Mechanical strain activates *BNP* gene transcription through a p38/NF-κB–dependent mechanism

Faquan Liang and David G. Gardner

Metabolic Research Unit and Department of Medicine, University of California–San Francisco, San Francisco, California 94143, USA

Address correspondence to: David G. Gardner, Metabolic Research Unit and Department of Medicine, Box 0540, University of California–San Francisco, San Francisco, California 94143-0540, USA. Phone: (415) 476-2729; Fax: (415) 476-1660; E-mail: gardner@itsa.ucsf.edu.

Application of mechanical strain to neonatal rat ventricular myocytes in culture evokes changes in gene expression reminiscent of those that occur with hypertrophy in vivo, such as stimulation of brain natriuretic peptide (*BNP*) gene expression. Here, we show that a major component of strain-dependent *BNP* promoter activation results from stimulation of p38 mitogen-activated protein kinase (MAPK) in the cardiac myocyte. Strain increased p38 activity in a time-dependent fashion. The p38 inhibitor SB203580 led to a reduction of approximately 60% in strain-activated human *BNP* (*hBNP*) promoter activity. Cotransfection of wild-type p38 increased both basal and strain-dependent promoter activity, while cotransfection with MKK6AL, a dominant-negative inhibitor of p38 MAPK kinase, resulted in partial inhibition of either p38- or strain-activated *hBNP* promoter activity. p38 MAPK increased *hBNP* promoter activity through activation of the transcription factor NF-κB. Activation of the *hBNP* promoter by either p38 or strain was mediated by DNA elements present in the 5′ flanking sequence of the gene. Mechanical strain promoted assembly of NF-κB components on these DNA elements in vitro. Thus, induction of the *hBNP* promoter by mechanical strain depends, at least in part, on stimulation of p38 and subsequent activation of NF-κB. This activation may play an important role in signaling the increased *BNP* gene expression that accompanies hemodynamic overload and cardiac hypertrophy in vivo.

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Introduction

Sustained hemodynamic overload elicits a series of functional and structural changes in the ventricular myocyte that culminate in cardiac hypertrophy. This is viewed as a compensatory response that, over the short term, results in improved cardiac performance; however, protracted exposure to the hypertrophic stimulus often results in an alteration in phenotype that leads to progressive heart failure. Clinical hypertrophy has, in fact, been linked to increased mortality independent of associated cardiovascular risk factors (1).

At the cellular level, hypertrophy is thought to develop in response to a combination of mechanical (i.e., load-dependent) and neurohumoral stimuli, such as angiotensin II (AII), endothelin (ET), and adrenergic agonists. In cultured neonatal rat cardiac myocytes, both mechanical (2–4) and biochemical (5–8) stimuli effect a series of changes in gene expression that closely parallel those seen in the hypertrophied heart in vivo (9). This includes the sequential activation of immediate early genes (e.g., protooncogenes like *c-jun*, *c-fos*, and *c-myc*), a fetal gene program (e.g., atrial natriuretic peptide [ANP], brain natriuretic peptide [BNP], skeletal α-actin, and β-myosin heavy chain) that is typically quiescent in the nonhypertrophied adult ventricular myocardium, and the structural sarcomeric genes that contribute the protein infrastructure associated with hypertrophy (e.g., cardiac α-actin and myosin light chain-2) (9).

The signaling cascades underlying the hypertrophic phenotype remain only partially understood. Protein kinase C (10), calcium/calmodulin (11), calcineurin (12–13), nonreceptor protein tyrosine kinases (14, 15), the Janus kinase/STAT system (16, 17), small G proteins (e.g., Ras, Rac, Rho, Cdc42) (18–21), and the various mitogen-activated protein kinases (i.e., ERKs, JNKs, and p38 MAPKs) (22–38) have each been implicated as playing a role in signaling hypertrophy. The MAPKs in particular have been the focus of considerable attention. Hypertrophic stimuli have been shown to activate ERK (22–24), JNK (30, 32), and p38 (34, 37, 38) in either cultured ventricular myocytes or intact myocardial tissue. Whereas interference with the ERK pathway has been shown to block some aspects of hypertrophy-dependent gene expression, it does not affect the morphological changes accompanying hypertrophy (27); in some instances (29) it has been dissociated from the hypertrophic program completely. JNKs have also been linked to hypertrophy (28–32), although recent studies have raised questions about the importance of these kinases in signaling various aspects of the phenotype (37). Activation of p38 by upstream kinases (e.g., MKK6) (35) or hypertrophic agonists (38) has been associated with effects on myocyte growth. Of note, the nature of the effect appears to be isoform specific. Overexpression of activated MKK3 (MKK3bE) elicited both characteristic hypertrophic changes and increased apoptosis in ade-

noviral vector–infected neonatal myocytes (33). The hypertrophic response was amplified by coinfection with wild-type p38β, whereas apoptosis was increased with wild-type p38α. It should be noted that, as with the ERKs and JNKs, some published studies argue against a major role for p38 in hypertrophy (32). Thus, whereas several signaling pathways appear to be involved, at present there is no consensus regarding the identity of the pathway or pathways that dominate initiation and maintenance of the hypertrophic phenotype in the cardiac myocyte.

