Distinct Roles of Mitogen-activated Protein Kinase Pathways in GATA-4 Transcription Factor-mediated Regulation of B-type Natriuretic Peptide Gene

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The expression of cardiac hormones, atrial natriuretic peptide and B-type natriuretic peptide, is induced by cardiac wall stretch and responds to various hypertrophic agonists such as endothelin-1. In cardiac myocytes, endothelin-1 induces GATA-4 binding to the B-type natriuretic peptide gene, but the signaling pathways involved in endothelin-1-induced GATA-4 activation are unknown. Mitogen-activated protein kinase pathways are stimulated in response to various extracellular stimuli, and they modulate the function of several transcription activators. Here we show that inhibition of p38 kinase with SB203580 inhibited endothelin-1-induced GATA-4 binding to B-type natriuretic peptide gene and serine phosphorylation of GATA-4. Inhibition of extracellular signal-regulated protein kinase with MEK1 inhibitor PD98059 reduced basal and p38-induced GATA-4 binding activity, but it had no significant effect on endothelin-1-induced GATA-4 binding activity. Overexpression of p38 kinase pathway, but not extracellular signal-regulated kinase or c-Jun N-terminal protein kinase, activated GATA-4 binding to B-type natriuretic peptide gene and induced rat B-type natriuretic peptide promoter activity via proximal GATA binding sites. In conclusion, these findings demonstrate that activation of p38 kinase is necessary for hypertrophic agonist-induced GATA-4 binding to B-type natriuretic peptide gene and sufficient for GATA-dependent B-type natriuretic peptide gene expression.

Cardiac hypertrophy is a physiologic process adapting heart to increased hemodynamic workload. In early stages, hypertrophy is a compensatory mechanism, but if prolonged, it leads to pathologic myocyte hypertrophy characterized by increase in cell size, enhanced sarcromeric organization, and induction of the fetal gene program (1). Myocyte hypertrophy can be induced by pressure or volume overload and by different neurohumoral factors, including endothelin-1 (ET-1),1 angiotensin II, and α1-adrenergic agonists (2). At the genetic level, activation of a program of immediate early genes, such as c-fos, c-jun, and c-myc, is the first detectable response to hypertrophic stimuli. This is followed by alterations in contractile protein compositions, including reactivation of β-myosin heavy chain, skeletal α-actin, and myosin light chain-2 genes (3, 4). Hypertrophy also results in induction of noncontractile protein genes such as atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP), which are known members of the mammalian cardiac natriuretic peptide system (5–7). ANP and BNP defend against increased hemodynamic load by decreasing blood pressure, regulating fluid homeostasis by increasing salt and water excretion, and regulating several hormones, such as angiotensin II, ET-1, and vasopressin (5, 8). In the normal adult heart, ANP is mainly synthesized in the atria, whereas BNP is abundant in cardiac atria and ventricles where its gene expression is rapidly up-regulated in response to cardiac wall stretch. Indeed, the induction of BNP gene expression is one of the earliest myocyte-specific markers of hemodynamic stress-induced hypertrophic response (5, 9–11).

Several signaling pathways, including intracellular calcium, protein kinase C, nonreceptor protein tyrosine kinases, and calcineurin are implicated in the initiation and maintenance of myocyte hypertrophy (12–14). There is also considerable evidence that activation of the mitogen-activated protein kinase (MAPK) cascades can lead to a hypertrophic response in myocytes. MAPK pathways can be divided into three subclasses; the extracellular signal-regulated protein kinase (ERK) pathway, the c-Jun N-terminal protein kinase (JNK) pathway, and the p38 kinase pathway (15). Each MAPK pathway consists of three or more levels and multiple isoforms, giving the signaling system potential to distinguish different extracellular stimuli. The MAPKs, ERK, JNK, and p38 MAPK, have been shown to be inducible by a variety of hypertrophic stimuli, including mechanical stretch, ET-1, and other GPCR (G protein-coupled receptor) agonists (15, 16). Cardiac-restricted MEK1 (an upstream kinase of ERK pathway) overexpression in vivo has been shown to lead to concentric hypertrophy in transgenic mice (17), and most studies have found that ERK is associated with ET-1-induced cardiomyocyte hypertrophy (15, 16, 18, 19).

