Src and Rac Mediate Endothelin-1 and Lysophosphatidic Acid Stimulation of the Human Brain Natriuretic Peptide Promoter

Quan He, Margot C. LaPointe

Abstract—Brain natriuretic peptide (BNP) gene expression accompanies cardiac hypertrophy and heart failure. The vasoconstrictor endothelin-1 (ET) may be involved in the development of these diseases. ET has also been shown to activate phospholipase A$_2$ (PLA$_2$), and the resulting metabolites are important second messengers. We studied how ET and PLA$_2$ metabolites regulate BNP gene expression. The human BNP (hBNP) promoter (from $-1818$ to $+100$) coupled to a luciferase reporter gene was transferred into neonatal ventricular myocytes (NVMs), and luciferase activity was measured as an index of promoter activity. ET induced BNP mRNA in NVMs as assessed by Northern blot. It also stimulated the hBNP promoter, an effect completely inhibited by actinomycin D. To test the involvement of different PLA$_2$ isoforms, transfected cells were treated with various PLA$_2$ inhibitors before stimulation with ET. Only Ca$^{2+}$-independent PLA$_2$ blockade prevented ET-stimulated hBNP promoter activity. The PLA$_2$ metabolite lysophosphatidic acid (LPA) also activated the hBNP promoter, but arachidonic acid itself did not. ET regulation of the hBNP promoter is pertussis toxin–sensitive. The nonreceptor tyrosine kinase Src and the small GTPase Rac mediate the effects of both ET and LPA in stimulation of the hBNP promoter. We studied the involvement of cis elements in ET-stimulated hBNP promoter activity. Deletion of BNP promoter sequences from $-1818$ to $-408$ and from $-408$ to $-40$ reduced the effect of ET by 60% and 80%, respectively. Moreover, ET-stimulated luciferase activity was reduced by 50% when the proximal GATA element was mutated. These data suggest that (1) ET activates the hBNP promoter through a transcriptional mechanism; (2) LPA, perhaps generated by iPLA$_2$, is involved in the effect of ET; (3) Src and Rac mediate ET and LPA stimulation of the hBNP promoter; and (4) ET regulation of the hBNP promoter targets both distal and proximal cis elements. (Hypertension. 2001;37[part 2]:478-484.)

Key Words: cardiomyocytes ■ genes ■ brain natriuretic peptide ■ endothelin ■ phospholipases

Brain or b-type natriuretic peptide (BNP) has been extensively studied because of its natriuretic, diuretic, and vasodilator properties. BNP expression is upregulated during cardiac hypertrophy, heart failure, and other cardiovascular diseases.$^1$ Immunoassay of this peptide in blood makes it a convenient and reliable biochemical marker of left ventricular dysfunction.$^2$ Recent reports show that BNP-overexpressing transgenic mice are hypotensive, while BNP knockout mice develop cardiac fibrosis,$^3,4$ suggesting that BNP has multiple compensatory functions in the cardiovascular system.

Endothelin-1 (ET), a 21-amino-acid peptide, is a potent vasoconstrictor expressed in endothelial cells, cardiomyocytes, and vascular smooth muscle cells. Plasma ET is elevated in heart failure, and ET receptor antagonists have been shown to have beneficial effects on both cardiac hypertrophy and heart failure.$^5,7$ There are at least 2 known ET receptors: ET$_A$ and ET$_B$. The ET$_A$ receptor mediates vasoconstriction, whereas the ET$_B$ receptor may induce vasodilatation by releasing nitric oxide or prostaglandins.$^8$ The ET$_A$ receptor is the predominant type in neonatal ventricular myocytes (NVMs) and is coupled to both Go$_q$ and Go$_q^o$.$^8,9$ ET is a hypertrophic growth factor in NVMs and induces expression of several cardiac genes, including atrial natriuretic factor (ANF), BNP, and cardiac $\alpha$- and $\beta$-myosin heavy-chain genes.$^{10,14}$ ET activates a number of different signaling pathways, including phospholipase C, protein kinase C (PKC), and mitogen-activated protein kinases (MAPKs), and is coupled to the nonreceptor tyrosine kinase Src and small GTPases.$^{14,16}$ Src and the small GTPases Ras and Rac have been implicated in the regulation of cardiac hypertrophy and the expression of ANF and other genes.$^{14,17,18}$ We previously showed that Rac and Src are involved in interleukin-1$\beta$ (IL$\beta$), isoproterenol (ISO$\beta$), and dibutyryl cAMP–induced activation of the hBNP promoter.$^{19,20}$ So we questioned whether they would also be involved in the effects of ET.