We have recently shown that application of mechanical strain to neonatal rat ventricular myocytes in vitro results in increased expression of the *BNP* gene product and secretion of the encoded protein (4). This increase in gene expression results, in large part, from increased *BNP* gene promoter activity and seems to depend upon intact ERK and JNK signaling cascades. It is noteworthy that approximately half of the increase in promoter activity represents a primary response to the strain stimulus (39). The remainder results from activation of a local autocrine/paracrine system (or systems). This system links increased angiotensinogen- and angiotensin-converting enzyme gene expression to local generation of angiotensin II and subsequently of

endothelin. Endothelin receptors are present on cardiac myocytes (40), and presumably signal the increase in *BNP* promoter activity (39).

In this study, we have examined the ability of mechanical strain to stimulate p38 activity in the neonatal rat ventricular myocyte model, and have explored the relationship of this activation to the subsequent increase in *BNP* gene promoter activity. Our findings suggest that p38 plays a major role in signaling strain-dependent activation of this promoter, and that it does so at least in part through specific *cis*-acting sites — shear stress response elements (SSREs) — present in the 5′ flanking sequence of the *BNP* gene.

Methods

Materials. SB203580 was a gift from J. Lee of SmithKline Beecham Pharmaceuticals Inc. (King of Prussia, Pennsylvania, USA). PD98059 was obtained from Research Biochemicals International (Natick, Massachusetts, USA). Bovine myelin basic protein (MBP) was purchased from Upstate Biotechnology Inc. (Lake Placid, New York, USA). Polyclonal antibodies against p38, p50, and p65 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). Anti-ACTIVE p38 polyclonal antibody and the luciferase assay system were purchased from Promega Corp. (Madison, Wisconsin, USA). NEN Life Science Products Inc. (Boston, Massachusetts, USA) provided [γ-^{32}P]ATP. The enhanced chemiluminescence (ECL) Western blotting detection system was purchased from Amersham Life Sciences Inc. (Arlington Heights, Illinois, USA). Other reagents were obtained through standard commercial suppliers.

Cell culture and application of mechanical strain. Ventricular myocytes were prepared from neonatal rat hearts (1–2 days old) by alternate cycles of 0.05% trypsin digestion and mechanical disruption as described previously (41). Cells (10^6) were cultured on collagen-coated Flex plates (Flexcell International Corp., McKeesport, Pennsylvania, USA) in DME-H21 medium containing 10% BCS (HyClone Laboratories Inc., Logan, Utah, USA), 2 mM glutamine, 10 U/mL penicillin, and 100 mg/mL streptomycin. A glass cloning cylinder (1 cm in diameter) was placed in the middle of each well to preclude cell attachment, thereby placing the majority of adherent cells on the outer 75% of the culture surface where distension is maximal (42). The medium was changed 24 hours before initiation of the experiment. Cells were subjected to cyclical strain (60 cycles/minute) on the Flexcell Strain apparatus (Flexcell International Corp.) at a level of distension sufficient to promote an increment of approximately 20% in surface area at the point of maximal distension on the culture surface (42).

Plasmid construction and site-directed mutagenesis. The construction of –904 and –1,595 *hBNP* luciferase (hBNPLuc) has been described previously (43). The wild-type p38α expression vector pCMV-FLAG-p38MAPK (44) was provided by R. Davis (University of Massachusetts, USA). MKK6AL pcDNA3, a dominant-negative MKK6 mutant (45), was provided by J.R. Woodgett (Universtiy of Toronto, Toronto, Canada). The latter replaces Ser 207 and Thr 211 in MKK6 with
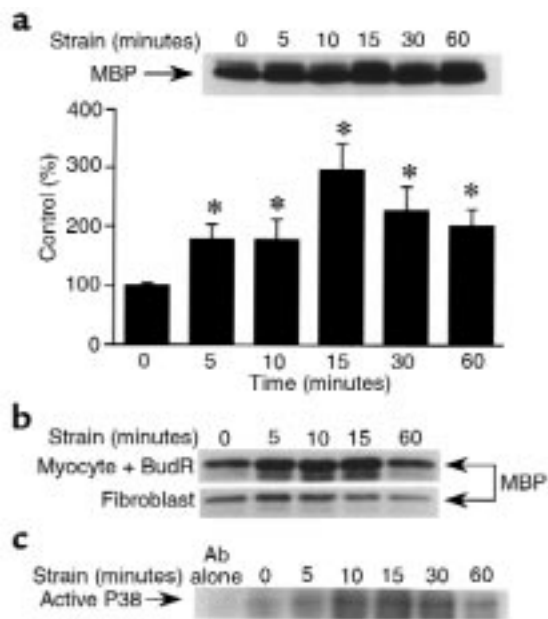

