Isoproterenol and cAMP regulation of the human brain natriuretic peptide gene involves Src and Rac

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Isoproterenol and cAMP regulation of the human brain natriuretic peptide gene involves Src and Rac. Am J Physiol Endocrinol Metab 278: E1115–E1123, 2000.—Brain natriuretic peptide (BNP) gene expression and chronic activation of the sympathetic nervous system are characteristics of the development of heart failure. We studied the role of the β-adrenergic signaling pathway in regulation of the human BNP (hBNP) promoter. An hBNP promoter (−1818 to +100) coupled to a luciferase reporter gene was transfected into neonatal cardiac myocytes, and luciferase activity was measured as an index of promoter activity. Isoproterenol (ISO), forskolin, and cAMP stimulated the promoter, and the β2-agonist ICI 118,551 abrogated the effect of ISO. In contrast, the protein kinase A (PKA) inhibitor H-89 failed to block the action of cAMP and ISO. Pertussis toxin (PT), which inactivates Gαi, inhibited ISO- and cAMP-stimulated hBNP promoter activity. The Src tyrosine kinase inhibitor PP1 and a dominant-negative mutant of the small G protein Rac also abolished the effect of ISO and cAMP. Finally, we studied the involvement of M-CAT-like binding sites in basal and inducible regulation of the hBNP promoter. Mutation of these elements decreased basal and cAMP-induced activity. These data suggest that β-adrenergic regulation of hBNP is PKA independent, involves a Gαi-activated pathway, and targets regulatory elements in the proximal BNP promoter.

cardiomyocytes; gene regulation; adrenergic signaling; M-CAT elements; protein kinaseA

BRAIN NATRIURETIC PEPTIDE (BNP), originally isolated from porcine brains, is constitutively expressed in the adult heart and is primarily a ventricular hormone, in contrast to atrial natriuretic peptide, which is highly expressed in adult atria (8). BNP has vasodilator, natriuretic, and diuretic properties. BNP gene expression is induced in the infarcted heart and elevated during the development of heart failure (26), and circulating plasma BNP is a marker of left ventricular dysfunction in these pathophysiological states (3, 27, 35).

Adrenergic signaling pathways, mediated by both α1- and β-adrenoreceptors (α1 or β-AR), regulate gene expression and growth of cardiac myocytes (reviewed in Refs. 9, 15, 37). When activated by norepinephrine binding, α1-ARs couple to the G protein Gα1, resulting in activation of serine/threonine kinases, such as protein kinase C (PKC) (reviewed in Ref. 32) and p42/44 mitogen-activated protein kinase (MAPK) (9, 15). The α1-AR agonist phenylephrine (PE) has been shown to increase rat BNP mRNA (13) as well as activate the rat BNP promoter (34).

The β-AR couples primarily to Gαq proteins, resulting in activation of adenylyl cyclase, elevation of intracellular cAMP, and activation of protein kinase A (PKA) (9). Chronic activation of the sympathetic nervous system occurs during the development of heart failure, resulting in major alterations in β-adrenergic signaling. Both decreased β1 receptors and increased Gαq protein content contribute to these changes (9). Moreover, β-ARs can also couple to pertussis toxin-sensitive Gαi, protein in cardiac myocytes (36). Cross-coupling of β-AR to Gαi has been studied extensively in COS-7 and HEK-293 cells, and results indicate that the βγ-subunit of Gαi activates the nonreceptor tyrosine kinase Src, resulting in Ras-dependent activation of p42/44 MAPK (1, 6, 7, 23, 24). Such alterations in β-AR signaling also play a role in the increased protein synthesis that accompanies hypertrophic growth of cardiac myocytes. Recent studies indicate that activation of β-ARs with either norepinephrine (38) or the β-agonist isoproterenol (ISO) (40) stimulates protein synthesis in neonatal ventricular myocytes (NVM). Moreover, ISO-induced protein synthesis involves PKA as well as coupling of the receptor to Gαq, the tyrosine kinase Src, the small G protein Ras, and p42/44 MAPK (40). To our knowledge, no one has addressed whether a similar mechanism is involved in β-adrenergic regulation of cardiac-specific gene expression. Because BNP is a marker gene for cardiac hypertrophy, infarction, and heart failure, we hypothesized that its regulation by a β-AR agonist would involve a similar mechanism.