In addition to phospholipase C, phospholipase A$_2$ (PLA$_2$) is involved in signal transduction.$^{21}$ Hydrolysis of the ester bond at the sn-2 position by PLA$_2$ releases unsaturated fatty acids,
such as arachidonic acid (AA), and other phospholipids, such as lysophosphatidylcholine (LPC). Subsequent removal of the choline head group of LPC yields lysophosphatidic acid (LPA). AA serves as a precursor for the synthesis of (1) prostaglandins and thromboxanes by cyclooxygenase (COX), (2) leukotrienes, including 5-hydroxyeicosatetraenoic acid (5-HETE), 12-HETE, and 20-HETE, by lipooxygenase (LO), and (3) cytochrome P450 monoxygenase (CYP450) products. AA, LPC, and LPA have been shown to exert multiple biological effects and may act as second messengers. ET evokes AA release by vascular smooth muscle cells. ET activation of PLA₂ is coupled to Gα₁. In addition, Rac activates PLA₂, whereas PLAP₂ and its metabolites activate Rac. Based on these reports, PLA₂ metabolites may be involved in ET regulation of BNP and may signal through Src and Rac.

Congestive heart failure is characterized by elevation of vasoactive peptides, including ET and BNP. The important question is how these factors interact during the development of cardiac hypertrophy and heart failure. Although a number of studies have shown that ET stimulates the synthesis and secretion of natriuretic peptides by cardiac myocytes, it is still not clear how ET signaling pathways target the hBNP promoter. We transferred the hBNP promoter coupled with the luciferase reporter gene into NVMs and measured luciferase activity as an index of hBNP promoter activity. We found that ET regulation of the hBNP promoter involved the PLA₂ metabolite LPA and that both ET and LPA activated the promoter through Src and Rac. We also found that ET regulation of the hBNP promoter targeted both proximal and distal cis elements.

Methods

Cell Culture

Ventricular myocyte–enriched cultures were generated from Sprague-Dawley rat pups (Charles River) as described previously. Myocytes were separated from myocardial fibroblasts by differential plating. NVMs were plated for 40 hours in DMEM containing 10% FBS (GIBCO) and 0.1 mM/L bromo-deoxyuridine to inhibit proliferation of contaminating fibroblasts. Cultures were maintained under serum-free conditions with DMEM supplemented with 2 mM/L glutamine, 5 mg/L insulin and transferrin, and 2.5 mg/L selenium. After 24 hours under serum-free conditions, cells were treated with the appropriate agent for 24 hours and then lysed for assay of luciferase and protein. Inhibitors were added for 1 hour before treatment with ET or LPA. The dosage was based on a survey of the literature and preliminary data. All studies were approved by the Henry Ford Hospital Committee for the Care of Experimental Animals and performed in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Northern Blot

BNP mRNA was detected by Northern blot as described previously. The signal was measured by laser scanning densitometry. BNP mRNA was normalized to GAPDH mRNA for quantification of fold increase versus untreated controls.

Transfection and Luciferase Assay

Transfection was performed and luciferase activity was assayed as described previously. 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 hBNP promoter and luciferase cDNA hybrid constructs (hBNPLuc), 1 μg was transfected per 3×10⁶ cells. In cotransfection experiments, 10 μg of dominant-negative mutant Ras or Rac was used. After transfection, the cells were aliquoted into 3 wells of a 12-well plate, and 40 hours later the medium was changed to serum-free DMEM. After 24 hours in serum-free medium, cells were treated with the appropriate agents for 24 hours and then harvested, lysed, and assayed for luciferase activity (Luciferase Assay System; Promega) 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. Data were expressed as mean±SEM and analyzed by t test or 1-way ANOVA, with multiple pairwise comparisons made by the Student-Newman-Keuls method. P<0.05 was considered significant.