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1The abbreviations used are: ET-1, endothelin-1; MAPK, mitogen-activated protein kinase; ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal protein kinase; GPCR, G protein-coupled receptor; MEF2, myocyte enhancer factor-2; ATF2, activating transcription factor-2; αMHC, α-myosin heavy chain; cTnC, cardiac troponin C; MBP, myelin basic protein; CSFM, complete serum-free medium; Oct-1, octamer-1; EMSA, electrophoretic mobility shift assay; FOG-2, friend of GATA-2; NF-AT3, nuclear factor of activated T lymphocytes 3; MEK1, MAPK/ERK kinase 1; MKP-1, MAPK phosphatase-1.
Regulation of GATA-4 Binding by p38 MAPK

The p38 MAPK family consists of six isoforms, of which p38α and p38δ are the predominant isoforms present in the heart (20). Activation of p38 has also been shown to lead to cardiomyocyte hypertrophy in vitro (21, 22). Activated MAPKs phosphorylate a number of substrates, including nuclear transcription factors such as myocyte enhancer factor-2 (MEF2), activating transcription factor-2 (ATF2), ATF6, and downstream kinases such as p38-regulated/activated kinase (23–26). However, the precise roles of different MAPKs and their downstream targets in hypertrophic signaling are not known.

The GATA family of transcriptional factors contains six mammalian members (reviewed in refs. 27, 28). GATA proteins, which contain a DNA binding domain composed of two evolutionarily conserved zinc fingers (N- and C-terminal), bind to consensus sequence 5’- (AT)GATA(A/G)-3’ and its variants (29). Cardiac transcription factor GATA-4 has been shown to play a nonredundant role for the cardiac muscle development during embryogenesis (30, 31). In postnatal cardiac myocytes, it has been reported that the expression of several cardiac genes, including α-myosin heavy chain (αMHC) and cardiac troponin C (cTnC), is directed into cardiac myocytes via GATA-4 binding elements on the promoter region (32, 33).

Interestingly, analysis of the ANP and BNP promoter regions has also revealed binding sites for GATA-4 (34, 35). There are data demonstrating possible involvement of GATA-4 in the hypertrophic signaling in cardiac myocytes (36–40). Recently, we have reported that pressure overload of rat heart activates GATA-4 and that the activation is mediated by ET-1 (41). In the present study, to identify molecular mechanisms mediating ET-1-induced BNP gene expression and activation of GATA-4, we focused on the role of MAPK signaling in cultured rat neonatal cardiac myocytes.

EXPERIMENTAL PROCEDURES

Chemicals—A PhosphoPlus p38 MAPK antibody kit was purchased from New England BioLabs Ltd. (Hitchin, Hertfordshire, UK). GATA-4, GATA-5, and GATA-6 polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphoserine antibody and anti-phosphothreonine antibody and body were from Zymed Laboratories Inc. (San Francisco, CA). Anti-phosphothreonine antibodies used were obtained from Alexis Corp. (San Diego, CA), Zymed Laboratories Inc. (San Francisco, CA) and Santa Cruz Biotechnology (Santa Cruz, CA). Bovine myelin basic protein (MBP) was purchased from Upstate Biotechnology (Lake Placid, NY). (γ-32P)ATP, [α-32P]dCTP, [3H]eucine, ECL plus Western Blotting Substrate, Hyperfilm MP and Hyperfilm MP/II (ECL) kinase enzyme assay system were purchased from Amersham Biosciences, Inc. (Bucks, UK). A p38 in vivo kinase assay kit (Mercury) was from CLONTECH (Palo Alto, CA). Luciferase and β-galactosidase reagents were purchased from Promega (Madison, WI), and FuGENE 6 transfection reagent from Roche Molecular Biochemicals (Mannheim, Germany).

Cell Culture and Transfection—Cells were prepared from 2- to 4-day-old Sprague-Dawley rats (42). Cells were plated at the density of 2 × 10^6/cm^2 onto Falcon wells from 15 to 60 mm in diameter. Following a 16-h incubation, myocytes were subjected to liposome-mediated transfection with FuGENE 6 for 6 h. To control the transfection efficiency, reporter plasmids were cotransfected with pRSV (Rous sarcoma virus) promoter driven reporter plasmids were used in other experiments. After transfection, cells were washed twice with Dulbecco’s modified Eagle’s medium and cultured in complete serum-free medium (CSFM). When appropriate, ET-1 100 nm (Sigma Chemical Co.) was added to control medium on a third day in culture. Previously, this concentration of ET-1 has been shown to induce cardiomyocyte hypertrophy in vivo (16, 18, 19). On the fourth day, myocytes were lysed, and luciferase and β-galactosidase activity assays were performed using Luminoscan (Labsystems). All experiments were repeated at least three times.