In our studies, we used transient transfection of the human BNP (hBNP) promoter coupled to a luciferase reporter gene to determine the signaling pathways by which ISO and cAMP regulate the hBNP promoter. In addition, we mutated putative regulatory elements in the proximal hBNP promoter to test their involvement in β-adrenergic regulation. Our data indicate that ISO and cAMP activate the hBNP promoter and that proximal regulatory elements are involved in this response. Moreover, the effect of ISO and cAMP is mediated by Gαi, the tyrosine kinase Src, and the small G protein Rac.

MATERIALS AND METHODS

Cell culture Ventricular myocyte-enriched cultures were generated from Sprague-Dawley rat pups (Charles River,
Kalamazoo, MI) as described previously (21). Myocytes were separated from myocardial fibroblasts by differential plating. NVM were plated for 40 h in DMEM (GIBCO) containing 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 10% fetal bovine serum (HyClone), and 0.1 mM 5′-bromo-2′-deoxyuridine to inhibit proliferation of contaminating fibroblasts. Cultures were maintained under serum-free conditions, with DMEM supplemented with 5 mg/l insulin and transferrin and 2.5 mg/l selenium. After 24 h under serum-free conditions, cells were treated with the appropriate agent for 24 h and then lysed for assay of luciferase and protein. Inhibitors were added for 1 h before treatment with ISO or cAMP. The dosage was based on a survey of the literature and preliminary studies. All studies were approved by the Henry Ford Hospital Committee for the Care of Experimental Animals and were performed in accord with the National Research Council Guide for the Care and Use of Laboratory Animals.

Northern blot. BNP mRNA was detected by Northern blot, as described previously (20). RNA was measured by laser scanning densitometry. BNP mRNA was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA for quantitation of the multiples of increase vs. untreated controls. Quantitation of the multiples of increase vs. untreated controls.

Plasmid constructions and mutagenesis. Chimera hBNP-luciferase reporter gene constructs have been described previously (21). PCR was used to generate mutations in the hBNP proximal promoter. Oligonucleotides included restriction sites at their 5′ and 3′ borders to facilitate subcloning (Hind III on the sense primer and BamHI on the antisense primer are not included in the following sequences). Putative regulatory elements were identified on the basis of sequence comparison with consensus elements. The muscle cardiac troponin T (M-CAT)-like element at position −124 is referred to in the text as 124BNP and the element at −97 as 97BNP. Whereas the 124BNP element extends from −124 to −118 on the sense strand (5′-CATTCCC-3′), the 97BNP element extends from −91 to −97 on the antisense strand (5′-CATTCCG-3′). The activating protein (AP)-1-like element at position −111 has an additional base pair (T at −108) vs. the consensus element (5′-TGCAG/CTCA-3′) and is referred to as 111BNP. Mutated 124BNP (M124) has three bp changes (5′-GCTACC-3′). Mutated 97BNP (M97) has three bp changes (5′-CTTACT-3′).

124 BNp was mutated through PCR by use of a sense strand oligonucleotide containing mutated bases (in bold): 5′-GCTGGTACCGCGCCCTGATCTCA-3′ (−127/−104). The antisense oligonucleotide was located between +83 and +100 (5′-GGGACTTGGGAGCTGCT-3′). PCR was performed with the full-length hBNP 5′ flanking sequence as a template. Standard reagents were obtained from Promega. PCR products of the expected size were cut with Hind III and BamHI and subcloned into a digested −127hBNP-luciferase vector. The M124 mutation was also introduced into the full-length hBNP promoter (−1818hBNPluc). −1818(M124)hBNPluc was generated by PCR by use of −1818hBNPluc as a template, with the sense oligonucleotide 5′-CCAACTTAGGACCCCGGAGA-3′ (−283/−264) and the antisense oligonucleotide 5′-TCAGGGCCCGGGTAC-3′. The resulting PCR product was digested with Ava II and Apa I and subcloned into −1818hBNPluc cut with the same restriction enzymes to generate −1818(M124)hBNPluc.