Figure 1
Mechanical strain activates p38 in cultured neonatal rat ventricular myocytes. After 48 hours of culture, cells were subjected to cyclical strain for the times indicated. Cells were then collected, lysed, and assayed for p38 kinase activity using either an immune complex kinase assay (**a** and **b**) or Western blot analysis for the activated form of the enzyme (**c**). (**a**) Time course of p38 induction in conventional myocyte cultures; pooled data from 3 independent experiments. (**b**) p38 induction in cultures further enriched for myocytes after 48 hours of treatment with bromodeoxyuridine (BudR; 0.1 mM) or nonmyocytes (primarily fibroblasts) collected from the same neonatal hearts and expanded in culture over a 2-week period. Representative of duplicate experiments. (**c**) Cells were subjected to mechanical strain for varying periods of time, then assayed for active p38 using Western blot analysis. Representative of duplicate experiments. Data are presented as mean ± SD. *$P < 0.01$ vs. control.
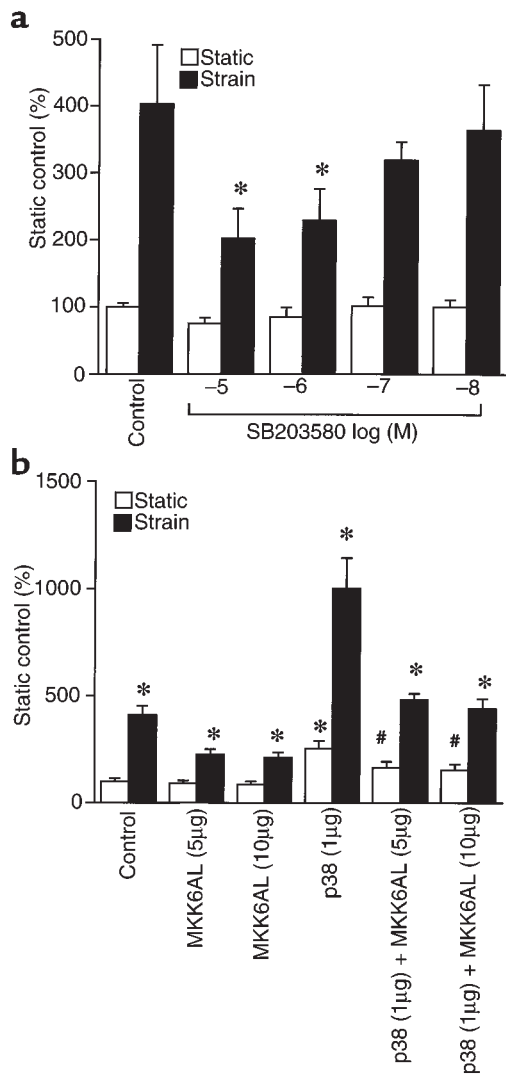
a

b

Figure 2
Effects of SB203580, MKK6AL, and wild-type p38 on strain-dependent *hBNP* promoter activity in neonatal rat ventricular myocytes. (**a**) Cells were transfected with 1 µg of –1,595 *hBNP* luciferase. After 24 hours of culture, cells were subjected to cyclical strain for 48 hours in the presence of different concentrations of SB203580. (**b**) Cells were cotransfected with 1 µg of –1,595 *hBNP* luciferase and 2 concentrations of MKK6AL with or without 1 µg of pCMV-FLAG-p38MAPK. After 24 hours of culture, cells were subjected to cyclical strain for 48 hours. Data are presented as mean ± SD of 4 separate experiments. *$P < 0.01$ vs. strain control.