ROS-1 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Cells were plated onto plates 100 mm in diameter and transfected with 1 μg of GATA-4 expression plasmid and 0.1 μg of expression plasmids for p38α, MERK, JNK1, and pUC19 using FuGENE 6 reagent. Forty-eight hours after transfection, cells were harvested and subjected to nuclear protein extraction. We thank Dr. Jukka Hakkola (Department of Pharmacology and Toxicology, University of Oulu) for the gift of COS-1 cells and for helpful advice on the project.

Kinase Assays—After treatment with appropriate agonists, myocytes (5 × 10^6) were washed with phosphate-buffered saline at room temperature and collected by scraping into 500 μl of lysis buffer, which consisted of 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, 1 μg/ml peptatin, 1 μg/ml aprotinin, 1 mg/ml phenylmethylsulfonyl fluoride, 2 mM DTT, and 50 mM NaF. Extracts were further lysed with sonication, and supernatant was collected after centrifugation. Western blot assays for p38 were performed using the PhosphoPlus p38 MAPK antibody kit. Samples (20–40 μg) were loaded onto SDS-PAGE and transferred to nitrocellulose filters. The membranes were blocked in 5% nonfat milk and then incubated with indicated primary antibody overnight at 4°C. Phospho-p38 and total p38 were detected by enhanced chemiluminescence. For a second Western blot, the membrane was stripped for 30 min at 60°C in stripping buffer (62.5 mM Tris (pH 6.8), 2% SDS, 100 mM β-mercaptoethanol). For immunocomplex kinase assay, endogenous p38 was immunoprecipitated with specific antibody at 4°C overnight, followed by protein G-Sepharose precipitation. Immunoprecipitates were washed twice with lysis buffer containing 25 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 25 mM β-glycerophosphate, 25 mM NaF, and 1% Triton X-100. Lysates were once more washed with kinase buffer containing 25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, 1.0 mM Na3VO4, and 10 mM MgCl2. The activity of the immunocomplex was assayed at 30°C for 15 min in 30 μl of kinase buffer in the presence of 2 μCi of [γ-32P]ATP and 20 μg of MBP as substrate. The reactions were terminated, and the reaction contents were electrophoresed on 15% SDS-polyacrylamide gels followed by PhosphorImager analysis to determine the phosphorylation level of MBP. The effect of p38 inhibitor SB203580 on p38 activity was measured by in vivo kinase assay.

For ERK assays, cells were collected with buffer containing 10 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EGTA, 2 mM DTT, 1 mM Na3VO4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 μg/ml pepstatin, and 5 mM benzamidine. Extracts were sonicated, and the supernatant was collected after centrifugation. 15 μl of protein extract was incubated at 30°C for 15 min with 10 μl of substrate buffer containing specific ERK-substrate peptide in the presence of 1 μCi of [γ-32P]ATP. Each reaction was terminated and blotted onto separate peptide binding proteins using Zymed antibody kit. GATA-4 antibody was first bound to Nitrocellulose membrane, and then washing was repeated with 75 mM orthophosphoric acid repeatedly. Incorporated radioactivity was measured with a scintillation counter (Rackbeta II, LKB Wallac).