We first created the 97BNP mutation (−97 M-CAT mutation or M97) in −127hBNPluc with the following oligonucleotides: mutant sense strand, 5′-GGCCCCTTATGGTGCTGATA-3′; mutant antisense strand, 5′-AGACTAAGGGCTCCCTGAC-3′; and the wild-type sense strand: 5′-GCTTATTTCGCCGCCC-3′ (−127/−113) and antisense oligonucleotide (+83/+100). −127(M97)hBNPluc was digested with Apa I and BamH I and then subcloned into −1818hBNPluc to generate −1818(M97)hBNPluc. All constructs were sequenced with the fmol DNA sequencing kit (Promega, Madison, WI). Expression vectors encoding the dominant-negative mutants of Ras, Rac, and Raf have been described previously (14).

Transfection and luciferase assay. Transfection was performed, and luciferase activity was assayed as described previously (21). Briefly, freshly isolated ventricular myocytes were transiently transfected in PBS-glucose by electroporation at 280 V and 250 μF with a Bio-Rad gene pulser. For the hBNPluc constructs, 1 μg was transfected per 3×10⁶ cells. In cotransfection experiments, 10 μg of the dominant-negative mutant of Ras, Raf, or Rac were used. After transfection, the cells were aliquoted into 3 wells of a 12-well plate and, 40 h later, the medium was changed to serum-free DMEM. After 24 h in serum-free medium, cells were treated with the appropriate agents for 24 h and then harvested, lysed, and assayed for luciferase activity (Luciferase Assay System, Promega) with an OptoComp 1 luminometer (MGM) according to the manufacturer’s protocol. Duplicate aliquots of cell lysate from triplicate wells were assayed and averaged. Luciferase activity was normalized to protein levels, as described previously (21). At least two separate preparations of each plasmid were used for each experiment and group. Data were expressed as means ± SE and were analyzed by one-way ANOVA, with multiple pairwise comparisons made by the Student-Newman-Keuls method. P < 0.05 was considered significant.

Nuclear extract. Crude nuclear extracts were prepared from cultured NVM (6×10⁶ cells per 15-cm tissue culture dish) maintained as described for the transfection studies. Cells were washed in PBS, scraped into PBS, and pelleted. Cells were lysed in buffer A (10 mM HEPEs (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.3 mM sucrose, 1 mM diethiothreitol (DTT), 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 µg/ml each of antipain, chymostatin, and leupeptin). The nuclei were pelleted and protein was extracted in buffer B (10 mM HEPEs (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM PMSF, 1 mM DTT, and 1 µg/ml each of antipain, chymostatin, leupeptin, and pepstatin). Nuclear protein was diluted to 5 mg/ml, aliquoted, and stored at −70°C.

Electrophoretic mobility shift assay. To detect binding of nuclear protein to putative cognate binding sites, we used the electrophoretic mobility shift assay (EMSA; Gel Shift Assay System, Promega). Oligonucleotides were synthesized, and their complementary strands were annealed before their use as probe or competitor. An 0.0175-pmol 5′-end-labeled double-stranded oligonucleotide was used as a probe. Binding reactions were carried out in buffer containing 50 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol, 10 mM Tris·HCl (pH 7.5), 0.05 mg/ml poly (d-dC), and 5–10 µg of cardiomyocyte nuclear protein. Unlabeled oligonucleotides (1.75 pmol) used as competitors of binding were preincubated with nuclear protein before the labeled probe was added. DNA-protein complexes were separated out on a 4% nondenaturating acrylamide gel in 0.5× TBE buffer. After electrophoresis, the gel was dried and exposed to film for 1–3 days. The sequences of the oligonucleotides used in EMSA are as follows (regulatory elements printed in boldface):