Site-directed mutagenesis was carried out with the QuikChange kit (Stratagene, La Jolla, California, USA) using conditions recommended by the manufacturer. In brief, mixtures containing 10–50 ng of –904 *hBNP* luciferase, 2 complementary mutagenic primers, dNTPs, and *Pfu* DNA polymerase were added to the PCR buffer. PCR was carried out for 16–18 cycles using 30 seconds of denaturation at 95°C, 1 minute of annealing at 55°C, and 2 minutes/kb of extension at 68°C. After PCR, 1 µL of *Dpn I* was added to the reaction to cut parental DNA template, and 5 µL of this digest was used for transformation. Several candidate clones were identified by restriction mapping and were sequenced using a DNA sequence kit and [α-^{35}S]dATP obtained from Amersham Life Sciences Inc. The oligonucleotides for site-directed mutagenesis (mutagenized primers) were as follows (mutagenized bases are identified by lowercase letters): mSSRE-1: 5′-GGTCGGCTCTGCCC-tacagtCACCTCCCACGTCG-3′; mSSRE-2: 5′-GAAAG-GTCTCGGAtacagtTTGTCCTTCGTCC-3′; mSSRE-3: 5′-GAGCATAGGGAAAtacagtGGAGGTCTCTTGTC-3′; mSSRE-2,3: 5′-GTGAGAGCATAGGGAAAtacagtGGAt-acagtTTGTCCTTGCTCCACG-3′.

Transfection and luciferase assay. Freshly prepared ventricular myocytes were transiently transfected with the indicated reporters and expression vectors by electroporation (Gene Pulser; Bio-Rad Laboratories, Richmond, California, USA) at 280 mV and 250 µF. DNA content in individual cultures was normalized with pUC 18 plasmid. After transfection, cells were plated and cultured as described above. Cells were harvested and lysed in 100 µL of cell culture lysis reagent (Promega Corp.). The protein concentration of each cell
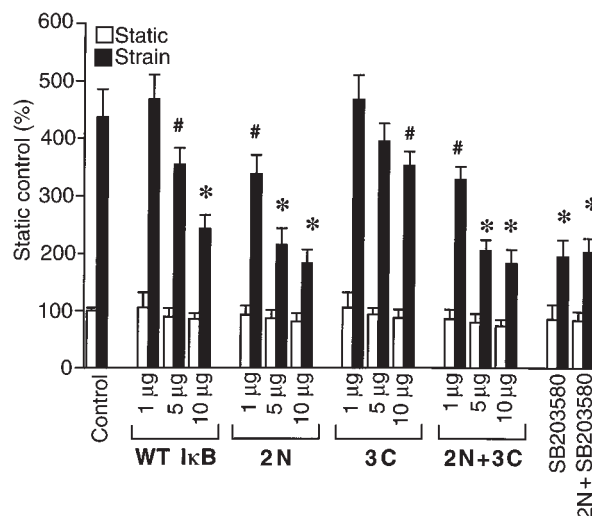

Figure 3
Effects of IκBα mutants on strain-dependent *hBNP* promoter activity in neonatal rat ventricular myocytes. Cells were cotransfected with 1 µg of –1,595 *hBNP* luciferase and different concentrations of either wild-type (WT) IκBα or the mutant indicated (2N, 3C, or 2N + 3C). After 24 hours of culture, cells were subjected to cyclical strain for 48 hours. A separate subgroup was treated with 10 µM SB203580 before strain. The data are expressed as mean ± SD of 4 separate experiments. *$P < 0.01$ vs. strain control. #$P < 0.05$ vs. strain control.