Nuclear Protein Extraction and Electrophoretic Mobility Shift Assay—Nuclear extracts from myocytes were prepared as described previously (43). Protein concentration from each sample was determined by using Bradford assay (44) (Bio-Rad Laboratories). Double-stranded oligonucleotides corresponding to GATA binding sites were inserted into the pFAST vector, and Zyma rat BNP promoter was used for analysis of GATA DNA binding activity and a previously described oligonucleotide for measurement of Octamer-1 (Oct-1) DNA binding activity (45). Both probes were sticky-end-labeled with [α-32P]dCTP by Klenow enzyme. For each reaction mixture (20 μl) 6 μg of nuclear protein and 2 μg of poly(dI-dC) was used in a buffer containing 10 mM HEPES (pH 7.9), 1 mM MgCl2, 50 mM KCl, 1 mM DTT, 1 mM EDTA, 10% glycerol, 0.025% Nonidet P-40, 0.25 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of leupeptin, pepstatin, and aprotonin. Protein phosphatase inhibitors NaF (50 mM) and Na3VO4 (1 mM) were also added to the mixture. Reaction mixtures were incubated with a labeled probe for 20 min followed by nondenaturating gel-electrophoresis on 5% polyacrylamide gel. Subsequently, gels were dried and exposed in a PhosphorImager screen and analyzed with ImageQuaNT (Molecular Dynamics). To confirm DNA sequence specificity of the protein-DNA complex formation, competition experiments with 10-, 50-, and 100-molar excesses of nonradiolabeled oligonucleotides with intact or mutated binding sites were performed. For competition and supershift experiments appropriate oligodeoxynucleotides or antibodies were added to the reaction mixture 20 min before addition of nuclear extract. GATA-4 Phosphorylation Analysis—To determine the GATA-4 phosphorylation state, GATA-4 was immunoprecipitated using a Seize X Protein G immunoprecipitation kit. GATA-4 antibody was first bound and immobilized to Protein G according to the manufacturer’s instructions. Nuclear extracts were then applied to immobilized antibody support, unbound proteins were washed out, and finally GATA-4 protein was eluted. Samples were loaded onto SDS-PAGE and subjected to...
FIG. 1. Activation of p38 MAPK by ET-1. A, effect of ET-1 on activation of p38 MAPK. Cardiac myocytes were treated with ET-1 at the concentration of 100 nM for 15 min at 37 °C and 5% CO₂. After ET-1 exposure, cells were washed and lysed. Cell lysis was centrifuged, and supernatants were subjected to SDS-PAGE and immunoblotted with antibody specific for phospho-p38 (Thr180/Tyr182) to detect the activated p38 kinase. To quantitate the total amount of p38 kinase protein, samples were immunoblotted with antibody specific for p38 kinase. Bars represent two separate experiments done in duplicates and are expressed as a -fold change versus untreated control. *, p < 0.05 compared with untreated control.

B, effect of ET-1 on p38 kinase activity. Cardiac myocytes were cultured in culture plates 50 mm in diameter and treated with 100 nM ET-1 for 5–60 min at 37 °C and 5% CO₂, followed by washing and lysing of the cells. Subsequently, cell lysates were centrifuged and protein concentration of supernatant was measured. 100 μg of cellular protein was subjected to immunoprecipitation with antibody specific for p38 MAPK. After addition of 2 μCi of [γ-³²P]ATP, immunoprecipitated p38 was incubated with 20 μg of MBP in reaction buffer at 30 °C for 15 min. After termination of reaction, proteins were resolved on SDS-PAGE gels, followed by autoradiography and densitometric scanning for incorporated radioactivity.

C, effect of ET-1 on p38 kinase substrate (ATF2) transactivation. Cardiac myocytes were cultured on 24-well cell culture plates and cotransfected with 0.1 μg of a p38 kinase pathway-specific transactivator vector fused to the tetracycline repressor protein (pTetR-ATF2), 0.9 μg of reporter vector containing the luciferase gene under the control of a tetracycline-responsive element and 0.5 μg of RSV-promoter driven β-galactosidase plasmid. After transfection, cells were washed and incubated overnight with complete serum-free medium. The next day, ET-1, p38...
Western blotting. The primary antibody indicated was incubated at 4 °C overnight. Antibody binding was detected with a peroxidase-conjugated goat anti-rabbit or bovine anti-goat IgG and enhanced chemiluminescence.

Plasmids—Rat BNP promoter fragment was generated by PCR, with rat genomic EMBL3-α-cleaved as a template and using the following primers: sense 5′-GGATTTGAACCTAGG-3′ with Kpn1, MluI-linker, antisense 5′-CACCTGTCTCGAAACG-3′ with BamHI-linker. Subsequently, PCR product was digested with Kpn1 and BamHI and cloned to the Kpn1-BglII site of pGL3-Basic plasmid (Promega) resulting in a (∆5kbp/+4) BNP promoter construct. (∆5kbp/+4) BNP-pGL3 construct was used to produce a (∆534/+4) BNP-pGL3 by nested deletion (Amersham Biosciences, Inc.). Site-directed mutations to two adjacent (−91 and −80 bp) GATA sites of (∆534/+4) BNP-pGL3 were prepared (Stratagene), and the resulting construct is referred to here as cent (91 and 80 bp) GATA sites of (∆534/+4) BNP-pGL3. The kinetics of p38 activation was measured by immunocomplex kinase assay. Endogenous p38 was immunoprecipitated with anti-p38 antibody, and its activity was measured using MBP as a substrate. As shown in Fig. 1B, ET-1 induced a rapid increase in p38 activity, which was maximal at 15–20 min. The pyridinyl imidazole SB203580 has been shown to be a potent inhibitor of p38α and p38β MAPKs (48). To verify the inhibition of p38 by SB203580 in cardiac myocytes, we applied in vivo kinase assay, which uses ATF2 as a substrate. Treatment with ET-1 (100 nM) for 24 h increased p38 activity by 3.4-fold, and activity was totally inhibited by p38 inhibitor SB203580, which also decreased basal activity of p38 MAPK by 50% (Fig. 1C). In contrast, treatment of myocytes with a potent MEK1 inhibitor PD98059 increased basal p38 activity but had no effect on ET-1-induced p38 activity (Fig. 1C).