1) −131/−113 hBNP (124BNP) (21): 5′-AGGGGCTCATTCCCGGGCC-3′;
2) −17/−15 hBNP (M124) (21): 5′-GGCCCCTTATGGTGCTGATA-3′;
3) −97 M-CAT (cardiac troponin T) (10): 5′-GCTTATTTCGCCGCCC-3′;
4) −83/−100 hBNP (M97) (21): 5′-GGGACTTGGGAGCTGCT-3′;
3) GT-IIIC (SV40 enhancer) (31): 5'-CCAGCTGTGGAATGTGTGTT-3';
(4) – 1 Orb/88 hBNP (97BPN) (21): 5'-GGCCCGGAATGTTGGG-3';
(5) Myc-Max (E-box): 5'-AAGCACAGCAGTTGTGCGTGTCTTCCC-3'.

Chemicals. Endothelin (ET)-1 was obtained from Peninsula (San Carlos, CA), dibutyryl cAMP, dibutyryl cGMP, and forskolin from Sigma (St. Louis, MO), and forskolin and H-89 from Biomol (Plymouth Meeting, PA). The Myc-Max oligonucleotide containing the consensus E-box element was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and the Sp1 oligonucleotide was obtained from Promega. All other routine chemicals and supplies were obtained from Fisher and Sigma.

RESULTS

Effect of ISO and cAMP on BNP mRNA and the hBNP promoter. To test whether β-adrenergic signaling regulates BNP mRNA in rat NVM, myocytes were treated with either 100 µM ISO or a stable form of cAMP, dibutyryl cAMP (dbcA, 1 mM). Northern blots showed that ISO and dbcA stimulated BNP mRNA 3.6 ± 0.5-fold and 3.8 ± 1.5-fold, respectively (Fig. 1).

To test the effect of a β-AR agonist on activation of the hBNP promoter, transfected myocytes were treated with ISO, which stimulated the promoter in a dose-dependent fashion (Fig. 2A). In addition, both the direct adenylly cyclase activator forskolin and dbcA were stimulatory (Fig. 2B), whereas 1 mM dibutyryl cGMP had no effect (data not shown). The effect of ISO was inhibited 70% by 10 µM propranolol, a β1/β2-AR antagonist (Fig. 2C). The specific β2-AR antagonist ICI 118,551 (10 µM) inhibited ISO-stimulated hBNP luciferase activity by >85%, whereas the β1-AR antagonist metoprolol (10 µM) had no effect (Fig. 2D). Either 50 µM ICI or metoprolol gave the same results as 10 µM (data not shown).

The stimulatory effect of ISO and dbcA was not inhibited by H-89 (0.1 or 1.0 µM), a selective PKA inhibitor (data not shown). At 10 µM, H-89 inhibited the effects not only of ISO and cAMP but also of the α1-agonist PE and the cytokine interleukin-1β (IL) (data not shown). Thus, in this cell culture system, high-dose H-89 had nonspecific effects on other kinases.

Effect of the Goi inhibitor PT and the Src tyrosine kinase inhibitor PP1 on regulation of the hBNP promoter. We next tested whether the signaling mechanism for ISO- and cAMP-stimulated hBNP promoter activity involves cross-coupling to Goi. When myocytes were treated with 500 ng/ml pertussis toxin (PT), which inactivates Goi, ISO- and cAMP-stimulated hBNP promoter activity was decreased by 40% and 76%, respectively (Fig. 3A). This effect was specific to Goi-activated pathways, as PT had no effect on IL activation of the hBNP promoter and little effect on PE activation (data not shown). The fact that PT failed to completely inhibit ISO-stimulated hBNP promoter activity may be due to several factors: 1) 100 µM ISO activates both β1- and β2-ARs, and stimulation of both might have some antagonistic effects; 2) although there are more β1-ARs on cardiac myocytes, only β2-ARs couple to Goi; 3) β2-ARs preferentially activate the hBNP promoter, and because they are less abundant than β1-ARs, they may be preferentially downregulated by 24 h of treatment with ISO.

Because the Goi-subunit of Gi has been shown to activate the nonreceptor tyrosine kinase Src (24), we tested the Src inhibitor PP1 on ISO- and cAMP-stimulated promoter activity and found that it completely eliminated both (Fig. 3B). Thus our data suggest that β2-AR activation initiates coupling to Goi and activation of Src.