alanine and leucine, respectively. These changes preclude phosphorylation and activation of the mutant kinase and permit it to function in a dominant-negative mode. Wild-type IκBα and IκBα mutants 2N, 3C, and 2N+3C (46) were provided by J. Hiscott (McGill University, Montreal, Quebec, Canada). NF-κB TKLuc, harboring a single copy of an NF-κB binding site [5′-AGGGACTTTCCGCTGGG-ACTTTCC-3′] upstream from a core (position –45 to +53), thymidine kinase promoter/luciferase reporter, came from D. Leitman (University of California–San Francisco, San Francisco, California, USA). TKLuc contains 109 bp of promoter sequence from the thymidine kinase gene (position –109 to +53) linked to the luciferase reporter.
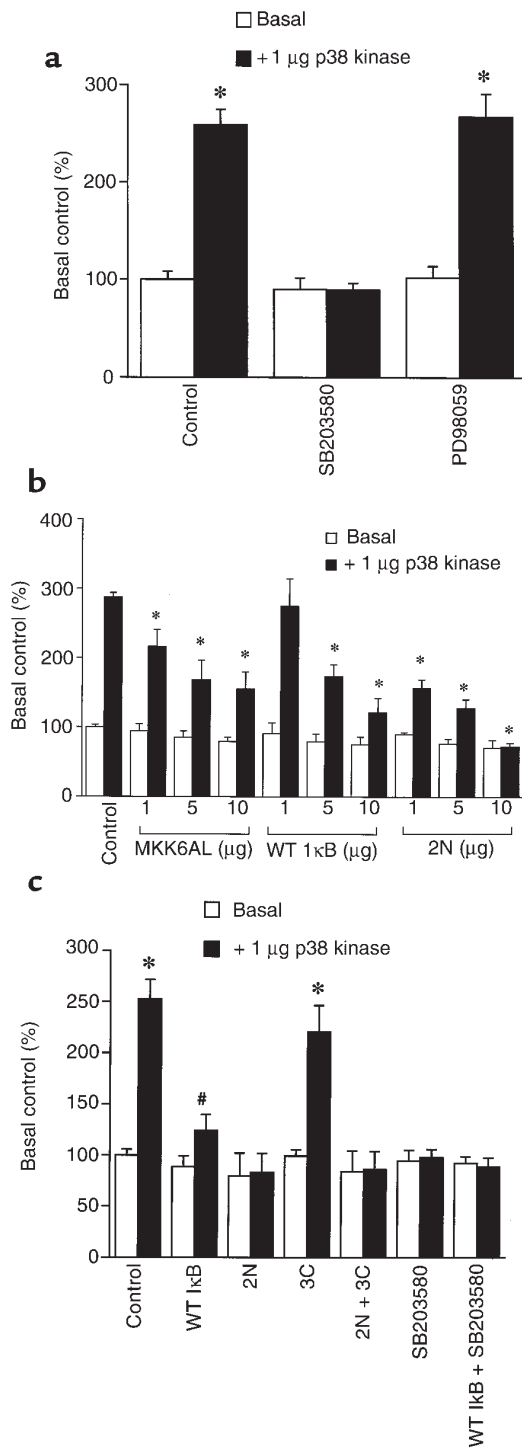
Figure 4

Effects of IκBα mutants and MKK6AL on p38-activated *hBNP*Luc or NF-κB TKLuc activity in neonatal rat ventricular myocytes. (**a**) Cells were cotransfected with 1 μg of –1,595 *hBNP* luciferase and 1 μg of pCMV-FLAG-p38MAPK. After 24 hours of culture, cells were treated with 10 μM SB203580 or 10 μM PD98059. (**b**) Cells were cotransfected with 1 μg of –1,595 *hBNP* luciferase, 1 μg of pCMV-FLAG-p38MAPK, and different concentrations of MKK6AL, WT IκBα, or the 2N mutant of IκBα. (**c**) Cells were cotransfected with 10 μg of NF-κB TKLuc, 1 μg of pCMV-FLAG-p38MAPK, and 10 μg of WT IκBα or one of the relevant mutants (2N, 3C, or 2N + 3C). A separate subgroup was treated with 10 μM SB203580. Data are presented as mean ± SD of 4 separate experiments. *$P < 0.01$ vs. basal control. #$P < 0.05$ vs. basal control.

extract was measured using Coomassie protein reagent (Pierce Chemical Co., Rockford, Illinois, USA). Cell lysates were processed (20 μg protein per sample) and assayed for luciferase as described using a commercially available kit (Promega Corp.). To ensure reproducibility, experiments were repeated 3–5 times.

Immunoprecipitation and kinase assay. Cells were harvested in 1 mL of lysis buffer (20 mM Tris-HCl at 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 PMSF, 1.5 μg/mL aprotinin, and 1 μg/mL pepstatin) and centrifuged at 12,800 *g* for 30 minutes. Two hundred micrograms of supernatant protein was incubated with 1 μg of anti-p38 antibody and 10 μL protein G–Sepharose (Amersham Pharmacia Biotech Inc., Piscataway, New Jersey, USA) for 2 hours at 4°C. The immunoprecipitates were recovered by centrifugation, and then washed 3 times with cell lysis buffer and once with a kinase reaction buffer (25 mM HEPES at pH 7.4, 10 mM MgCl$_2$, 10 mM MnCl$_2$, and 1 mM DTT) without ATP. Twenty micrograms of myelin basic protein was then added to the immunoprecipitates in 30 μL of kinase reaction buffer containing 2 μCi [γ-^{32}P]ATP. Reactions were incubated for 15 minutes at 30°C. Total reaction contents were electrophoresed on 15% SDS-polyacrylamide gels that were then dried and subjected to autoradiography. Autoradiographic signals were quantified using the NIH Image program.