As noted previously, ERK is activated by several GPCR agonists in cardiac myocytes (49). To examine the regulation of ERK by ET-1, we applied an assay, which measures transfer of a phosphate group to a peptide highly selective for ERK (p42/44 MAPK). As reported previously (12, 16), ET-1 at the concentration of 100 nM was a strong activator of p42/44. This response was maximal at 5 min and declined to almost basal level within 35 min (Fig. 2). MEK1 inhibitor PD98059 (20 μM) was sufficient to abolish ET-1-induced ERK activation by 80% measured at 5 min (data not shown).

**Effect of ERK and p38 Inhibition on ET-1-induced Protein Synthesis**—Activation of de novo protein synthesis, a major

cytes (referred after this as myocytes) by various extracellular stimuli such as pro-inflammatory cytokines interleukin-1α and tumor necrosis factor-α (47). It has also been shown that hypertrophic agonists ET-1 and phenylephrine (PE) stimulate p38 activity in myocytes (18). To establish the activation of p38 by ET-1 in the present study, we used an antibody selective to a multiply phosphorylated form of p38 for Western blot analysis. Phosphorylation of p38 was imminent and peaked at 15 min (Fig. 1A). The kinetics of p38 activation was measured by immunocomplex kinase assay. Endogenous p38 was immunoprecipitated with anti-p38 antibody, and its activity was measured using MBP as a substrate. As shown in Fig. 1B, ET-1 induced a rapid increase in p38 activity, which was maximal at 15–20 min. The pyridinyl imidazole SB203580 has been shown to be a potent inhibitor of p38α and p38β MAPKs (48). To verify the inhibition of p38 by SB203580 in cardiac myocytes, we applied in vivo kinase assay, which uses ATF2 as a substrate. Treatment with ET-1 (100 nM) for 24 h increased p38 activity by 3.4-fold, and activity was totally inhibited by p38 inhibitor SB203580, which also decreased basal activity of p38 MAPK by 50% (Fig. 1C). In contrast, treatment of myocytes with a potent MEK1 inhibitor PD98059 increased basal p38 activity but had no effect on ET-1-induced p38 activity (Fig. 1C).

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**Effect of ERK and p38 Inhibition on ET-1-induced Protein Synthesis**—Activation of de novo protein synthesis, a major

inhibitor SB203580, and ERK inhibitor PD98059 (final concentrations of 100 nM, 20 μM, and 20 μM, respectively) were added, and cells were incubated with or without 2 μg/ml tetracycline hydrochloride at 37 °C and 5% CO₂ for 24 h, followed by luciferase and β-galactosidase assays. Reporter activity obtained in the presence of tetracycline was subtracted from luciferase activity of cells without tetracycline treatment to confirm the specificity of the TetR-ATF2-dependent transactivation. Each bar represents results of 4–6 separate experiments obtained from three independent cell cultures. *, p < 0.05 compared with untreated control cells. ***, p < 0.01 compared with untreated control cells. ###, p < 0.001 compared with ET-1 treated cells.
Inhibition of activity was significantly decreased (Fig. 5B). The ERK pathway with PD98059 had no effect on ET-1-induced inhibition of GATA-4 binding was dose-dependent (Fig. 5A). Mutation of either of the GATA binding sites showed that GATA-4 binds equally well to both GATA-6, followed by addition of 32P-labeled double-stranded oligonucleotide corresponding to (Δ-68/97) of rat BNP promoter binding site (Fig. 4B). Mutation of either of the GATA binding sites showed that GATA-4 binds equally well to both sites, but the binding activity is reduced to about a half of that observed with a probe having both sites intact (data not shown). This data agree with previous findings by Thuerauf et al. (50) indicating that at least one of the GATA sites is required to confer full GATA-4-inducible transcription.

