Mechanical Strain Increases Expression of the Brain Natriuretic Peptide Gene in Rat Cardiac Myocytes*

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Faquan Liang, Jianming Wu, Miklos Garami‡, and David G. Gardner§

From the Metabolic Research Unit and the Department of Medicine, University of California, San Francisco, California 94143

Using a device that applies cyclical strain (1 Hz) to ventricular cardiocytes cultured on collagen-coated silicone elastomer surfaces, we have demonstrated strain-dependent increases in brain natriuretic peptide (BNP) secretion, BNP mRNA levels, and expression of a transiently transfected −1595 human BNP-luciferase reporter. When actinomycin D (10 μM) was introduced concomitantly with the strain stimulus, the strain-induced increase in BNP mRNA was eliminated, and the decay of transcripts was identical in the control and strained cells, indicating the lack of independent effects on transcript stability. Strain-dependent −1595 human BNP-luciferase activity was completely inhibited by chloroquine, 2-aminopurine, genistein, and W-7 and only partially or not at all by KN-62, wortmannin, and H-89. The effects of these individual agents paralleled their effects on mitogen-activated protein kinase (MAPK) activity, but not c-Jun N-terminal kinase (JNK) activity, in these cells. Overexpression of wild-type MAPK and, to a lesser extent, JNK increased strain-dependent BNP promoter activity, whereas dominant-negative mutants of MAPK kinase, JNK kinase, or Ras completely blocked strain-dependent reporter activity. These findings provide the first demonstration that mechanical strain can increase myocardial gene expression through a transcriptional mechanism and suggest important roles for MAPK and JNK in mediating this effect.

The natriuretic peptides comprise a family of vasoactive hormones that play an important role in the regulation of cardiovascular and renal homeostasis (1). Their natriuretic and vasodepressor properties suggest that they represent endogenous antagonists of the various systems (e.g. renin-angiotensin system and sympathetic nervous system) that support arterial blood pressure under basal conditions and, at times, contribute to the pathophysiology of cardiovascular disease.

Atrial natriuretic peptide (ANP),† the prototype of the group, is produced primarily in the atria of the heart. ANP is expressed in the cardiac ventricle during development and early neonatal life (2, 3). Expression decays as the animal ages and remains quiescent in the adult unless the ventricle is subjected to hemodynamic overload (i.e. mechanical strain that leads to increased wall stress and subsequently to hypertrophy of the myocardium), as occurs with systemic arterial hypertension or congestive heart failure (4–7).

Brain natriuretic peptide (BNP) is also produced in the heart. Despite the nomenclature, relatively little BNP is expressed in the mammalian brain (the exception being the porcine brain, where the peptide was identified originally). Expression of BNP in the heart is lower than that of ANP under basal conditions, and the atrial/ventricular ratio of expression is considerably less than that seen with ANP (8). Ventricular expression of BNP is activated in a fashion similar to ANP in pathophysiological states associated with hemodynamic overload (9, 10). At some stages of advanced congestive heart failure, circulating BNP levels may actually surpass those of ANP, implying that BNP contributes a significant fraction of circulating natriuretic peptide activity under these conditions (11).

Thus, both ANP and BNP gene expression are linked to pathophysiological stimuli associated with hypertrophy in the whole animal. In vitro, the neonatal rat cardiac myocyte model responds to the activation of a number of extracellular (e.g. phenylephrine, angiotensin II, and endothelin) (14–17) and intracellular (e.g. protein kinase C, Ras, and c-Jun) (18–20) signaling systems with an increase in cell size, stimulation of protein synthesis, and/or activation of a genetic program (e.g. activation of c-jun and c-fos followed by increased ANP and BNP expression) that parallels that seen in the hypertrophic myocardium in vivo. Similarly, passive mechanical strain of cardiac ventricular myocytes in vitro, simulating that observed in the hemodynamically overloaded myocardium in vivo, has been shown to trigger the appearance of many of the same phenotypic markers of hypertrophy, including activation of c-fos, c-jun, and the ANP gene (21, 22). In the case of c-fos, this effect operates at a transcriptional locus and requires a serum response element present in the promoter of that gene (23). Of note, these studies have failed to demonstrate activation of ANP promoter-dependent transcription (22). In a parallel transgenic approach, neither Rockman et al. (24) nor Knowlton et al. (25) demonstrated activation of an hANP promoter-driven reporter in an in vivo model of hypertrophy. Therefore, at present, we have only a limited understanding of the mechanism(s) underlying enhanced expression of cardiac specific genes in the face of mechanical strain in vitro or hemodynamic overload in vivo.