Plasmid Constructs

Chimeric hBNP-luciferase reporter gene constructs and mutations of −97 MCAT, −124 MCAT, and −85GATA in hBNP luc constructs have been described previously. The polymerase chain reaction (PCR) was used to mutate an AP-1–like site in the hBNP proximal promoter. Oligonucleotides included restriction sites at their 5′ and 3′ borders to facilitate subcloning. The HIIII site on the sense primer and the BamHI site on the antisense primer are not included in the following sequences.) The AP-1–like site was mutated by using the following oligonucleotides: mutant sense 5′-GCCCTTTTCTTCAGAGGGCCG-3′ (−115/−96), mutant antisense 5′-TGAGAAAAGGGCCCGGAAT-3′ (−104/−123), wild-type sense 5′-GCGCAACGCCCGCAATTC-3′ (−198/−181), and wild-type antisense 5′-GGGACTCGAGGAGGTCT-3′ (+83/+100). The PCR product was cut with ApaI and BamHI to isolate the mutated fragment, which was subcloned into −1818hBNPLuc cut with the same enzymes to generate −1818(mAP1)hBNPLuc. Mutation of base pairs was verified by sequencing. An expression vector encoding the dominant-negative mutant of Ras (Ras N17) was obtained from Dr Michael Karin (University of California San Diego). Dominant-negative Rac (N17rac2) was obtained from Dr Melanie Cobb (University of Texas Southwestern Medical Center at Dallas).

Chemicals

ET-1 was obtained from Peninsula. Indomethacin, LPA, and pertussis toxin (PT) were obtained from Sigma Chemical Co. ONO-082 (ONO), metyrapone (MET), PPI, and baicalein (BAIC) were obtained from BIOMOL. Bromoeno lactone (BEL) and methyl arachidonyl fluorophosphonate (MAPF) were obtained from Cayman Chemical. Actinomycin D (ActD) was obtained from Calbiochem. All other chemicals and supplies were obtained from Fisher Scientific and Sigma Chemical Co.

Results

ET Induces hBNP Promoter Activity

ET increased hBNP promoter activity in a dose-dependent manner over the range of 10⁻⁸ to 10⁻⁶ mol/L in transfected NVMs (Figure 1A). To examine whether ET’s action involved a transcriptional mechanism, transfected cells were treated with the transcription inhibitor ActD 1 hour before treatment with ET. ActD totally inhibited ET-stimulated hBNP promoter activity but had no effect on basal activity (Figure 1B). When myocytes were treated with 10⁻⁸ mol/L ET for 24 hours and total RNA was subjected to Northern blotting, ET stimulated BNP mRNA 13.6±2.0-fold compared with control (Figures 1C and 1D).

PLA₂ And LPA Are Involved in ET Regulation of hBNP

ET evokes AA release by cultured vascular smooth muscle cells. Our previous work showed that AA and PLA₂...
Metabolites regulate inducible nitric oxide synthase in cardiac myocytes. In the present study, we tested whether hydrolysis of membrane phospholipids by PLA₂ was involved in ET (10⁻² mol/L) stimulation of the hBNP promoter. Transfected NVMs were treated with PLA₂ inhibitors 1 hour before ET. The Ca²⁺-independent PLA₂ (iPLA₂) inhibitor BEL abolished the action of ET. Neither the cytosolic PLA₂ (cPLA2) inhibitor MAFP nor the secretory PLA₂ (sPLA₂) inhibitor ONO had any inhibitory effect (Figure 2A).

One potential problem with working with BEL is that it reportedly inhibits Mg²⁺-dependent phosphatidic acid phosphohydrolase, reduces 1,2-diacylglycerol levels, and attenuates PKC activity. If PKC were a major regulator of ET-stimulated hBNP promoter activity, then the effect of BEL might be nonspecific. Thus, we treated NVMs with the specific PKC inhibitor GF109203X (10 μmol/L) in the presence of ET and found no effect on hBNP promoter activity, suggesting that PKC is not a mediator (data not shown); the effect of BEL is likely due to inhibition of iPLA₂.

Production of AA, LPC, or LPA by PLA₂ may mediate ET regulation of the hBNP promoter. We treated transfected NVMs separately with each compound and found that LPA activated the hBNP promoter 2.2-fold, whereas AA and LPC had no effect (Figure 2B). Thus, LPA, perhaps generated by a BEL-sensitive iPLA₂, participates in ET stimulation of the hBNP promoter.