We next pretreated the myocytes with SB203580, PD98059, or transfected the cells with the dominant negative form of JNK and then subjected the cells to ET-1 treatment. The induction of GATA-4 binding to BNP gene was completely inhibited by p38 inhibitor SB203580, and, moreover, this inhibition of GATA-4 binding was dose-dependent (Fig. 5A). Inhibition of the ERK pathway with PD98059 had no effect on ET-1-induced increase in GATA-4 DNA binding, but basal GATA-4 binding activity was significantly decreased (Fig. 5B). Inhibition of JNK pathway with dominant negative form of JNK had no effect on basal or ET-1-induced GATA-4 DNA binding (data not shown). The levels of GATA-4 mRNA did not change in neonatal cardiac myocytes treated with ET-1 for 4 h (41) suggesting that the increase in GATA binding activity was due to post-transcriptional mechanisms.

p38 MAPK Increases DNA Binding Activity and Phosphorylation of GATA-4—To further elucidate the role of p38 MAPK in the induction of GATA-4 DNA binding, the p38 protein levels were increased by transfecting the myocytes with a cytomegalovector and the ERK pathway with dominant negative form of JNK had no effect on basal or ET-1-induced GATA-4 DNA binding (data not shown).
lovirus (CMV) promoter-driven plasmid overexpressing p38α. Similarly, ERK and JNK pathways were studied by using CMV promoter-driven plasmids overexpressing MEK1 and MEKK1. Myocytes transfected with pUC-19 were used as control. p38 overexpression substantially evoked GATA-4 binding to BNP gene compared with control plasmid, which was abolished by p38 inhibitor SB203580 (Fig. 6A). ERK inhibition with PD98059 (20 μM) slightly decreased p38-induced GATA-4 binding to BNP gene. MEK1 or MEKK1 overexpression had no effect on GATA-4 DNA binding (Fig. 6A).

It has recently been shown that serine residues of GATA-4 are phosphorylated in response to PE and that the phosphorylation is ERK-dependent (39). We examined whether the p38β-induced increase in GATA-4 DNA binding activity was also due to changes in phosphorylation of GATA-4. Myocytes were transfected with plasmids overexpressing p38α, MEK1, MEKK1, or pUC19 (control). Subsequently, GATA-4 was immunoprecipitated from nuclear extracts, and Western blot analysis was performed. Immunoblotting with GATA-4 antibody showed that GATA-4 protein levels were unaffected (Fig. 6B). Overexpression of MEK1 and p38α exhibited a marked increase in serine phosphorylation of GATA-4, whereas overexpression of JNK pathway (MEKK1) had no effect. p38β-induced serine phosphorylation of GATA-4 was inhibited by p38 inhibitor SB203580 and also by ERK inhibitor PD98059 consistently with the finding that p38α-induced GATA-4 binding was also depressed with PD98059. It is, therefore, likely that various serine residues of GATA-4 are differently phosphorylated by ERK and p38 MAPK. Forced expression of p38, MEK1, or MEKK1 did not induce threonine phosphorylation (five different antibodies used) or tyrosine phosphorylation of GATA-4. These results indicate that in cardiac myocytes p38 MAPK and ERK preferentially activate serine phosphorylation of GATA-4.

MAPK Regulation of GATA-4 DNA Binding in COS-1 Cells—To further investigate the role of MAPKs in the regulation of GATA-4, we used COS-1 cells transiently expressing GATA-4 and cotransfected the cells with plasmids overexpressing p38α, MEK1, or JNK1. Control cells, cotransfected with pUC19, showed modest GATA-4 binding activity to BNP promoter (Fig. 7). p38α overexpression resulted in 4-fold increase in GATA-4 binding activity, whereas MEK1 or JNK1 overexpression had no effect on GATA-4 binding to BNP gene promoter. Oct-1 binding activity was not affected with transient expression of different plasmids.

p38 MAPK Regulation of a GATA-dependent Promoter—Because BNP expression is an important genetic marker of myocyte hypertrophy, we tested whether p38 overexpression would be sufficient to stimulate BNP promoter activity. Myocytes were cotransfected with (Δ-534bp/+4bp) BNP promoter plasmids and p38α expression plasmid or pUC19 plasmid (control). p38α overexpression stimulated 4-fold increase in promoter activity (Fig. 8). The mutation of two proximal GATA binding sites at −91 and −80 bp of (Δ-534bp/+4bp) BNP promoter abolished p38α-induced increase in promoter activity. Cotransfection with a plasmid expressing either MEK1 or MEKK1 induced both the BNP and the mutated constructs similarly (data not shown).