Similarly, while several signal transduction systems have been shown to be activated by mechanical strain (20, 23, 26–28), controversy exists as to which of these is most important for the development of hypertrophy. Nonreceptor tyrosine kinases (29), Ras (20, 23, 30), Raf (31), MAPK (23, 27, 30–33),
SAPK (28), protein kinase C and Rsk (23, 30) have each been implicated in signaling one or more components of the hypertrophic phenotype; however, no consensus exists as to which of these plays the dominant role in triggering the growth response. MAPK, for example, is known to be activated by both the biochemical agonists of hypertrophy as well as mechanical strain (23, 27, 30–32), and dominant-negative mutants of MAPK and MAPK kinase (MEK), which lies immediately upstream from MAPK in the effector cascade (34), suppress phenylephrine induction of a transiently transfected ANP promoter (31). Moreover, antisense oligonucleotides directed against MAPK have been shown to reduce phenylephrine-dependent increments in ANP mRNA levels and ANP promoter activity while, at the same time, suppressing sarcomerogenesis and increments in cell size that typically accompany hypertrophy induced by this agent (33). However, Post et al. (32) recently reported that activation of MAPK does not routinely parallel the development of hypertrophy in the cultured myocyte model, and in their hands, dominant-negative mutants of the MAPK pathway failed to suppress phenylephrine-mediated activation of a transfected ANP promoter.

This study documents activation of BNP gene expression by passive mechanical strain in the neonatal cardiac myocyte model. This increased expression is due to enhanced transcription of the BNP gene, with little contribution from changes in transcript stability. This provides the first demonstration that mechanical strain can increase expression of a myocardial specific gene through a transcriptional mechanism.

EXPERIMENTAL PROCEDURES

Materials—[α-32P]dCTP, [γ-32P]ATP, and [3H]acetyl coenzyme A were purchased from NEN Life Science Products. A BNP radioimmunoassay kit was obtained from Peninsula Laboratories, Inc. (Belmont, CA). Anti-ERK2 (C-14) and anti-JNK1 (C-17) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Hemagglutinin (HA) antibodies were obtained from Berkeley Antibody Co. (Berkeley, CA). Bovine myelin basic protein was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY), and glutathione S-transferase-c-Jun was prepared as described (35). Chelerythrine, genistein, 2-amino-4-morpholine, W-7, KN-62, wortmannin, and actinomycin D were purchased from Sigma. PD98059 was purchased from Research Biochemicals International (Natick, MA). H-89 was from Seikagaku Co. (Tokyo, Japan), and the luciferase assay system was from Promega (Madison, WI).

Other reagents were obtained through standard commercial suppliers.

Cell Culture and Stretch—Ventricular myocytes were prepared from 1-day-old neonatal rat hearts by alternate cycles of 0.05% trypsin digestion and mechanical disruption as described previously (18). Cells (1 × 10^5) were cultured on collagen-coated Flex plates (Flexcell International Corp., McKeesport, PA) in Dulbecco’s modified Eagle’s H21 medium containing 10% enriched calf serum (Gemini Bioproducts, Calabasas, CA), 2 mM glutamine, 10 units/ml penicillin, and 100 mg/ml streptomycin. The medium was changed 24 h prior to initiation of the experiment. Cells were stretched by cyclical strain (60 cycles/min) on the Flexcell Strain apparatus at a level of distension sufficient to promote an ~20–25% increment in area surface at the point of maximal distension on the culture surface (36).

Radioimmunoassay for BNP Secretion—The culture medium of each well was collected and centrifuged to remove cellular debris; the supernatant was taken for assay. Radioimmunoassays were performed according to the instructions provided by the manufacturer using rabbit antiserum specific for rat BNP-32 and 125I-labeled rat BNP-32. Goat anti-rabbit IgG and normal rabbit serum were used to separate bound and free ligand.

RNA Isolation and Northern Blot Analysis—Total RNA was isolated from the cultured ventricular cells by the guanidinium thiocyanate/phenol/chloroform method (37). Fifteen μg of RNA was size-fractionated on a gel containing 2% formaldehyde, transferred to nitrocellulose filter, and hybridized with a 32P-labeled 640-base pair fragment of rat BNP cDNA. Blots were subsequently washed and rehybridized with a 32P-labeled 1.3-kilobase pair glyceraldehyde-3-phosphate dehydrogenase cDNA to normalize the blots for differences in RNA loading and/or transfer to the filter. Autoradiography was performed with an intensifying screen at −70 °C for 6–24 h. Autoradiographic signals were quantified by laser densitometry. Normalized data are presented as the ratio of BNP to glyceraldehyde-3-phosphate dehydrogenase signal.