AA metabolites, including prostanoids produced by COX, leukotrienes produced by LO, and dihydroxy/epoxyeicosa-
trienoic acids produced by CPY450, possess a remarkably wide spectrum of biological properties in the cardiovascular system. We tested whether AA metabolites were involved in ET regulation of the hBNP promoter but found that neither the COX inhibitor indomethacin nor the CYP450 inhibitor metyrapone significantly reduced ET stimulation of the hBNP promoter. In contrast, the LO inhibitor baicalein significantly inhibited the effect of ET by 70% (Figure 3). Thus, ET stimulation of a lipoxygenase product may mediate activation of the hBNP promoter.

ET Regulation of hBNP Promoter Is Pertussis Toxin–Sensitive
ET A receptors can couple to Gi protein. Coupling to the Bγ subunit of Gi, has been shown to activate the nonreceptor tyrosine kinase Src. To test whether Gi was involved in ET regulation of the hBNP promoter, transfected NVMs were pretreated with 500 ng/mL PT for 1 hour and then coincubated with ET. PT partially inhibited ET-induced hBNP promoter activation (Figure 4). In addition to the ET receptor, the LPA receptor can couple to Gi. In contrast to ET, PT had no effect on LPA activation of the hBNP promoter (data not shown).

Src and Rac Mediate ET and LPA Regulation of the hBNP Promoter
To test the involvement of Src in ET activation of the hBNP promoter, we treated transfected NVMs with PPI, a specific inhibitor. Although PPI reduced basal hBNP promoter activity, it also decreased ET-stimulated activity to control levels (Figure 5A).

Rac, a GTPase of the Rho family, is an essential element of the signaling pathway leading to cardiac myocyte hypertrophy and can be activated by PLA2. Because we have shown that Rac mediates both IL-1β and ISO stimulation of the hBNP promoter, we tested whether Rac was a mediator of ET regulation of hBNP. When a dominant-negative mutant of Rac (dnRac) was cotransfected with hBNP luc and the transfected NVMs were stimulated with ET, hBNP promoter activity was reduced; however, a dominant-negative mutant of Ras had no effect (Figure 5B).

Based on the fact that ET regulation of the hBNP promoter involved LPA, we next tested whether Src and Rac mediated LPA-stimulated hBNP promoter activity. We found that the Src inhibitor PPI and dnRac abolished the stimulatory effect of LPA (Figures 6A and 6B).

ET Targets Both Distal and Proximal cis Elements in the hBNP Promoter
We have detected 4 cis elements in the hBNP proximal promoter region (− 97 MCAT, − 124 MCAT, − 85 GATA, and − 111 AP-1) via mutational analysis. Our studies indicate that − 97 MCAT is involved in IL and ISO activation of the hBNP promoter, and − 85 GATA is involved in the effect of ISO. We tested whether these cis elements and other regions of the hBNP promoter were involved in the effect of ET. Using deletions of the 5′flanking sequence of the hBNP promoter, we found that deletion of the sequence from −1818 to −408 and from −408 to −40 significantly decreased ET-induced hBNP promoter activity by 60% and 80%, respectively (Figure 7A). Regarding the proximal cis elements, mutation of the GATA element at position −85 resulted in 50% reduction of ET-induced hBNP promoter activity, whereas mutation of the MCAT and AP-1 elements had no effect (Figure 7B). These data indicate that the proximal GATA element and more distal cis elements are targets of ET stimulation.

Discussion
Our data demonstrate that ET regulates the hBNP promoter via a transcriptional mechanism and that mediators of this effect include PLA2, an LO product, LPA, Src tyrosine kinase, and Rac. Based on the use of pharmacological inhibitors, LPA generation may occur after ET stimulation of a BEL-sensitive iPLA2. LPA and ET seem to activate Src and Rac through different mechanisms, as part of the effect of ET is pertussis toxin–sensitive (involving Gi), whereas the effect of LPA is not. In addition, both proximal (GATA) and distal cis elements in the hBNP promoter region respond to ET.

Activation of PLA2 results in the release of a number of lipid mediators, including AA, LPC, and LPA. The present results indicate that the inhibition of iPLA2 activity with BEL results in a 70% decrease in ET-stimulated hBNP promoter
activity. We have previously shown that AA is released by iPLA₂ in IL-stimulated cardiac myocytes. In vascular smooth muscle cells, ET releases AA through a Ca²⁺-dependent process, suggesting that there are cell-type specific differences in phospholipid metabolism by PLA₂ isoforms.