Effect of dominant-negative small G proteins on regulation of the hBNP promoter. Studies indicate that β-AR stimulation of Src results in activation of the Ras-Raf-MEK-p42/44 MAPK pathway (23, 24, 38). We tested whether overexpression of a dominant-negative mutation of the small G protein Ras (dnRas) would inhibit ISO and cAMP stimulation of hBNP promoter activity but found that it had no effect (data not shown). In addition, a dominant-negative form of Raf also had no effect. These dominant-negative signaling molecules effectively inhibit regulation of the hBNP promoter in cardiac myocytes; indeed, we found that dnRas inhibits IL stimulation of the hBNP promoter (14), and dnRaf inhibits ET-1 activation of the hBNP promoter (He and LaPointe, unpublished data).

Another member of the small G protein family of effector molecules is Rac, which normally couples to MAPK pathways involved in the stress response, such as c-J un kinase (JNK) and p38 MAPK. Overexpression...
of dnRac inhibited the effect of both ISO and cAMP on the hBNP promoter (Fig. 4). dnRac had no effect on luciferase activity in untreated cardiac myocytes (data not shown). Thus our data suggest that the small G protein Rac is involved in ISO and cAMP regulation of the hBNP promoter.

Role of M-CAT-like elements in basal and cAMP-inducible expression of the hBNP promoter. We have shown that the proximal region of the hBNP promoter (−127 to −40) is highly active in ventricular myocytes (21). Putative regulatory elements in this region include two M-CAT-like elements (124BNP and 97BNP). Many cardiac muscle-specific genes are regulated by M-CAT elements (17, 25, 31, 33, 34). To see whether 124BNP and 97BNP are important for basal regulation of the hBNP promoter in ventricular myocytes, they were mutated individually in −1818hBNP. Mutation of 124BNP and 97BNP resulted in a 60 and 96% decrease in luciferase activity, respectively (Fig. 5).

Using EMSA, we next tested whether 124BNP and 97BNP bind to a family of proteins called M-CAT-binding factors (MCBF or TEF) (Fig. 6). A radiolabeled oligonucleotide containing the 97BNP element was bound to nuclear protein (lane 2), and binding was competed for by a 100-fold molar excess of unlabeled 97BNP (lane 3) as well as consensus M-CAT (14) (lane 4) and GT-IIC (40) (lane5). 124BNP did not compete for binding at 100-fold molar excess (lane 6) but did so at
the highest concentration (500x, lane 8), although the intensity of the band was not different from the noncompetitive control Sp1 (lanes 12–14).

Gupta et al. (11) have shown that the cardiac tropo-

nin T M-CAT element binds the E-box binding protein Max. This has not been tested for the BNP promoter. EMSA showed that the E-box binding site (CANNTG) did not compete with 97BNP for binding to nuclear proteins (Fig. 6; lanes 9–11). Similar results were obtained with a noncompetitive control oligonucleotide (Sp1), as seen in lanes 12–14.

We next tested whether cAMP regulates the hBNP promoter through M-CAT-like sites, because cAMP has been shown to target a similar element in the α-MHC promoter, composed of overlapping M-CAT and E-box sequences (12). Mutation of 97BNP resulted in a 68% decrease in dbcA-induced hBNP promoter activity, whereas mutation of 124BNP decreased dbcA's effect by 36% (Fig. 7). Mutation of an element located at -111 (TGATCTCA), which has a sequence similar to both AP-1 (TRE) and cAMP response (CRE) sites, did not significantly inhibit dbcA induction of the hBNP promoter (data not shown). ISO also targeted the 97BNP element but not 124BNP (Fig. 7).

To test whether 97BNP and 124BNP are common targets for hypertrophic growth factors, we stimulated myocytes with ET and PE. ET (10 µM) stimulated -1818hBNPLuc 4.2-fold; however, mutations in 97BNP and 124BNP did not significantly affect promoter activation by ET (data not shown). PE (50 µM) activated the hBNP promoter 12-fold, and this effect was not altered by mutation of 97BNP and 124BNP (data not shown).
Thus 97BNP is a target of ISO- and cAMP-dependent signaling pathways in cardiac myocytes and not a common target for hypertrophic growth factors.

