Endothelin-1 Specific Activation of B-type Natriuretic Peptide Gene via p38 Mitogen Activated Protein Kinase and Nuclear ETS Factors* 

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SUMMARY

Terminally differentiated cardiac myocytes adapt to mechanical and neurohumoral stress via morphological changes of individual cells accompanied by reactivation of fetal pattern of gene expression. Endothelin-1, a powerful paracrine mediator of myocyte growth, induces similar changes in cultured cardiac myocytes as those seen in hypertrophied heart in vivo. By using of rat B-type natriuretic peptide promoter, we identified a novel ETS binding sequence, on which nuclear protein binding is activated in endothelin-1 treated cultured cardiac myocytes. This sequence binds ETS-like gene-1 transcription factor and mediates endothelin-1-specific activation of transcription, but not responses to increased calcium signaling via L-type calcium channels, angiotensin II treatment or mechanical stretch of myocytes. Interestingly, endothelin-1 activated signaling converges via p38 mitogen activated protein kinase dependent mechanism on ETS binding site, whereas this element inhibits extracellular signal-regulated kinase activated transcription. In conclusion, given the fundamental role of the interaction of mitogen activated protein kinases and ETS factors in regulation of eukaryotic cell differentiation, growth and oncogenesis, these results provide the unique evidence of a endothelin-1 and mitogen activated protein kinase regulated ETS factor pathway for cardiac myocytes.
INTRODUCTION

Terminally differentiated cardiac myocytes, due to their inability to divide, adapt to increased mechanical load and the activation of neurohumoral system by hypertrophy. Initiation of hypertrophic growth is accompanied by a rapid and transient expression of immediate early genes (e.g. c-jun, c-fos and Egr-1) followed by activation of a pattern of cardiac genes, including atrial and B-type natriuretic peptide (ANP\(^1\), BNP) and \(\beta\)-myosin heavy chain (\(\beta\)-MHC), whereas relative expression of a subset of genes is simultaneously decreased (i.e. \(\alpha\)-myosin heavy chain, \(\alpha\)-MHC) (1, 2). Natriuretic peptides, released into circulation by cardiac myocytes, defend cardiovascular system against increased hemodynamic load and decrease blood pressure by increasing salt and water excretion and by promoting vasodilation (3). However, when both mechanical and neurohumoral stress of myocytes are sustained, adaptive mechanisms fail leading to maladaptation and eventually cardiac dysfunction (i.e. congestive heart failure) (4). Emerging evidence suggests that a number of humoral factors, such as angiotensin II (Ang II) and endothelin-1 (ET-1), participate in this adaptive process and modify the growth of cardiac myocytes (5). Production of ET-1 is increased in humans with cardiac hypertrophy and congestive heart failure, and ET-1 plasma concentrations correlate with the clinical severity of the cardiac failure (6).

There are two distinct G-protein coupled receptor (GPCR) subtypes for ET-1, \(\text{ET}_A\) and \(\text{ET}_B\) receptors, both of which are widely expressed in a variety of tissues including myocardium (7). Activation of GPCRs catalyzes exchange of GTP for GDP within the heterotrimeric G-protein complex releasing activated \(\text{G}\alpha\)-GTP and \(\text{G}\beta\gamma\) subunits, each of which has regulatory functions (8). Binding of ET-1 to its receptors induces \(\text{G}\gamma_{11}\)-protein dependent stimulation of phospholipase C, leading to formation of inositol 1,4,5-triphosphate (IP\(_3\)) and diacylglycerol (DAG) (9), which activate the release of calcium from intracellular stores and protein kinase C (PKC) (10), respectively. Altered calcium handling of myocyte, a feature of human heart failure (11), may result in activation of \(\text{Ca}^{2+}\)/calmodulin dependent kinase (CaMK) and phosphatase (i.e. calcineurin) pathways (12), both of which are implicated in endothelin-1 induced signaling cascades (13). In addition, signaling pathways downstream of ET receptors involve small G-
proteins (Ras, RhoA and Rac) accompanied by activation of mitogen activated protein kinases (MAPKs) (14).

The mechanisms connecting ET-1 induced cytosolic signaling to nuclear targets are poorly understood in cardiac myocytes. ET-1 has been reported to activate nuclear factor-κB (NFκB) transcription factor (15) and via MAPK mediated phosphorylation also GATA-4 transcription factor (16). Physical and functional interactions of GATA-4 with its cofactors, nuclear factor of activated T lymphocytes (NFAT) and serum response factor (SRF), are induced in ET-1 treated cardiac myocytes (17, 18). However, inhibitory strategies targeted either against GATA-4 protein synthesis or DNA binding affinity have produced differential results in ET-1-induced hypertrophic growth of cardiac myocytes, suggesting an increasing complexity of ET-1 dependent pathways (19, 20). To elucidate the nuclear mechanisms of ET-1 activated cell signaling, we studied rat BNP (rBNP) as a cardiac myocyte-specific target gene for ET-1, and identified a novel signaling mechanism initiating from cell membrane ET_{A} receptor and leading to p38 MAPK and ETS factor dependent induction of rBNP gene.
EXPERIMENTAL PROCEDURES