Western blot analysis. Immunoprecipitation using the anti-ACTIVE® p38 polyclonal antibody was carried out as described above. Immunoprecipitates were boiled for 5 minutes, electrophoresed on 10% SDS-polyacrylamide gels, and electrophoretically transferred onto Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). The membranes were incubated with rabbit anti-ACTIVE® p38 polyclonal antibody (1:2,000 dilution) in TBST buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20, and 5% nonfat dried milk) for 2 hours at room temperature with agitation, and then washed 3 times with TBST buffer. Membranes were later incubated with horseradish peroxidase–conjugated goat anti-rabbit antibody for 1 hour at room temperature and washed 3 times in TBST buffer. The blots were soaked for 1 minute in ECL Detection Reagent and exposed to ECL Hyperfilm (Amersham Pharmacia Biotech) for varying lengths of time. Signals were identified and quantified using NIH Image.

Preparation of nuclear extracts. Ventricular myocytes were subjected to mechanical strain for varying lengths of time. Nuclear extracts were prepared using a modification of the method of Han and Brasier (47). Cells were harvested and lysed with buffer A (10 mM HEPES at pH 7.9, 1.5 mM MgCl$_2$, 10 mM KCl, 0.5% NP-40, 1 mM DTT, 1 mM PMSF, 5 μg/mL leupeptin, and 5 μg/mL aprotinin) on ice for 10 minutes. Lysates were centrifuged for 5 minutes at 4°C. Particulates were resuspended in buffer B (1 M sucrose, 10 mM HEPES at pH 7.9, 1.5 mM MgCl$_2$, 10 mM KCl, 1 mM DTT, and the above protease inhibitors) and centrifuged at 12,800 *g* for 3 minutes at 4°C. Pelleted nuclei were resuspended in buffer C (20 mM HEPES at pH 7.9, 420 mM NaCl, 1.5

Group	Static	Strain
Control	100 ± 3	107 ± 11
PD98059 (10 μM)	98 ± 15	94 ± 12
SB203580 (10 μM)	96 ± 17	93 ± 13
p38 (5 μg)	105 ± 19	108 ± 12
WT IκB (10 μg)	94 ± 15	93 ± 14
2N (10 μg)	102 ± 16	101 ± 16
3C (10 μg)	91 ± 7	96 ± 10
2N + 3C (10 μg)	93 ± 8	93 ± 6

Cells were transfected with 10 μg of –109 TKLuc alone or in combination with 5 μg
of pCMV-FLAG-p38MAPK or 10 μg of the IκB mutants. After 24 hours of culture, cells
were subjected to strain for 48 hours in the presence or absence of 10 μM SB203580
or 10 μM PD98059. Data represent mean ± SD of 3 separate experiments.

mM MgCl$_2$, 0.2 mM EDTA, 25% glycerol, and the above protease inhibitors) and kept on ice for 30 minutes. Nuclear suspensions were centrifuged at 12,800 *g* for 15 minutes and the supernatant was saved. Nuclear extracts were stored at –80°C until use.

Electrophoretic mobility shift assay. Oligonucleotides used for electrophoretic mobility shift assay were as follows (SSRE sequences are underlined, and mutagenized bases are identified by lowercase letters): wild type: 5′- GTGAGAGCATAGGGAAAGGTCTCG-GAGGTCTCTTGTCCTTGCTCCACG-3′, and mutant: 5′-GTGAGAGCATAGGGAAAtacagtGGAtacagtTTGTC-CTTGCTCCACG-3′. Nuclear extracts (10 μg) were incubated in binding reaction buffer (10 mM HEPES at pH 7.9, 50 mM KCl, 0.2 mM EDTA, 2.5 mM DTT, 10% glycerol, and 0.05% NP-40) containing 0.5 μg poly(dI-dC) and ^{32}P-endlabeled, double-stranded, wild-type oligonucleotide at room temperature for 30 minutes. For competition experiments, a 10- or 100-fold molar excess of unlabeled, double-stranded oligonucleotide was added to the binding reaction. For immunoperturbation experiments, nuclear extracts were preincubated on ice for 1 hour with 1 μg polyclonal antibody directed against NF-κB1 (p50) or RelA (p65). All samples were resolved on 5% nondenaturing polyacrylamide gels. Gels were dried and exposed to x-ray film.

Statistics. Data were analyzed by ANOVA using the Newman-Keuls test to assess significance.