Plasmid Constructions—The construction of −1595 hBNP-luciferase (38) and −1150 hANP-chloramphenicol acetyltransferase (39) has been described previously. Dominant-negative Ras (N17) was provided by W. Fantl, and glutathione S-transferase-c-Jun by J. Hambleton. HA-MAPK and HA-SAPK expression vectors and dominant-negative MEK and SEK were provided by M. Karin.

Transfection and Luciferase Assay—Freshly prepared ventricular myocytes were transiently transfected with the indicated reporters and expression vectors (Gene-Pulsor, Bio-Rad) at 280 nV and 250 microfarads. Individual cultures were normalized for DNA content with pUC18. After transfection, cells were plated in 6-well collagen-coated Flex plates at a density of 1 × 10^5 cells/well in Dulbecco’s modified Eagle’s H21 medium containing 10% enriched calf serum. The medium was changed 24 h after plating, and cyclical strain was applied. Cells were harvested and lysed in 60 μl of 250 mM Tris and 0.1% Triton X-100. The protein concentration of each cell extract was measured using Coomassie protein reagent (Pierce). Cell lysates were processed for either
Strain-activated BNP Gene Expression

RESULTS

Mechanical strain, applied in cyclical fashion (1 Hz with 30 s of strain followed by 30 s of membrane relaxation), increased BNP release into the culture media (Fig. 1A). This effect was first seen after 6 h of incubation and was maximal (~160% of that in the static cultures) after 48 h of incubation.

This was accompanied by an increase in BNP gene expression (Fig. 1B). Steady-state levels of the BNP transcript were differentially increased in the stretched versus static cultures after 6 h of treatment, and this difference persisted throughout the remainder of the experiment (48 h). BNP mRNA levels were not significantly changed in the static cultures during the course of the experiment.

To probe the mechanism underlying the elevation in BNP transcript levels, we transfected a chimeric reporter (~1595 hBNP-luciferase) containing 1595 base pairs of hBNP 5'-flank- ing sequence into freshly isolated neonatal ventricular myocytes. The transfectants were cultured for 24 h and then subjected to cyclical strain for 48 h in the presence of chelerythrine (5 μM), PD98059 (10 μM), 2-aminoquinoline (5 μM), genistein (100 μM), W-7 (10 μM), KN-62 (5 μM), wortmannin (1 μM), or H-89 (100 μM). Each drug was administered to the myocytes 1 h prior to application of the strain stimulus. The data are presented as means ± S.D. from four separate experiments. *, p < 0.01 versus static control; #, p < 0.05 versus static control.

Effect of various kinase inhibitors on strain-induced ~1595 hBNP-luciferase activities. Twenty μg of ~1595 hBNP-luciferase was transfected into ventricular myocytes. After 24 h of culture, the cells were subjected to strain for 48 h in the presence of chelerythrine (5 μM), PD98059 (10 μM), 2-aminoquinoline (5 μM), genistein (100 μM), W-7 (10 μM), KN-62 (5 μM), wortmannin (1 μM), or H-89 (100 μM). Each drug was administered to the myocytes 1 h prior to application of the strain stimulus. The data are presented as means ± S.D. from four separate experiments. *, p < 0.01 versus static control; #, p < 0.05 versus static control.

FIG. 3. Effects of various kinase inhibitors on strain-induced ~1595 hBNP-luciferase activities. Twenty μg of ~1595 hBNP-luciferase was transfected into ventricular myocytes. After 24 h of culture, the cells were subjected to strain for 48 h in the presence of chelerythrine (5 μM), PD98059 (10 μM), 2-aminoquinoline (5 μM), genistein (100 μM), W-7 (10 μM), KN-62 (5 μM), wortmannin (1 μM), or H-89 (100 μM). Each drug was administered to the myocytes 1 h prior to application of the strain stimulus. The data are presented as means ± S.D. from four separate experiments. *, p < 0.01 versus static control; #, p < 0.05 versus static control.