Chemicals- Specific antibodies raised against transcription factors of Activator Protein-1 (AP-1) complex (c-fos, c-jun, jun-B, jun-D), NFATc3, NFATc4, ETS-like gene-1 (Elk-1), Ser383-phosphorylated Elk-1, ETS-1/-2 (DNA binding domain), Fli-1, GATA-4, NFκB (p50, p65), Nkx-2.5 and SRF were from Santa Cruz Biotechnology (San Diego, CA). Specific antibodies against signal transducers and activators of transcription-1 and -3 (STAT-1 and -3) were purchased from Upstate Biotechnology (Lake Placid, NY). FuGENE 6 transfection reagent was from Roche Molecular Biochemicals (Indianapolis, IN). ET-1, Ang II and Bay K8644 were obtained from Sigma Chemical Co. (St. Louis, MO), BQ610 from Peninsula Laboratories Inc. (San Carlos, CA) and bosentan was a kind gift from Dr. M. Clozel (Actelion Pharmaceuticals Ltd., Basel, Switzerland). Anti-rabbit IgG antibody linked to horseradish peroxidase (Anti-Rabbit IgG-HRP, New England Biolabs Ltd, Hertfordshire, UK) and ECL+Plus reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK) were used for western blot detection.

Plasmids and oligonucleotides- All oligonucleotides were from Sigma Chemical Co. except that NFκB and SIE (shift-inducible element) electrophoretic mobility shift assay (EMSA) probes were from Santa Cruz Biotechnology. Plasmid for Rous Sarcoma virus-promoter linked to β-galactosidase gene (RSV-βgal) and pGL3-Basic plasmid expressing luciferase gene were purchased from Clontech (Palo Alto, CA). Mutations (Quick-Change Site-Directed Mutagenesis, Stratagene, La Jolla, CA) to –534 bp 5’-flanking region of rBNP promoter driven pGL3-Basic plasmid (–534BNP-Luc) (20) were introduced using oligonucleotides (coding strand, mutated nucleotides in bold) of 5’-GCTACCAGAGTGCCCAGCCTCCGTGCAGCCCGGCC-3’, 5’-CTGGAAGTGTTTTTGACAGTTTACCCCATAAAGCCCGCCC-3’ and 5’-GCGAGGAATGTGTCTTGCAAATCAGATGCAACCCACCCCTAC-3’, for ETS binding sequence at –498 bp, AP-1 binding sequence at –373 bp and tandem GATA binding sequence at –90/–81 bp of rBNP promoter (GenBank Accession Number M60266), respectively. All mutations were confirmed by nucleotide sequencing (ABI-Prism 7700, Perkin-Elmer,
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Foster City, CA). Plasmids encoding wild type and dominant negative mutant of MEK1 (pCMV-MEK1, pCMV-MEK1(K97M)) were a kind gift from Dr K. L. Guan (University of Michigan, Ann Arbor, USA), a dominant negative MKK6 (pCMV-MKK6AL) from Dr. J. R. Woodgett (University of Toronto, Canada) and plasmids expressing of p38α MAPK (pCMV-FLAG-p38α) and wild type and dominant negative mutant c-Jun N-terminal kinase-1 (pCDNA-FLAG-JNK1, pCDNA-FLAG-JNK1(APF)) from Dr. R. Davis (University of Massachusetts, USA).

Cell culture and transfection- Neonatal rat ventricular myocytes were prepared from 2-4 days old Sprague-Dawley rats and transfected with liposome-mediated transfection (20). The Animal Use and Care Committee of the University of Oulu approved the experimental design. Briefly, after preparation cells were plated at the density of 2 x 10^5 /cm² on cell culture plates (Falcon) or on flexible bottomed collagen I-coated elastomere plates (Bioflex, Flexcell Int.) for stretch experiments, and cultured overnight with Dulbecco’s modification of Eagle’s medium/ Hams’s F12 medium (1:1) containing 10 % of fetal bovine serum. Transfection of cells (if designated) was performed on the second day in culture. Cells were exposed to 3 µl of FuGENE 6 and 1.5 µg of DNA (1 µg of Luc, 0.5 µg of β-gal plasmids) per ml of complete serum free medium (CSFM) for 6 hours and cultured thereafter in CSFM. Cells on Bioflex plates were exposed to cyclic mechanical stretch. Frequency of cyclic stretch was 0.5 Hz with pulsation of 10-25 % elongation of cells for 24 hours. Cells were stretched by applying a cyclic vacuum suction under Bioflex plates by computer controlled equipment (FX-3000, Flexcell Int.). In co-transfection experiments, cells were grown on 24-well plates and transfected with 0.45 µg intact –534BNP-luc or with –534BNP-luc containing mutation of EBS and 0.05 µg expression plasmid (see Plasmids and Oligonucleotides) of dnMEK1, dnMKK6, dnJNK1, MEK1, p38α, JNK1 or pMT2 plasmids with 0.25 µg RSV β-gal and 1.5 µl FuGENE 6 per ml. Empty control plasmid pMT2 was used to equalize the amount of transfected DNA between experimental groups. All experiments were started subsequently on third day and cells were harvested according to the experiment during the third or fourth day in culture. Reporter gene activities were measured by using luciferase and β-galactosidase (to correct transfection efficiency) assays.
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Luciferase activity levels were 9.2 ± 1.0 (average ± SEM) fold higher than those of β-galactosidase activity. Both luciferase and β-galactosidase levels were at the linear range of assay measurement scale.