DISCUSSION

We explored the signaling mechanisms by which the β-AR agonist ISO and cAMP activate the hBNP promoter. Our data show that 1) ISO stimulation of the hBNP promoter is mediated primarily by β2-ARs; 2) ISO and cAMP activate the hBNP promoter through Ga_i-, Src-, and Rac-dependent pathways; 3) the effect of ISO and cAMP on promoter activity does not appear to involve PKA; and 4) ISO and cAMP target 97BNP, which is an M-CAT-like element.

Both Ga_i- and β2-adrenergic signaling pathways induce hypertrophy of cardiac myocytes, including increased protein synthesis, upregulation of early response genes, and fetal gene expression (e.g., the natriuretic peptide genes) (4, 5, 9, 15). Our results indicate that ISO and cAMP regulation of the hBNP promoter has both similar and distinct properties compared with β2-AR signaling in nonmyocyte cell lines (1, 6, 7, 23, 24) and β2-AR regulation of protein synthesis in myocytes (40). The similarities include the involvement of Ga_i and the tyrosine kinase Src. The major differences are that, in our studies, 1) the small G protein Rac seems to be a major effector, rather than Ras, and 2) the effect of ISO and cAMP seems to be independent of PKA.

In the nonmyocyte cell lines, cAMP couples the β2-adrenoreceptor to activation of Ras. Thus an important question derived from our data is how ISO and cAMP couple to Rac. We know of no studies showing that either β2-AR signaling or cAMP directly activates Rac; however, Kawasaki et al. (18) identified a cAMP-binding protein that can activate the small G protein Rap1 independently of PKA. Thus cAMP may regulate a factor that controls Rac activation. On the basis of studies of β2-AR overexpression (1, 6, 7, 23, 24), it is also possible that cAMP acts directly or indirectly to activate Src kinase, which in turn activates Rac. Of interest, both Src and Rac have been implicated in the pathway leading to cardiac myocyte hypertrophy in...
vitro (22, 29). Although Rac normally activates the stress pathways c-j un kinase (JNK) and p38 MAPK, such mechanisms may not be involved in ISO and cAMP regulation of the hBNP promoter, as dominant-negative inhibitors of both pathways had no effect (unpublished data). In addition, we have previously shown that the cytokine IL activates the hBNP promoter in a Rac- and p38 MAPK-dependent fashion, but neither p42/44 nor J NK seems to be involved (14). Because hypertrophy, ischemic injury, and heart failure are characterized by production of inflammatory cytokines and catecholamines and upregulation of the BNP gene, the small G protein Rac could well be an important transducer of multiple receptor-mediated signaling pathways activated during these pathophysiological events.

As mentioned above, our data also suggest that ISO and cAMP activation of the hBNP promoter is independent of PKA. The PKA inhibitor H-89 is very specific, with an inhibitor constant or Ki of 0.048 µM for PKA vs. 32 µM for PKC. In these studies, we used low doses (0.1 and 1 µM), which had no effect on ISO and cAMP stimulation of hBNP promoter activity. In contrast, 10 µM of H-89 inhibited other factors, including IL and PE, suggesting nonspecific effects. Whether β2-AR responses in cardiac myocytes are dependent on cAMP and PKA has been debated. Studies suggest that the inotropic effect of β2-AR is dissociated from cAMP/PKA signaling (2). However, more recently it has become apparent that β2-AR responses depend on very localized increases in cAMP and affect sarcolemmal L-type Ca2+ channels but not cytoplasmic contractile regulatory proteins (19, 39). In fact, the components of cAMP signaling, including G proteins, adenyl cyclase, and PKA regulatory subunits, are colocalized to caveolae (specialized plasma membrane vesicles) in many types of cells (30). Thus it is possible that our studies with H-89 failed to show a response because the inhibitor was unable to target the proper PKA isoform. Another possibility is that activation of the β2-AR produces intracellular signals in addition to cAMP, as Pavoine et al. (28) have recently shown. In their studies, β2-AR activation was coupled to the release of arachidonic acid by the cytosolic isofrom of phospholipase A2 (cPLA2). Additional studies are needed to determine whether β2-AR regulation of the hBNP promoter is dependent on additional intracellular signals, such as arachidonic acid and its metabolites.