**DISCUSSION**

MAPKs, ERK, JNK, and p38, regulate a broad range of biological functions in response to extracellular stimuli. Each MAPK pathway is a complex formation, which provides multiple alternatives to distinguish between different signals. On the other hand, cross-talk between MAPK pathways is known to exist at several levels, i.e. MEKK1 (an upstream kinase of JNK pathway) activating both ERK and p38 MAPK pathways (21, 51), therefore influencing the interpretation of the results when studying the specific cellular roles of MAPKs. In the present study, we investigated the role of MAPK signaling in hypertrophic gene expression induced by ET-1 in cardiac myo-
cytes. ET-1 rapidly activated p38 MAPK, in agreement with several previous papers suggesting involvement of p38 MAPK in ET-1-induced hypertrophic response (16, 18). We also found that ET-1-induced de novo protein synthesis of neonatal rat ventricular myocytes was inhibited by pharmacological blockade of p38 MAPK (SB203580), but not with blockade of ERK signaling (PD98059). This finding disagrees with the previous results showing that SB203580, which blocks the activity of p38 by binding to the ATP binding site of p38 MAPK (52), had no effect on ET-1-induced protein synthesis or sarcomere organization (19). The reason for these discrepant findings remains to be established but may be related to differences under experimental procedures, such as the duration of experiments and inhibitor concentration.

As reported previously (12, 16), p42/44 MAPK was also rapidly activated by ET-1. Inhibition of ERK pathway with PD98059 has been proposed to inhibit also p38 to some extent (18), but we found no inhibition of ET-1-induced p38 activity by PD98059 at the concentration of 20 μM (Fig. 1C). On the other hand, ERK inhibition induced basal p38 activation about 2-fold, but it had no additional effect on ET-1-induced p38 activity. Previously, a higher dose of PD98059 (50 μM) has been shown to increase basal levels of phosphorylated p38 MAPK (18). Furthermore, in a recent study constitutive active MEK1 (an upstream kinase of ERK pathway) was shown to inhibit p38 MAPK activity and p38-induced phosphorylation of TATA-binding protein (53). This inhibitory response was suggested to be mediated by MAPK phosphatase-1 (MKP-1), which has been shown to block ET-1-induced activation of the MAPKs (53, 54).

Studies using MEK1/2 inhibitor or overexpression of dominant negative form of MEK1 have shown that ERK is necessary for...
the stimulation of \textit{MKP-1} mRNA expression \cite{55}. Therefore, blockade of ERK for 24 h in the present experiments is likely to inhibit \textit{MKP-1} expression and thus result in increased p38 activity. On the other hand, hypertrophic agonists have been shown to activate \textit{MKP-1} through mechanisms involving Ca$^{2+}$, protein kinase C, and diacylglycerol \cite{56, 57}. Therefore, lack of additive effect with PD on ET-1-induced p38 activity is likely to result of ET-1-induced activation of \textit{MKP-1}. Another mechanism involved may be the substrate specificity of \textit{MKP-1}, because it has been shown to preferentially block the activation of p38 MAPK \cite{58}.

A large number of transcription factors, including GATA-1–4 \cite{39, 59–61} have been shown to exist within cells as phosphoproteins. The GATA-4 protein has at least seven potential sites for serine phosphorylation by MAPKs, and the phosphorylation was increased after $\alpha$-agonist stimulation via ERK pathway \cite{39}. A novel finding in our studies is the differential regulation of GATA-4 binding activity by MAPKs. The present results indicate that p38 MAPK and ERK are involved in the regulation of GATA-4 binding activity. Blockade of ERK pathway, although increasing p38 MAPK activity, lead to decreased phosphorylation of serine residues in GATA-4 and decreased basal binding activity, but it had no effect on ET-1-induced increase in GATA-4 DNA binding. ERK overexpression lead to phosphorylation of the serine residues of GATA-4 protein, but it was not sufficient to increase GATA-4 binding to BNP gene. Blockade of p38 pathway similarly decreased phosphorylation of serine residues in GATA-4 and, in contrast to ERK inhibition, totally abolished ET-1-induced GATA-4 binding to BNP gene. It is remarkable that p38 overexpression not only phosphorylated serine residues in GATA-4 protein, but also increased GATA-4 binding to BNP promoter. Interestingly, p38-induced increase, but not ET-1-induced increase, in GATA-4 DNA binding activity was partially inhibited by MEK1 inhibitor PD98059. This is likely to result from other mechanisms induced by ET-1, such as other kinases or transcription factors, which can compensate for the inhibited ERK pathway. Studies on MAPKs in COS-1 cells transiently expressing GATA-4 further supported the essential role of p38 MAPK in the regulation of GATA-4 DNA binding activity. Our findings together indicate the preferential but distinct roles of ERK and p38 MAPK signaling pathways in regulation of GATA-4 transcription factor binding activity. The present results show that blockade of p38 MAPK pathway abolishes hypertrophic agonist-induced GATA-4 binding to BNP gene, whereas inhibition of ERK pathway only disrupts GATA-4 binding activity in nonstimulated myocytes.