Northern blot analysis and radioimmunoassay for BNP- RNA was isolated from cardiac myocytes by the guanidine thiocyanate-CsCl method (21). Northern blots were hybridized with specific cDNA probes for rat BNP, rat ANP and rat ribosomal 18S (20) labeled with [32P]dCTP (Amersham-Pharmacia) with a T7 Quick Prime Kit (Amersham-Pharmacia). To measure the secretion of BNP, samples of incubation medium were subjected to radioimmunoassay of BNP (20).

EMSA- Nuclear extracts from cardiac myocytes (22) were prepared and protein concentration from each sample was colorimetrically determined (BioRad Laboratories). Double stranded-oligonucleotide probes were sticky-end-labeled with [32P]-dCTP by Klenow enzyme (22). For each reaction mixture (20), 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, 1mM DTT, 1mM EDTA, 10 % Glycerol, 0.025 % NP-40, 0.25mM PMSF and 1 µg/ml of each leupeptin, pepstatin and aprotinin. Reaction mixtures were incubated with a labeled probe for 20 min followed by gel-electrophoresis (250 V, for 1.5-1.75 hr normally and for supershift 2.5 hr) on 5 % polyacrylamide gel. Subsequently, gels were dried and exposed in a PhosphorImager screen and analyzed with ImageQuant (Molecular Dynamics, Amersham Biosciences, CA). To confirm DNA sequence specificity of the protein-DNA complex formation, competition experiments with 10-, 50- and 100-molar excesses of unlabeled oligonucleotides with intact or mutated binding sites were performed. In addition, DNA binding activity assays of Octamer-1 (Oct-1) transcription factor were run in parallel to control the specificity of the altered DNA binding activity (16). For competition and supershift experiments appropriate competitor oligonucleotides or antibodies were added to reaction mixture 20 minutes before labeled probe.

Immunoblot- Western blot analysis was done using standard reagents and protocol (16). Briefly, 20 µg of nuclear proteins were used for SDS-polyacrylamide gel electrophoresis, followed by western blotting utilizing antibodies against phospho-Elk-1 (Ser383) and Elk-1 (binds both phosphorylated and
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dephosphorylated Elk-1). Finally, blots were developed with goat anti-Rabbit IgG-HRP and enhanced luminescence reagents followed by exposure of film for 1 to 3 minutes (Hyperfilm ECL, Amersham Pharmacia Biotech).

*Sarcomeric organization*-Cardiac myocytes were grown on collagen-coated glass coverslips and exposed to ET-1 (100 nM) for 24 hours. α-actin filaments of fixed myocytes were labeled with Alexa Fluor™ 488 Phalloidin (Molecular Probes Inc., Eugene, OR) (20). Representative images were taken with laser confocal microscope (LSM 510, Zeiss, Jena, Germany).

*Statistical analysis*- Results are expressed as mean ± SEM. To determine the statistical significance between two groups and for analysis of multiple groups, Student's t-test and one-way ANOVA followed by LSD post hoc test were used, respectively. Differences at the 95 % level were considered statistically significant.
RESULTS

ET-1 activates BNP gene transcription via a novel ETS binding sequence- Cultured neonatal ventricular myocytes serve as a useful experimental model for the study of myocyte growth and the intracellular mechanisms involved. Primary neonatal ventricular myocytes (referred to as cardiac myocytes) used in this study respond to a variety of stimuli by the activation of hypertrophic growth program virtually identical to that of developing neonatal and the pathologic adult myocardium (5). ET-1 activated cardiac genes associated with hypertrophic growth programme, such as ANP and BNP (Fig. 1A), of which BNP synthesis was augmented through ETₐ receptor dependent mechanism (Fig. 1B) (23). In addition, cardiac myocytes subjected to ET-1 develop hypertrophy including activation of protein synthesis (16, 20) and increased cell size accompanied by reorganization of sarcomeres (Fig. 1C).