Our study also indicates that basal and cAMP-inducible regulation of the hBNP promoter targets cis elements in the proximal promoter. We identified two M-CAT-like elements in the proximal promoter of the hBNP gene, 97BNP and 124BNP, by sequence homology to consensus elements. Mutation of these elements within 1818 bp of the hBNP 5' flanking sequence indicated that each contributes to basal expression, but disruption of 97BNP almost completely eliminates promoter activity, whereas disruption of 124BNP has only a partial effect. Another difference is that 97BNP was able to bind M-CAT binding factors (MCBF or TEF), as determined by competition with unlabeled M-CAT and GT-IIC consensus oligonucleotides; in contrast, 124BNP was only able to compete for binding at high concentrations. On the basis of comparison with two noncompetitive binding sites (E-box and Sp1), it is unlikely that 124BNP is an M-CAT element.

cAMP regulation of the hBNP promoter involves both 97BNP and 124BNP, neither of which is associated with an E-box element, distinguishing the hBNP promoter from α-myosin heavy chain (α-MHC) (12). Just as with basal regulation of the promoter, cAMP-inducible regulation is more dependent on 97BNP than on 124BNP. PKA and the CRE/AP-1-like element in the proximal hBNP promoter do not mediate the effect of cAMP. Because dnRac inhibits cAMP stimulation of the hBNP promoter, it is most likely that a Rac-dependent signaling molecule is involved in regulating the activity of a factor(s) binding to 97BNP. 97BNP seems to be an important target for other signaling pathways, as previous studies from our laboratory have shown it to be targeted by both IL signaling and p38 MAPK (14). Additional studies are needed to determine whether a particular kinase signaling pathway mediates the effect of Rac, or whether other Rac-mediated signals, such as reactive oxygen species, are involved in regulation of the hBNP promoter.

M-CAT elements are also important in α-adrenergic inducible regulation of cardiac-specific genes (16, 17, 33, 34). Our data indicate that neither PE nor ET targets the 97BNP M-CAT-like element, in contrast to studies on the rat BNP promoter, which indicate that PE targets the M-CAT element via PKC- and Ras-dependent mechanisms (34). Although both rat and human BNP genes contain M-CAT and GATA elements in their proximal promoters, the elements are not arranged in the same fashion. The proximal rat BNP promoter contains one M-CAT element upstream from two GATA elements, whereas the hBNP promoter contains one M-CAT-like element, one GATA element, and one AP-1-like element (21). Thus functional differences in regulation of the two genes may be the result of interactions between proteins binding to M-CAT sites and adjacent cis elements. In support of this concept, we found that mutation of the GATA element at −85 in the hBNP promoter resulted in a 70% decrease in cAMP-stimulated promoter activity (He and LaPointe, unpublished observations), suggesting that multiple interactions are required for cAMP’s effect.

In summary, our studies indicate that ISO and cAMP regulate the hBNP promoter through a pathway utilizing Gαi,Src, and Rac, targeting an M-CAT-like element in the proximal hBNP promoter. In addition, a second regulatory element, 124BNP, contributes in part to basal and cAMP-inducible regulation of promoter activity. Coupled with our previous data indicating that the cytokine IL upregulates the hBNP promoter in part by targeting the 97BNP M-CAT-like element (14), it would appear that multiple signaling pathways are focused on modulating the activity of regulatory factors that interact with this region of the hBNP promoter. Because cytokines are induced and β-adrenergic signaling is altered in the failing heart, studies on regulation of
the hBNP gene may provide insights into the molecular mechanisms underlying a number of pathophysiological conditions, such as hyper trophy, ischemic injury, and heart failure.

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REFERENCES