Results

We used a conventional immune complex kinase assay to quantitate p38 activity after application of mechanical strain to neonatal rat ventricular myocytes in vitro. The strain stimulus effected a time-dependent increment in p38 activity (Figure 1a). The increase was first noticeable after 5 minutes, peaked at 15 minutes, and remained elevated after 60 minutes of mechanical strain. Activation of p38 was seen in myocyte-enriched cultures treated with bromodeoxyuridine, and was seen to a much lesser extent in fibroblasts cultured from the same neonatal hearts (Figure 1b). This indicates that the observed p38 induction occurs predominantly in the

myocytes (as opposed to the nonmyocytes) in these cultures. Activation of p38 was confirmed by Western blot analysis using an antibody that selectively recognizes the activated form of the kinase. Again, the induction was apparent at 5 minutes, peaked at 10–15 minutes, and was still apparent after 1 hour of exposure to the strain stimulus (Figure 1c).

Next, we asked whether this increment in p38 activity could be linked to the strain-dependent increment in *hBNP* promoter activity that we have documented previously in this system (4, 39). As shown in Figure 2a, SB203580 effected a partial (~60%), but dose-dependent reduction in strain-activated *hBNP* promoter activity. The p38 inhibitor had no effect on basal expression of this promoter. Cotransfection of MKK6AL, a dominant-negative mutant of MKK6 (an activating kinase positioned upstream from p38) also resulted in partial reduction in strain-activated *hBNP* promoter activity (Figure 2b). Again, the mutant had no effect on basal activity. Forced expression of wild-type p38 increased both basal and strain-dependent *hBNP* promoter activity, whereas MKK6AL effected a partial reversal of each. Independent experiments confirmed that the effects of SB203580 and MKK6AL were largely confined to the p38 (as opposed to the JNK) signaling pathway (data not shown). Collectively, these data indicate that the strain-dependent increase in p38 activity noted above participates directly in the activation of *hBNP* gene transcription.

It has been shown that p38 signals transcriptional activity through activation of NF-κB in some (48, 49) but not all (50) systems. Activation of NF-κB is determined in part by the phosphorylation status of the inhibitory protein IκB. IκB associates with NF-κB in the cytoplasmic compartment, precluding activation and entry of the latter into the nucleus. Activation of NF-κB occurs after phosphorylation of IκB on key serine residues (Ser 32 and Ser 36) at the amino terminus of the molecule. Phosphorylation marks IκB for ubiquination and subsequent degradation through the proteasome pathway (51). This frees NF-κB to enter the nuclear compartment, associate with its cognate recognition element, and stimulate transcription.