In the context of rBNP promoter, previous studies have indicated that proximal 5´-flanking region of 535 bp is sufficient to confer the induction by GPCR agonists (20, 24). Proximal –534 bp of BNP promoter contains multiple potential cis-acting elements (25), such as GATA elements. Proximal GATA elements participate to the activation of rBNP gene transcription by α-adrenergic agonist phenylephrine (24), but not by ET-1 (20). Therefore, we utilized computer based search for novel cis-acting elements of rat BNP gene and identified an ETS binding sequence (EBS) and AP-1 binding sequence at –498 bp and –373 bp of rBNP promoter, respectively. In order to characterize the role of these elements, we introduced site-directed mutations to EBS and AP-1 binding sequences, and also to –90/–81 bp tandem GATA elements of rBNP promoter (Fig. 2A). Endothelin-1 activated proximal –534 bp rBNP promoter dose-dependently via ETₐ receptor and the induction was inhibited by mutation of EBS (Fig 2B-C). The combination of mutations of AP-1 and/or GATA elements with the mutation of EBS did not further decrease the inhibition seen with rBNP promoter containing mutated EBS (data not shown). Importantly, the induction of rBNP transcription in myocytes by mechanical stretch or Ang II was unaffected by the mutation of EBS (Fig. 2D). To investigate this ET-1 responsive element further, we studied the complex formation of cardiac nuclear proteins with EBS of rBNP promoter.
ET-1 activates specific complex formation of cardiac nuclear proteins with EBS. Previously, it has been demonstrated that exposure of cardiac myocytes to hypertrophic agonists selectively activate transcription factors followed by specific DNA binding affinity and eventually activation of transcription (16, 26). To analyze whether there is specific binding activity on EBS of rBNP promoter by cardiac nuclear proteins, we utilized EMSA, and when designated, performed the protein-DNA binding reactions in the presence a 100-molar excess of unlabeled competitor oligonucleotides (Fig. 3A). We found that three specific shifts (complexes C1-3) were constantly formed, of which C1 and C2 were migrating closely to each other separately from a faster migrating complex C3. Competitor oligonucleotides with mutated nucleotides from −497 bp (mut 2) to −491 bp (mut 8) failed to compete against intact EBS and were found essential for formation of complexes C1-3 (i.e. 5´-cCCGGAAGtg-3´), suggesting similarity of binding proteins in these complexes. Interestingly, a labeled high affinity ETS binding sequence (SC1) (27) formed complexes similar to C1-3 (Fig. 3A). The core sequence for ETS factor binding of SC1 (Table 1) is similar to EBS of rBNP promoter. When SC1 oligonucleotide was used as a unlabeled competitor against nuclear protein binding on EBS of rBNP promoter, SC1 dose-dependently inhibited protein binding on EBS and the competition was abolished by single nucleotide mutation of SC1 (i.e. 5´-CCGaAAGTG-3´) (Fig. 3B). A NFκB binding oligonucleotide (Table 1), as an another potential GGA-sequence binding element, was not able to compete against binding on EBS of rBNP promoter (Fig. 3B). Moreover, with EBS of rBNP, nuclear proteins extracted from adult rat heart (left ventricle) formed identical complexes C1-3 in EMSA (data not shown), suggesting that ETS factors binding on EBS of rBNP are present in adult heart.

In view of specific complex formation of cardiac nuclear proteins with EBS of BNP promoter, we analyzed whether ET-1 treatment of cardiac myocytes had any effect on the nuclear protein binding activity on EBS (Fig. 4A-D). Initially, a rapid and transient increase in formation of complex C2 was detected at 5 and 15 minutes. Later, a sustained increase of EBS binding activity was observed at 3 hours for complexes C1 and C3, which remained elevated at 24 hours. In agreement with our previous studies (16), all the nuclear extracts were tested in parallel for Octamer-1 binding activity, which was not altered.
by ET-1 suggesting a specific activation of EBS binding (data not shown). Taken together, ET-1 treatment of cardiac myocytes initiated a biphasic activation of protein binding on the EBS of rBNP and these specific complexes closely resembled complex formation of nuclear proteins with a high affinity ETS binding sequence.

*Elk-1 binds on EBS of rBNP promoter*—Functional ETS factor binding regulatory *cis*-sequences have been characterized extensively in both viral and eukaryotic genes, but there are exceptionally few reports of the cardiac genes regulated by ETS factors (28). Gupta et al. have reported a repressor role for 3′-flanking EBS resulting in myocyte-restricted expression of α-MHC gene (29), while other studies available have described an interaction of serum response element (SRE) with an EBS in the activation of c-fos gene in cardiac myocytes (30, 31). EBS of rBNP differs significantly from those reported previously: (i) –2.2 kb of 5′-flanking region of rat BNP gene contains no potential SRE for TCF-SRF factor synergy (18) and (ii) EBS functions as an activator element of rBNP transcription exclusively by ET-1. In order to identify the transcription factors binding on EBS, we used specific antibodies added to binding reaction prior radioactively labeled EBS and subsequent EMSA. We utilized antibodies for NFκB (p50 and p65), AP-1 complex (c-fos, c-jun, jun-B, jun-D), Nkx-2.5, SRF, ETS-1/-2 (DNA binding domain), Fli-1 (a member of ETS transcription factor family) and activated Ser383-phosphorylated Elk-1 (Phospho-Elk-1). We found that ET-1 activated C2 complex was supershifted by antibody raised against Phospho-Elk-1 (Fig. 5A). In contrast, none of these antibodies had any effect on GATA-4 binding activity on –90/−81 bp rBNP GATA binding site in EMSA run in parallel (data not shown).

Given the cumulative evidence demonstrating that Elk-1 transcription factor is regulated via site-specific phosphorylation (32), we further investigated activation of Elk-1 by using western blot analysis. The site-specific phosphorylation (Ser383) of Elk-1 transiently increased at 5 minutes and 15 minutes in cardiac myocytes treated with ET-1 (Fig. 5B). Therefore, Elk-1 phosphorylation is temporally related to increased formation of complex C2 in EMSA (Fig. 4B). In addition, antibodies raised against NFATc3, NFATc4, GATA-4, STAT-1 and -3 and competitor oligonucleotides of AP-1, GATA-4 binding
sequences (–373 bp and –90 bp/–81 bp of rBNP) and Nkx-2.5 binding element (at –240 bp of rat ANP gene) or SIE failed to have any effect on complex formation of cardiac nuclear proteins with EBS (data not shown). In conclusion, ET-1 induces phosphorylation and binding of Elk-1 on EBS of rBNP (complex C2) and likely activates additional ETS related proteins whose identity we were unable to identify by using specific antibodies against ETS related or other transcription factors.

