thromb. Vasc. Biol.* **20**, 989–997.
- Shichiri, M., Kato, H., Marumo, F. and Hirata, Y. (1997) Endothelin-1 as an autocrine, paracrine apoptosis survival factor for endothelial cells. *Hypertension* **30**, 1198–1203.
- Suzuki, T. and Miyauchi, T. (2001) A novel pharmacological action of ET-1 to prevent the cytotoxicity of doxorubicin in cardiomyocytes. *Am. J. Physiol.* **280**, R1399–R1406.
- Molkentin, J. D., Kalvakolanu, D. V. and Markham, B. E. (1994) Transcription factor GATA-4 regulates cardiac muscle-specific expression of the α-myosin heavy-chain gene. *Mol. Cell. Biol.* **14**, 4947–4957.
- Rindt, H., Gulick, J., Knotts, S., Neumann, J. and Robbins, J. (1993) *In vivo* analysis of the murine β-myosin heavy chain gene promoter. *J. Biol. Chem.* **268**, 5332–5338.
- Kurabayashi, M., Komuro, I., Shibasaki, Y., Tsuchimochi, H., Takaku, F. and Yazaki, Y. (1990) Functional identification of the transcriptional regulatory elements within the promoter region of the human ventricular myosin alkali light chain gene. *J. Biol. Chem.* **265**, 19271–19278.

- 18 Cheng, G., Hagen, T. P., Dawson, M. L., Barnes, K. V. and Menick, D. R. (1999) The role of GATA, CArG, E-box, and a novel element in the regulation of cardiac expression of the Na⁺–Ca²⁺ exchanger gene. *J. Biol. Chem.* **274**, 12819–12826
- 19 Nicholas, S. B. and Philipson, K. D. (1999) Cardiac expression of the Na⁺/Ca²⁺ exchanger NCX1 is GATA factor dependent. *Am. J. Physiol.* **277**, H324–H330
- 20 Herzig, T. C., Jobe, S. M., Aoki, H., Molkentin, J. D., Cowley Jr, A. W., Izumo, S. and Markham, B. E. (1997) Angiotensin II type_{1a} receptor gene expression in the heart: AP-1 and GATA-4 participate in the response to pressure overload. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 7543–7548
- 21 Lee, Y., Shiomi, T., Kasahara, H., Jobe, S. M., Wiese, R. J., Markham, B. E. and Izumo, S. (1998) The cardiac tissue-restricted homeobox protein Csx/Nkx2.5 physically associates with the zinc finger protein GATA4 and cooperatively activates atrial natriuretic factor gene expression. *Mol. Cell. Biol.* **18**, 3120–3129
- 22 Thuerlauf, D. J., Hanford, D. S. and Glembotski, C. C. (1994) Regulation of rat brain natriuretic peptide transcription. A potential role for GATA-related transcription factors in myocardial cell gene expression. *J. Biol. Chem.* **269**, 17772–17775
- 23 Gregory, T., Yu, C., Ma, A., Orkin, S. H., Blobel, G. A. and Weiss, M. J. (1999) GATA-1 and erythropoietin cooperate to promote erythroid cell survival by regulating bcl-x_L expression. *Blood* **94**, 87–96
- 24 Keinänen, R., Vartiainen, N. and Koistinen, J. (1999) Molecular cloning and characterization of the rat inducible nitric oxide synthase (iNOS) gene. *Gene* **234**, 297–305
- 25 Zhang, R., Min, W. and Sessa, W. C. (1995) Functional analysis of the human endothelial nitric oxide synthase promoter. Sp1 and GATA factors are necessary for basal transcription in endothelial cells. *J. Biol. Chem.* **270**, 15320–15326
- 26 O'Prey, J., Ramsay, S., Chambers, I. and Harrison, P. R. (1993) Transcriptional up-regulation of the mouse cytosolic glutathione peroxidase gene in erythroid cells is due to a tissue-specific 3' enhancer containing functionally important CACC/GT motifs and binding sites for GATA and Ets transcription factors. *Mol. Cell. Biol.* **13**, 6290–6303
- 27 Molkentin, J. D., Lu, J. R., Antos, C. L., Markham, B., Richardson, J., Robbins, J., Grant, S. R. and Olson, E. N. (1998) A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell* **93**, 215–228
- 28 Sussman, M. A., Lim, H. W., Gude, N., Taigen, T., Olson, E. N., Robbins, J., Colbert, M. C., Gualberto, A., Wiecek, D. F. and Molkentin, J. D. (1998) Prevention of cardiac hypertrophy in mice by calcineurin inhibition. *Science* **281**, 1690–1693
- 29 Morimoto, T., Hasegawa, K., Kaburagi, S., Kakita, T., Wada, H., Yanazume, T. and Sasayama, S. (2000) Phosphorylation of GATA-4 is involved in α_1 -adrenergic agonist-responsive transcription of the endothelin-1 gene in cardiac myocytes. *J. Biol. Chem.* **275**, 13721–13726
- 30 Claycomb, W. C., Lanson, N. A., Stallworth, B. S., Egeland, D. B., Delcarpio, J. B., Bahinski, A. and Izzo, N. J. (1998) HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 2979–2984
- 31 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
- 32 Suzuki, Y. J., Mizuno, M. and Packer, L. (1994) Signal transduction for NF- κ B activation: proposed location of antioxidant-inhibitable step. *J. Immunol.* **153**, 5008–5015
- 33 Towatari, M., May, G. E., Marais, R., Perkins, G. R., Marshall, C. J., Cowley, S. and Enver, T. (1995) Regulation of GATA-2 phosphorylation by mitogen-activated protein kinase and interleukin-3. *J. Biol. Chem.* **270**, 4101–4107
- 34 Arceci, R. J., King, A. A., Simon, M. C., Orkin, S. H. and Wilson, D. B. (1993) Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. *Mol. Cell. Biol.* **13**, 2235–2246
- 35 Bogoyevitch, M. A., Glennon, P. E. and Sugden, P. H. (1993) Endothelin-1, phorbol esters and phenylephrine stimulate MAP kinase activation in ventricular cardiomyocytes. *FEBS Lett.* **317**, 271–275
- 36 Bogoyevitch, M. A., Glennon, P. E., Andersson, M. B., Clerk, A., Lazou, A., Marshall, C. J., Parker, P. J. and Sugden, P. H. (1994) Endothelin-1 and fibroblast growth factors stimulate the mitogen-activated protein kinase signaling cascade in cardiac myocytes. The potential role of the cascade in the integration of two signaling pathways leading to myocyte hypertrophy. *J. Biol. Chem.* **269**, 1110–1119
- 37 Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T. and Saltiel, A. R. (1995) PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase *in vitro* and *in vivo*. *J. Biol. Chem.* **270**, 27489–27494
- 38 McWhinney, C. D., Hansen, C. and Robishaw, J. D. (2000) α_1 -Adrenergic signaling in a cardiac murine atrial myocyte (HL-1) cell line. *Mol. Cell. Biochem.* **214**, 111–119
- 39 Crossley, M. and Orkin, S. H. (1994) Phosphorylation of the erythroid transcription factor GATA-1. *J. Biol. Chem.* **269**, 16589–16596

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