In addition to the increase in the DNA binding activity, the functional consequences of GATA-4 phosphorylation may include changes in cellular localization and transcriptional activation. To define the role of GATA-4 binding on BNP gene expression, we introduced site-directed mutations to two adjacent GATA-sites at $-91$ and $-80$ bp of the proximal BNP promoter ($\Delta$-534bp/4bp). Previously, these GATA binding sites have been shown to direct cardiac myocyte-specific expression of rat BNP promoter and regulate basal promoter activity \cite{34, 50}. We found that $p38\alpha$ overexpression was potent in activating the proximal BNP promoter, but the mutation of GATA sites abolished $p38$-induced promoter activity. In contrast, overexpression of either MEK1 or MEK1K activated both the proximal BNP promoter and the mutated promoter. These results demonstrate that, in the context of proximal rat BNP promoter, p38- but not ERK-induced transcription is dependent upon a GATA binding site in the promoter.

The precise role of the third member of MAPK family, JNK, in hypertrophic response is even more controversial due to the lack of specific inhibitor for JNK. In cardiac myocytes, a dominant-negative JNK construct has been shown to inhibit PE-induced ANP expression, and some studies also find functional JNK pathway essential for hypertrophic response to ET-1 \cite{19, 51}. In our studies we found that inhibition of the JNK pathway with dominant negative mutant of JNK1 had no effect on basal or hypertrophic agonist-induced GATA-4 DNA binding activity. On the other hand, overexpression of MEK1K, an upstream kinase of JNK, induced proximal BNP promoter activity, but the induction was independent of GATA-4 binding in the promoter.

The mechanisms involved in GATA-4-induced tissue-specific gene expression are not well understood but may involve interactions between GATA-4 and other cell-restricted transcription factors \cite{27, 35, 62}. The ANP promoter is a known downstream target for the cardiac-specific transcription factor GATA-4, and for Ncx-2.5, which bind to adjacent sites in ANP promoter and synergistically activate ANP gene \cite{35, 63}. There is also evidence from an earlier study \cite{64} that MEF2 proteins are recruited by GATA-4 to synergistically activate ANP and $\alpha$MHC genes. Interaction of friend of GATA-2 (FOG-2) with GATA-4 has also been confirmed \cite{65, 66}: FOG-2 repressed expression of several GATA-4-dependent promoters, including ANP, BNP, and cTnC \cite{65, 67}. Ncx-2.5 and NF-AT3 (nuclear factor of activated T lymphocytes), in turn, bind to C-terminal zinc finger of GATA-4 resulting in synergistic transcriptional activation \cite{35, 38}. The mechanisms by which GATA-4 increases or represses the transcriptional activity with its cofactors remain unclear, but site-specific phosphorylation of GATA-4 protein by MAPKs may have an effect on the interactions of GATA-4 with its cofactors in cardiac myocytes. GATA-4 protein harbors a
strong MAPK recognition sequence (Pro-Val-Ser-Pro) at 102–105 residues and multiple Ser-Pro sequences (68). Given that p38-mediated Ser phosphorylation of GATA-4 is followed by increased DNA binding activity, mapping of these Ser phosphorylation sites of GATA-4 will be necessary to fully understand the regulation of GATA-4-mediated gene expression.

The heart adapts to increased demands for cardiac work by increasing muscle mass through the initiation of a hypertrophic response. Hypertrophic stimuli reach the nucleus via multiple signaling pathways within cardiac myocytes and elicit hypertrophic response. Hypertrophic stimuli reach the nucleus via the regulation of GATA-4-mediated gene expression. p38 MAPK has been implicated in multiple signaling pathways within cardiac myocytes and elicit trophic response. Hypertrophic stimuli reach the nucleus via the regulation of GATA-4-mediated gene expression.

p38-mediated Ser phosphorylation of GATA-4 is followed by increased DNA binding activity, mapping of these Ser phosphorylation sites of GATA-4 will be necessary to fully understand the regulation of GATA-4-mediated gene expression.

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Distinct Roles of Mitogen-activated Protein Kinase Pathways in GATA-4
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