**ET-1 activates rBNP transcription via EBS by p38 MAPK dependent mechanism** - Hypertrophic stimuli including GPCR agonists (e.g. ET-1, phenylephrine and Ang II) and mechanical stress activate MAPKs in cardiac myocytes (14, 16, 33). Elk-1 transcription factor is a known downstream target for MAPKs (34, 35) and thus we investigated their potential role in activation rBNP transcription via EBS by using expression plasmids for each MAPK pathway. A minimal amount of expression plasmids (0.05 µg) was selected to prevent quenching and to confirm that the effects of overexpression were specific for each MAPK cascade. Dominant negative (dn) kinases of MEK1 (upstream of ERK), MKK6 (upstream of p38) and c-jun N-terminal kinase-1 (JNK) were utilized to inhibit specifically each pathway. Interestingly, only the inhibition of p38 MAPK dependent mechanism attenuated the activation of rBNP transcription by ET-1 (Fig. 6A). Vice versa, the activation of p38 MAPK resulted in EBS dependent activation of rBNP promoter activity (Fig. 6B). Contrastingly, activation of ERK signaling by forced expression of MEK1 activated rBNP transcription only when signaling via EBS was blocked by mutation (Fig. 6B). Furthermore, pharmacological blockade of p38 MAPK pathway by p38 MAPK inhibitor (SB203580, 20 µM) prevented ET-1 (100 nM, 15 min) induced Ser383-phosphorylation of Elk-1 (data not shown).

In addition to MAPK cascades, ET-1 is a known activator of Ca\(^{2+}\) dependent signaling such as CaMK and calcineurin in cardiac myocytes (13). Therefore, we further examined whether activation of Ca\(^{2+}\) dependent pathways induced rBNP transcription similarly via the p38 MAPK and EBS dependent mechanism as ET-1. Cells were exposed to dihydropyridine agonist Bay K8644 to activate Ca\(^{2+}\) influx via L-type Ca\(^{2+}\) -channels (36). Bay K8644 induced rBNP transcription independently of EBS, and the induction was equally inhibited by dominant negative expression plasmids of each MAPK cascade (Fig. 6C). Similar results were obtained with physiological activation of L-type Ca\(^{2+}\)-channels using β1-
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adrenergic receptor stimulation by isoproterenol (data not shown). Conclusively, these results suggest that ET-1 activates \textit{rBNP} gene transcription via EBS in a p38 MAPK dependent mechanism, whereas induction of BNP gene by robust activation of Ca\textsuperscript{2+} dependent signaling likely requires activity of all three MAPK cascades in parallel. In contrast, intact EBS of rBNP promoter inhibits the activation of transcription by ERK pathway alone.
DISCUSSION

The founding member of ETS family in mammalians, ETS-1, was originally discovered due to homology to \( v\)-\( ets \), an oncogene of avian E26 ("E-Twenty-Six") retrovirus genome (37). ETS factors share a winged helix-turn-helix domain for DNA binding and they bind to 5′-GGAT/A-3′ ETS binding sequence (EBS) (27, 38). ETS factors have been implicated in transforming and tumour-associated products as well as cell-cycle and apoptosis regulator genes and they regulate hematopoiesis and the development of central nervous system, bone and cartilage, mammary glands and vasculature (28, 39). Therefore, the identification of target genes for ETS signaling has emerged to an major area of research interest.

Although there are multiple ETS factors in the heart (40), their functional role has not been revealed. Activation of c-\( fos \) promoter via Elk-1 together with SRF composes a regulatory mechanism of c-\( fos \) gene present in most eukaryotic cell types including cardiac myocytes (30, 31). In addition, ETS-domain transcription factors act through EBS containing no adjacent SREs, likely in complex with alternative co-factors, but only a single report is available of such a regulatory EBS acting on a gene expressed in myocytes (\( \alpha\)-MHC) (29). Yet, multiple genes activated during hypertrophic growth of cardiac myocytes contain potential EBS in their 5′-flanking regulatory region, such as genes for \( \beta\)-MHC and ANP (29). In the present study, we identified a novel EBS at –498 bp of rBNP promoter, constituting \( rBNP \) gene as a first cardiac target for ETS signaling that is activated during hypertrophic growth.

Importantly, treatment of myocytes with ET-1 activated specific complex formation between nuclear proteins and EBS of BNP. Similar activation of binding affinity on \( rBNP \) gene has been reported of GATA-4 transcription factor (16). Moreover, it is evident that activation of \( rBNP \) gene by forced expression of p38 MAPK alone requires both intact EBS and GATA elements (16) at the proximal promoter region. However, ERK is also capable to phosphorylate GATA-4 in a manner similar to p38 MAPK (16), but it appears that activation of rBNP promoter by ERK pathway is inhibited by signaling via EBS (schematic presentation in Fig. 7). This model of nuclear regulation may target parallel MAPK pathways in a stimulus dependent manner. Our hypothesis is supported by previous studies indicating that activation of rat BNP promoter during myocyte stretch appears to require intact GATA binding sequence
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at −90/−81 bp (25), but not EBS at −498 bp of rBNP. Taken together with the fact that ETS factors have been shown to regulate tissue-restricted gene expression by utilizing both gene activation and repression mechanisms (41, 42), this study provides unique evidence for the role of ETS transcription factors as a gatekeeper of MAPK mediated cardiac gene activation.