Although ET activates iPLA₂, its metabolite AA does not seem to mediate ET signaling, because AA itself does not activate hBNP promoter activity. In addition, neither the COX nor the CYP450 pathways are involved in ET regulation of the hBNP promoter. In contrast, the LO inhibitor baicalein decreased ET activation of hBNP promoter activity. LO products are thought to be involved in many intracellular signaling pathways, including activation of MAPKs and transcription factors, such as AP-1 and NF-κB. At this point, further studies are needed to more clearly define this pathway.

Interestingly, another PLA₂ metabolite, LPA, was able to directly activate the hBNP promoter. LPA has a number of biological effects, including cell proliferation, stress fiber formation, tumor cell invasion, and contraction of smooth muscle cells and fibroblasts. Signal transduction involves coupling of the LPA receptor to either Gᵢ or Gₛ with Gₛ activation of Src as a mechanism for LPA-induced cell proliferation. Because the Gₛ inhibitor PT had no effect on LPA stimulation of the hBNP promoter, we conclude that the effect of LPA is coupled to Gₛ. Coupling of the LPA receptor to Gₛ results in activation of tyrosine kinase activity and the small GTPase Rho, a Rac family member. In our study, the effect of LPA was partially abrogated by both an Src inhibitor and dnRac, suggesting that the LPA effect in NVMs proceeds from Gₛ through Src and Rac in regulation of the hBNP promoter.

ETₐ couples to both Gᵢ and Gₛ proteins. We found that ET couples in part to Gᵢ protein, and this is most likely responsible for the subsequent activation of PLA₂ and Src tyrosine kinase (through the βγ subunit). In vascular smooth muscle cells, ET stimulation of AA release is inhibited by pertussis toxin, demonstrating such a linkage of Gᵢ to PLA₂. As for ET activation of Src, several investigators have demonstrated the role of this pathway in the regulation of gene expression. ET regulation of c-fos transcription in mesangial cells and ANF transcription in NVMs involve activation of Src, which targets a CArG DNA sequence. Constitutively active Src also stimulates the expression of other cardiac genes, including skeletal muscle α-actin and β-myosin heavy chain. In these studies, the effect of Src on gene expression was mediated by signaling molecules downstream from the small GTPase Ras, such as MAPKs.

Although many studies indicate that Gₛ couples to Src, Ras, and the p42/44 MAPK pathway through its βγ subunits, we have reported that Rac is important in the regulation of the...
hBNP promoter by IL-1β and ISO. The present study indicates that Rac mediates the effects of both ET and LPA. At this point, we are unsure whether Rac is directly activated by Src and whether Rac directly or indirectly regulates the hBNP promoter. Regarding our previous studies on Rac regulation of the hBNP promoter, the 3 MAPKs do not seem to mediate this effect.

We previously studied the proximal promoter of the hBNP gene and the importance of cis elements in its basal and inducible regulation, including MCAT (−124 and −97), GATA (−85), and AP-1 (−111). In the present study, we have shown that the GATA element and unidentified elements upstream from it are involved in the response of ET. Kovacic et al demonstrated that ET-stimulated hANF promoter activity requires the CARG element located at position −422 in the proximal promoter region. We analyzed the hBNP distal promoter sequence and found a sequence similar to CARG located at −860 in the hBNP promoter. In addition, Liang et al have shown that a portion of the effect of ET on the hBNP promoter involves p38 MAPK operating through 3 NF-kB-binding sites positioned at −652, −633, and −162. Based on our deletion and mutation data, 50% of the effect of ET is mediated by the proximal GATA element. Whether the CARG element at −860 and 1 or more of the NF-kB-binding sites contribute to the remaining 50% has yet to be determined.

In conclusion, our results indicate that ET regulation of the hBNP promoter involves the generation of signals by 3 pathways: ET acting through an LO product, ET-Gαs-Src/Rac, and ET-PLA2-LPA-Src/Rac, of which the latter 2 seem to intersect at the level of Src or Rac. In combination with our previous work on the regulation of the hBNP promoter, our data indicate that Src and Rac are important signaling molecules for many agonists that induce gene expression in myocytes during hypertrophic growth or other pathological events (eg, in response to inflammatory stimuli). Because BNP is a good marker of left ventricular dysfunction and heart failure, understanding how BNP is regulated by signaling molecules that are chronically activated should be useful in understanding the underlying disease pathology.

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Quan He and Margot C. LaPointe

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