FIG. 2. Transcriptional and post-transcriptional effects of mechanical strain on BNP gene expression in neonatal rat ventricular myocytes. A, cells were transfected with 20 μg of ~1595 hBNP-luciferase and then collected at different intervals following application of the strain stimulus, and luciferase activity was quantified. B, cells were pretreated with actinomycin D (Act D; 10 μM) for 1 h to arrest transcriptional activity and then subjected to mechanical strain for varying periods of time. Controls (Ctl) were cultured in the static versus strain environment for 24 h in the absence of actinomycin D. Fifteen μg of total RNA was subjected to blot hybridization analysis as described in the legend to Fig. 1. Shown is a representative autoradiograph depicting the time course of BNP mRNA decay in the presence or absence of strain. C, BNP mRNA levels are expressed as the ratio of BNP to glyceraldehyde-3-phosphate dehydrogenase signal. The data presented represent means ± S.D. from four separate experiments. *, p < 0.01 versus static control. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Luciferase (30 μg of protein/sample) or chloramphenicol acetyltransferase (60 μg of protein/sample) measured as described previously (19, 38). To ensure reproducibility, experiments were repeated three to five times, using at least three different plasmid DNA preparations. Transfection efficiency, assessed from measurements of pRSVCAT activity, varied by <15% within a given experiment.

Immunoprecipitation and Kinase Assay—The cells were harvested in 1 ml of lysis buffer (20 mM Tris-HCl, pH 7.9, 137 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM EGTA, 10% glycerol, 10 mM NaF, 1 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1.5 μg/ml aprotinin, and 1 μg/ml pepstatin) and centrifuged at 12,000 × g for 30 min. Two-hundred μg of supernatant protein was incubated with 1 μg of anti-ERK2, anti-JNK1, or anti-HA antibody and 10 μl of protein G-Sepharose for 2 h at 4 °C. The immunoprecipitates were recovered by centrifugation and washed three times with cell lysis buffer and once with kinase reaction buffer (25 mM HEPES, pH 7.4, 10 mM MgCl2, 10 mM MnCl2, and 1 mM dithiothreitol) without ATP. Activities of MAPK and JNK were measured by adding 20 μg of myelin basic protein or 5 μg of glutathione S-transferase-c-Jun, respectively, to the immunoprecipitates in 30 μl of kinase reaction buffer containing 2 μCi of [γ-32P]ATP. Reactions were incubated for 15 min at 30 °C. The samples were electrophoresed on 15% SDS-polyacrylamide gels, which were then dried and subjected to autoradiography. Signals were identified and quantified by densitometric scanning of the autoradiographs.

Statistical Analysis—Data were evaluated by one-way analysis of variance with Newman-Keuls test for significance.
transcription start site. Of note, a transfected hANP-chloramphenicol acetyltransferase reporter was not activated by mechanical strain applied over a similar 48-h period (static: 1860 ± 628 cpm; strain: 2088 ± 513 cpm; n = 4), confirming the previous findings of Sadoshima et al. (22).

BNP mRNA is known to harbor a number of putative destabilizing regulatory elements within its 3′-untranslated region (40). It has been hypothesized, although not proven, that these elements are responsible for the short half-life (t1/2 ≈ 1 h) that this transcript exhibits in the intact cardiac myocyte (16). The abbreviated lifespan of this transcript, which clearly distinguishes it from ANP (half-life of 12–24 h) (16, 41), may represent a potentially important locus for regulating steady-state levels of BNP mRNA. Hanford et al. (16) demonstrated that the α-adrenergic agonist phenylephrine, a well described effector of hypertrophy in this in vitro model (14), not only increased BNP gene transcription, but stabilized the mature transcript as well. To assess the possibility of an independent effect of strain on transcript stability, we used actinomycin D to arrest transcription in these ventricular cardiocyte cultures and followed the decay of the BNP transcript in the presence or absence of mechanical strain. As shown in Fig. 2B, subjecting cells to cyclical strain in the absence of actinomycin D led to the expected increase in BNP mRNA levels. When actinomycin D was introduced concomitantly with the strain stimulus, the induction was lost; however, transcript levels fell steadily and equivalently in the static versus strained cultures. Thus, in contrast to observations made in the setting of phenylephrine-induced hypertrophy (16), the half-life of the BNP transcript (~12 h in this study) was unaffected by strain.