Elk-1, identified in protein complex binding on EBS of rBNP, is directly regulated via site-specific phosphorylation by MAPK pathways (34, 35) and conversely inactivated by dephosphorylation by phosphatases including calcineurin (43). Originally, studies with T cells demonstrated that calcineurin dephosphorylates NFAT leading to nuclear translocation (44), but Molkentin et al. described a role for calcineurin and NFAT dependent signaling pathway also in myocyte hypertrophy (45). In the present study, robust activation of calcium signaling via L-type calcium channels resulted in the activation of BNP promoter independently of EBS, and all three MAPK cascades were equally required. Therefore, increased calcium signaling, mechanical stretch and Ang II activate rBNP promoter via mechanisms that appear to uncouple the requirement of EBS of BNP gene. Calcineurin has been shown to direct signaling through JNK and ERK cascades (46, 47) and to inactivate signaling via Elk-1 through dephosphorylation (32). In addition, accumulation of intracellular calcium may lead to autoinhibition of DNA binding activity of ETS factors via site-specific CaMK mediated phosphorylation (48). In conclusion, recent studies (49, 50) combined with our results suggest an intimate relationship between MAPKs and Ca²⁺-dependent pathways in the development of cardiac hypertrophy.

It is noteworthy that pathophysiological effects of ET-1 extend beyond myocardium to the regulation of proliferation (51) and contraction (52) of vascular smooth muscle cells, most notable effects are seen in coronary arteries (53). Therefore, it is intriguing to speculate that ET-1 activated nuclear pathway described in the present study may not be limited to the regulation of cardiac gene expression, but might extend to involve additional ETS factors expressed in vasculature, such as ETS-1, the expression of which is activated by ET-1 in cultured vascular smooth muscle cells (54) and in human endothelial cells during tumour angiogenesis (55). Interestingly, ET-1 increases vascular endothelial growth factor (VEGF) production in ovarian cancer cells via ET₁ receptor (56), while ETS factors activate genes regulating
angiogenesis, such as *VEGF receptor R1* (57) and -2 (58). Furthermore, ET-1 is an endogenously produced growth factor implicated in tumour cell proliferation (59) and ET\textsubscript{A} receptor antagonism may prove useful treatment strategy in malignancies where this protein is overexpressed (60). Indeed, the ET-1 activated and MAPK regulated ETS factor signaling provides an attractive target for the ongoing study of cellular growth, proliferation and oncogenesis.

In summary, our results demonstrated that ET-1 activates *rBNP* gene via mechanisms distinct from those of increased calcium influx via L-type calcium channels, Ang II or mechanical stretch of myocytes. Importantly, ET-1 induced binding of ETS factors (Elk-1) on EBS of *rBNP* promoter and this element was required for the activation of transcription by ET-1. p38 MAPK dependent mechanism mediated activation of *rBNP* transcription via EBS, whereas activation of *rBNP* promoter via MEK1/ERK pathway was inhibited by intact EBS. In conclusion, given the fundamental role of the interaction of MAPKs and ETS factors in the regulation of eukaryotic cells, these results provide the unique evidence of a ET-1 specific and MAPK regulated ETS factor pathway for cardiac myocytes.
**Fig. 1. ET-1 induces hypertrophic phenotype of cardiac myocytes.** A, In cardiac myocytes treated with ET-1 (100 nM), ANP and BNP mRNA levels increased. After 24 hours of ET-1 treatment, total RNA was extracted and subjected to northern blot analysis. Northern blots were hybridized with specific cDNA probes for rBNP, rANP and rat ribosomal 18S labeled with [³²P]dCTP. ANP and BNP mRNA levels were measured and corrected with 18S mRNA levels. For each bar, average ± SEM of 6 separate experiments are shown. B, ET-1 activates BNP secretion via ETₐ receptor. Cardiac myocytes were treated with ET-1 alone (100 nM, 24 hours) or with specific ETₐ receptor antagonist (BQ610, 10 µM) or mixed ETₐ/B receptor antagonist (bosentan, 1 µM). After incubation, amount of immunoreactive-BNP (ir-BNP) in culture medium was measured with radioimmunoassay. ET-1 increased ir-BNP concentration, which was equally abolished by BQ610 or bosentan, suggesting an ETₐ receptor mediated mechanism. For each bar, average ± SEM of 4 separate experiments are shown. C, Activation of sarcomeric protein organization of cardiac myocytes by ET-1. Cardiac myocytes grown on collagen-coated glass coverslips were incubated with or without ET-1 (100 nM, 24 hours). After fixation of cells, α–actin filaments were labeled with Alexa Fluor 488 Phalloidin. Representative images of 5 separate experiments were taken with laser confocal microscope (LSM 510). *, p < 0.05 compared with untreated control cells and #, p < 0.05 compared with ET-1 treated cells.

**Fig. 2. Endothelin-1-specific activation of rBNP promoter via novel ETS binding sequence (EBS).** A, Schematic presentation of the binding sites of -534BNP-luc construct with the mutated nucleotides of EBS (-498 mut), AP-1 (-372 mut) and GATA (-90/-81 mut) motifs. B, Dose-dependent activation of rBNP promoter activity via ETₐ receptor by ET-1. Cardiac myocytes were transfected simultaneously with plasmids of -534BNP-luc and RSV β-gal by using cationic liposome delivery. Transfected cells were treated with increasing concentrations of ET-1 (0-100 nM) alone or in the presence of specific ETₐ receptor blocker BQ610 (10 µM). After 24 hours, cells were lysed and lysates were assayed for luciferase.
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and β-galactosidase activity to correct transfection efficiency. *, p < 0.05 compared with untreated control cells and #, p < 0.05 compared with 100 nM ET-1 treated cells. C, The effect of site-directed mutations on the induction of rat BNP promoter by ET-1. Cardiac myocytes were transfected with intact plasmid of –534BNP-Luc or with –534BNP-Luc containing site-directed mutations (EBS (–498 mut), AP-1 (–373 mut) or GATA (–90/–81 mut)) and RSV β-gal by using cationic liposome delivery. Transfected cells were subjected to ET-1 (100 nM, 24 hours) and subsequently cell lysates were assayed for luciferase and β-galactosidase activity to correct transfection efficiency. *, p < 0.05 compared with induction of –534BNP-Luc. D, The effect of EBS mutation on –534-BNP promoter activation by mechanical stretch and Ang II. Cardiac myocytes were transfected with intact plasmid of –534BNP-Luc or –534BNP-Luc with site-directed mutation of EBS and RSV β-gal by using cationic liposome delivery. Transfected cells were subjected to cyclical stretch (frequency of 0.5 Hz with elongation between 10 - 25 %) or Ang II (1 µM) for 24 hours and subsequently assayed for luciferase corrected with β-galactosidase activity. Data is shown as fold induction compared with control cells (transfected with same luciferase construct). For each bar, average ± SEM of 5 (B), 9 (C) or 10 (D) separate experiments are shown.

Fig. 3. Characterization of –498 BNP ETS binding sequence (EBS). A, Competition analysis of ETS binding sequence of rat BNP promoter and comparison with SC1 probe, B, Competition analysis for nuclear protein binding on ETS binding sequence of rBNP promoter with NFκB binding site and SC1 oligonucleotides. EMSA using double-stranded oligonucleotide corresponding to ETS binding sequence at –498 bp of rBNP promoter ((–498)BNP) and oligonucleotide corresponding to a high affinity ETS binding site (SC1) as a radioactively labeled probes was carried out with 100-molar excess of unlabeled competitor oligonucleotides (for sequence see Table 1). For binding reaction, 6 µg of a mixed nuclear protein extract from untreated and ET-1 (100 nM, 15 min and 24 hr) stimulated cardiac myocytes was used. Binding reactions with EMSA were repeated separately four times from different nuclear extracts and the three specific complexes invariably formed are indicated as C1, C2 and C3.
**Fig. 4. Activation of nuclear protein binding on EBS during ET-1 treatment.** *A*, Representative EMSA of C1-3 complex formation in ET-1 stimulated cardiac myocytes and the kinetics of *B*, C1 complex, *C*, C2 complex formation and *D*, C3 complex formation in EMSA using double-stranded oligonucleotide corresponding to ETS binding sequence of rBNP promoter ((–498) BNP) as a radioactively labeled probe was carried out with nuclear proteins of cardiac myocytes treated with 100 nM of ET-1. For binding reaction, 6 µg of nuclear protein extract of cardiac myocytes was used. The three specific complexes are indicated as C1, C2 and C3. For each bar, average ± SEM of 9 separate experiments for A and C or of 13-14 for B, are shown. *, *p* < 0.05 and **, *p* < 0.01 compared with untreated control cells.

**Fig. 5. Analysis of EBS binding proteins.** *A*, Elk-1 binds on EBS of rBNP and is a major protein in C2 complex. EMSA using double-stranded oligonucleotide corresponding to EBS of rBNP ((-498) BNP) as a radioactively labeled probe was used with 6 µg of nuclear proteins from cardiac myocytes treated with ET-1 (100nM, 15 min). For supershift reaction, 2.5 µl of specific antibodies raised against NFκB (p50 and p65), the members of AP-1 complex, Nkx-2.5, SRF, ETS-1/-2 (DNA binding domain), Fli-1 or Ser383-phosphorylated Elk-1 (P-Elk-1) were added to binding reaction. Supershifted C2 complex is indicated (SS). *B*, ET-1 increases phosphorylation of Elk-1. Nuclear proteins from control and ET-1 treated (100 nM) cardiac myocytes were subjected to SDS-PAGE. An antibody raised against Ser383-phosphorylated Elk-1 was used to reveal the amount of activated Elk-1 protein (P-Elk-1) and total amount of Elk-1 (Elk-1) was quantified by immunoblotting filters with another antibody raised against Elk-1 that binds both phosphorylated and dephosphorylated Elk-1 proteins. Representative western blot of 4-5 separate experiments for each time point is shown.
Fig. 6. The role of MAPKs and calcium dependent pathways in the activation of rBNP promoter. A, Effect of dominant negative (dn) mutants of MEK1, MKK6 and JNK1 on induction of BNP promoter by ET-1. B, Effects of dn mutants of MEK1, MKK6 and JNK1 and mutated EBS on activation of BNP promoter by L-type Ca\(^{2+}\)-calcium channel agonist Bay K8644. C, Effect of mutated EBS on activation rat BNP promoter by forced expression of MAPKs. Cardiac myocytes were grown on 24-well Falcon plates and each well was transfected with 0.45 \(\mu\)g intact –534BNP-luc or with –534BNP-luc containing site-directed mutation of EBS and 0.05 \(\mu\)g expression plasmid of dnMEK1, dnMKK6, dnJNK1, MEK1, p38\(\alpha\), JNK1 or pMT2 (empty control plasmid) with 0.25 \(\mu\)g RSV \(\beta\)-gal and 1.5 \(\mu\)l FuGENE 6. When indicated, transfected cells were subjected to treatment with ET-1 (100 nM) or Bay K8644 (1 \(\mu\)M) for 24 hours. Cell lysates were assayed for luciferase activity corrected with \(\beta\)-galactosidase activity. For each bar, average \(\pm\) SEM of 6 separate experiments are shown. *** , \(p < 0.001\) compared with control cells transfected with pMT2 plasmid.