Previous investigations have shown that strain activates several different signaling modalities in cardiac myocytes, including phospholipases C, A2 and D; protein kinase C; MAPK; MAPK kinase; MAPK kinase kinase; tyrosine kinase; Raf-1; p21ras; pp90RSK; and stress-activated kinase (23, 26–28). We employed a number of pharmacological agents to probe the mechanism underlying strain-dependent BNP gene transcription. As shown in Fig. 3, transcription was inhibited by antagonists of protein kinase C (chelerythrine), MAPK (2-aminopurine), MEK (PD98059), tyrosine kinase (genistein), and calmodulin (W-7). KN-62, a calcium/calmodulin kinase inhibitor, partially reversed the strain effect. Neither wortmannin, an inhibitor of phosphatidylinositol 3-kinase, nor H-89, a protein kinase A antagonist, affected reporter activity. Measurement of MAPK (Fig. 4A) using an immune complex kinase assay (42) revealed a pattern of inhibition that was very similar to that observed for BNP promoter activity (i.e. the group, KN-62, wortmannin, and H-89 were the least effective in reducing strain-dependent MAPK activity). Measurement of JNK/SAPK activity (Fig. 4B) using the same approach showed that several inhibitors that nearly completely suppressed strain-dependent BNP promoter activity had little or no effect on strain-dependent SAPK activity (e.g. chelerythrine, 2-aminopurine, and PD98059), whereas KN-62, which was only marginally effective in suppressing the hBNP promoter, almost completely blocked strain-dependent JNK/SAPK activity.
mediating the strain effect, we cotransfected dominant-negative mutants of Ras, MEK (MAPK kinase), or SEK (SAPK kinase) together with the hBNP-luciferase reporter and subjected the cultures to mechanical strain. As shown in Fig. 6, each of these mutants effected a dose-dependent reduction in strain-responsive reporter activity. At the higher concentration of the mutant vectors (5 μg), basal activity of the reporter was reduced moderately, whereas the strain-dependent increment was completely suppressed.

**DISCUSSION**

Mechanical stimuli have been shown to be potent regulators of gene expression in the cardiovascular system. Shear stress and cyclical mechanical strain represent important components of the normal homeostatic mechanisms that regulate gene expression in the endothelium (43-47), vascular smooth muscle (48), and myocardium (21, 22, 49). Aberration of this regulatory activity may contribute to the pathological changes that accompany hypertrophy.

We found that cyclical mechanical strain evoked increases in BNP secretion, steady-state levels of the BNP transcript, and activation of a transected BNP promoter-driven luciferase reporter in primary cultures of neonatal rat ventriculocytes. This provides the first demonstration that mechanical strain increases steady-state levels of a myocardial specific gene through a transcriptional mechanism.

Studies from Komuro et al. (21) and Sadoshima et al. (22) demonstrated stretch-dependent transcriptional activation of the ubiquitously expressed c-fos gene promoter. This activation required the participation of a serum response element positioned at approximately position −300 in the c-fos promoter (23). Other studies have demonstrated stretch-dependent increases in ANP secretion and gene expression (22, 49), but failed to document stimulation of ANP promoter activity, a finding that we have confirmed here. Analogous findings were obtained in an in vivo model of hypertrophy using a transgenic mouse bearing an hANP-driven T antigen reporter developed by Field (50). The 500 base pairs of hANP gene promoter included in this construct were sufficient to target expression of the transgene to the atrial myocardium, but failed to respond to hemodynamic overload in a ligated aorta model (24) despite a
robust stimulation of the endogenous mouse ANP transcript. Similar findings have been reported using transgenic mice bearing up to 3 kilobase pairs of rat 5′-flanking sequence linked to either chloramphenicol acetyltransferase or luciferase reporters (25). These findings imply either that strain- and/or load-dependent increments in steady-state ANP transcript levels depend exclusively on post-transcriptional mechanisms or, more likely, that the transcriptional regulatory elements responsible for conferring sensitivity to strain are located outside the 3 kilobase pairs of sequence included in these reporter constructs.

Somewhat to our surprise, strain had no effect on the stability of the BNP transcript. The data presented in Figs. 1 and 2 indicate that steady-state BNP mRNA levels and BNP promoter activity are increased to an equivalent degree by mechanical strain, implying that the former can be attributed entirely to increased mRNA synthesis. Furthermore, following suspension of RNA polymerase II activity with actinomycin D, the data presented in Figs. 1 and 2 indicate that steady-state BNP mRNA levels and BNP protein expression can be assigned with certainty. Thus, while the pharmacological studies imply a close tie between MAPK and the strain response, there are sufficient data to implicate both pathways in signaling the events leading to activation of the BNP promoter. Additional studies will be required before the relative contribution of each to the strain response can be assigned with certainty.

In summary, we have demonstrated that BNP gene promoter activity, which undergoes a selective and robust activation in cardiac hypertrophy, is stimulated by cyclical mechanical strain in vitro. This model may prove useful in dissecting the molecular events that underlie the changes in gene expression and protein synthesis that accompany hypertrophy in the intact animal.

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