Fig. 7. Schematic presentation of ET-1 activated signaling pathways converging on rBNP promoter. ET-1 activates rBNP gene transcription in a MKK6/p38 MAPK dependent manner via ETS binding sequence. Both p38 MAPK and MEK1/ERK phosphorylate GATA-4 transcription factor, while intact ETS binding sequence inhibits induction of rBNP gene transcription via ERK pathway, leading to exclusively p38 MAPK dependent mechanism.
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FOOTNOTES

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1 The abbreviations used are: ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; β-MHC, α-myosin heavy chain; Ang II, angiotensin II; ET-1, endothelin-1; GPRC, G protein coupled receptor; CAMK, calcium/calmodulin dependent kinase; MAPK, mitogen activated protein kinase; NFκB, SRF, serum response factor; STAT, signal transducers and activators of transcription; SIE, shift-inducible element; Oct-1, octamer-1; EMSA, electrophoretic mobility shift assay; EBS, ETS binding sequence; complete serum free medium (CSFM).
Table 1. Comparison of oligonucleotides used in EMSA.

| Binding site | 5’-coding strand-3’ |
|--------------|---------------------|
| EBS          | AGAGTGCGCCGAAAGTCGCTGTCAGCCCCG |
| SCI          | AGAGGGCCAGCCGAAAGTCGAGTGTGCCCG |
| SCI mut      | AGAGGGCCAGCCGAAAGTCGAGTGTGCCCCCG |
| NFκB         | AGTTGAGGGCTTTCCCCAGGC |
| NFκB mut     | AGTTGAGGCTTTCCCCAGGC |

Nucleotides corresponding to EBS at -498 bp BNP are marked with box, the point mutations in bold and 5’-overhangs in italics.
A

B

C

D

EBS

AP-1

2x GATA

Luc

BQ610

ET-1

ET-1 (nM) 0 1 10 100

Fold induction

0.5

1.0

1.5

2.0

2.5

3.0

Fold induction

0.5

1.0

1.5

2.0

2.5

3.0

Fold induction

0.5

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Fold induction

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Fold induction

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Fold induction

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3.0

Fold induction

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1.0

1.5

2.0

2.5

3.0

Fold induction
**(-498) BNP mutations:**

1. 5’ - CCCGGAAGTG -3`
2. MUT: TTTTTCTGC

**A**

| COMPETITOR | w/o protein | -498 BNP | -498 BNPmut 1 | -498 BNPmut 2 | -498 BNPmut 1 | -498 BNPmut 3 | -498 BNPmut 4 | -498 BNPmut 5 | -498 BNPmut 6 | -498 BNPmut 7 | -498 BNPmut 8 | -498 BNPmut 9 | -498 BNPmut 10 |
|-------------|-------------|----------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| C1          |            |          |               |               |               |               |               |               |               |               |               |               |               |               |
| C2          |            |          |               |               |               |               |               |               |               |               |               |               |               |               |
| C3          |            |          |               |               |               |               |               |               |               |               |               |               |               |               |

**B**

| COMPETITOR | w/o protein | NFkB mut (x100) | NFkB (x100) | SC1 mut | SC1 (x100) | SC1 (x50) | SC1 (x10) | (-498) BNP |
|-------------|-------------|-----------------|-------------|---------|------------|-----------|------------|------------|
| C1          |            |                 |             |         |            |           |            |            |
| C2          |            |                 |             |         |            |           |            |            |
| C3          |            |                 |             |         |            |           |            |            |
Fold activation (C1)

Fold activation (C3)

Fold activation (C2)

Fold activation (C3)
**Figure A**

| ANTI BODY | - | NFkB (p65) | NFkB (p50) | Jun-B | c-Jun | c-Fos | Jun-D | Nkx-2.5 | SRF | ETS-1/2 | Fli-1 | P-ELK-1 |
|-----------|---|-------------|------------|-------|-------|-------|-------|----------|-----|--------|-------|---------|

SS → C1 → C2 → C3 → (-498) BNP

**Figure B**

| Time  | control | 5 min | 15 min | 1 hr | 3 hr | 24 hr |
|-------|---------|-------|--------|------|------|-------|
| p-Elk-1 |         |       |        |      |      |       |
| Elk-1  |         |       |        |      |      |       |
Fold induction

**A**

- pMT2
- dnMEK1
dnMKK6
dnJNK1

**B**

- pMT2
- MEK1
p38
JNK1

**C**

- pMT2
- dnMEK1
dnMKK6
dnJNK1

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EBS

by guest on March 24, 2020 http://www.jbc.org/Downloaded from
ET-1

MKK-3/6
p38

MEK

ERK

ETS factor complex

ET  

Elk-1

P

GATA-4

BNP mRNA

EBS

WGATAR