To investigate the role of NF-κB in signaling the p38 effect, we introduced an hBNPLuc reporter into ventricular myocytes together with either wild-type IκB or an IκB phosphorylation-defective mutant (i.e., a constitutive suppressor of NF-κB activity) before applying the strain stimulus. Wild-type IκB led to a concentration-dependent reduction (~50%) in strain-associated, but not basal, reporter activity (Figure 3). 2N, an IκB mutant that is modified at 2 amino termi-
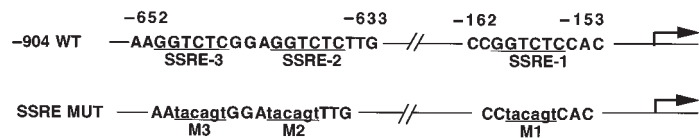
Figure 5
Identification of SSRE-like structures in the 5′ flanking sequence of the *hBNP* gene (–904 WT). Positioning of the 3 elements relative to the transcription start site are indicated. Putative SSRE sequences are underlined. Mutated bases in each of the 3 SSRE mutant reporters (SSRE MUT) are indicated in lowercase letters.
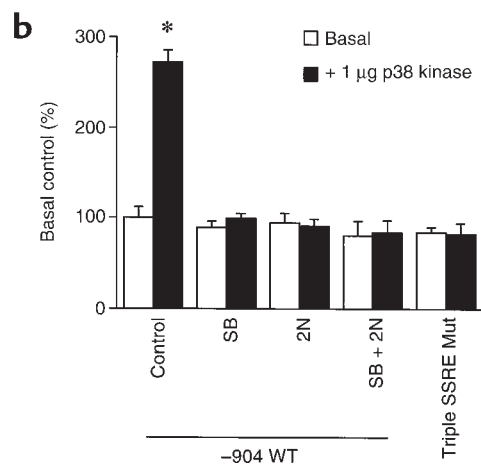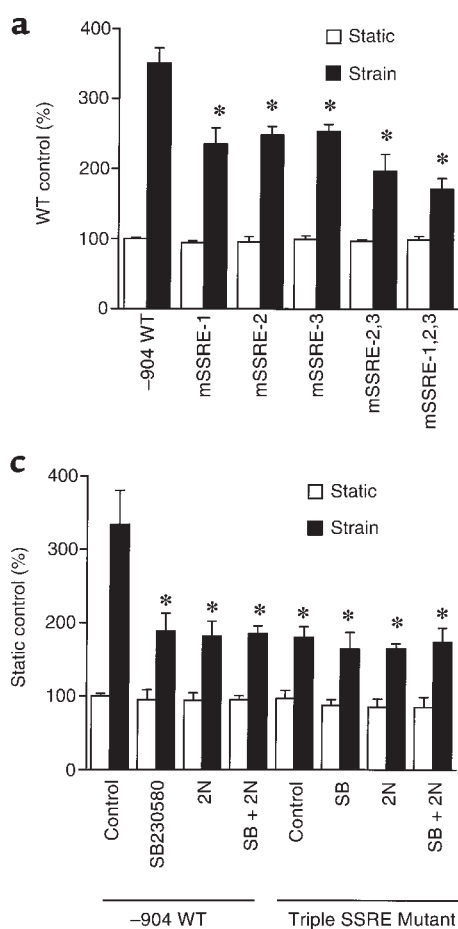
Figure 6
Effects of mutations in putative SSRE sequences on *hBNP* promoter activity. (**a**) Wild-type –904 hBNPLuc or site-directed mutants targeted at 1 or more of the SSREs described in Figure 5 were introduced into ventricular myocytes. After 24 hours, cells were subjected to mechanical strain for 48 hours; lysates were generated and analyzed for luciferase activity. (**b**) One microgram of wild-type –904 hBN-PLuc or the triple SSRE mutant was cotransfected into ventricular cells with 1 µg of pCMV-FLAG-p38MAPK alone or in combination with 10 µg of the 2N mutant. Where indicated, cells were treated with 10 µM SB203580 for 48 hours. (**c**) Cells were cotransfected with 1 µg of wild-type –904 hBNPLucc or a –904 hBNPLuc harboring a triple SSRE mutation, and 10 µg of the 2N mutant of IκBα. After 24 hours of culture, cells were subjected to mechanical strain for 48 hours in the presence or absence of 10 µM SB203580, as indicated. Seventy-two hours after transfection, cells were harvested and luciferase activity was measured. Data are presented as mean ± SD of 3 separate experiments.

nal serine residues (Ser 32 and Ser 36) that are thought to be the targets for the IκB kinase(s), effected a concentration-dependent reduction in stress-dependent *hBNP* promoter activity that was even more pronounced than that seen with wild-type IκB. 3C, an IκB mutant that targets Ser 283, Thr 291, and Thr 299 at the carboxyl terminus of IκB — residues that are not thought to be involved in the ubiquination/proteasome degradation pathway (46) — had essentially no effect, possibly reflecting the limited stability of this protein in intact cells. The combination mutant (2N+3C) was no more effective than 2N alone. Of interest, the maximal inhibition seen with the 2N mutant was roughly equivalent to that seen with SB203580, and the 2 were not additive, implying that p38 and NF-κB operate over shared pathways in contributing to the *hBNP* transcriptional response.

The p38-dependent increment (2.5- to 3-fold) in *hBNP* promoter activity (Figure 4a) was blocked completely by SB203580, but was unaffected by PD98059. The response was also partially inhibited by MKK6AL, by wild-type IκB, and even more effectively by the 2N mutant of IκB, which completely blocked the response to p38 (Figure 4b). A similar result was obtained when p38 was cotransfected with NF-κB TKLuc, a reporter plasmid harboring an NF-κB recognition sequence placed immediately upstream from the core thymidine kinase promoter (Figure 4c). A 2.5-fold induction of

this reporter was effected by p38. This was inhibited partially by wild-type IκB and was completely blocked by the 2N mutant and SB203580.

Nonspecific effects on transcriptional activity in these cultured myocytes were not seen after treatment with PD98059 or SB203580, or after cotransfection with p38 or the different IκB mutants. None of these perturbations had a significant effect on the activity of the transfected thymidine kinase promoter/luciferase reporter –109 TKLuc (see Table 1).

NF-κB has been implicated as playing a role in mediating shear stress activation of the PDGF receptor (52, 53). This activation involves the interaction of NF-κB with the so-called SSRE(s) in the proximal promoter of that gene. We scanned the *hBNP* 5′ flanking sequence for elements with potential homology to the SSRE, and identified 3 sites within 700 bp of the transcription start site. Two of these, positioned between –652 and –633, are arrayed as a direct repeat (Figure 5). To investigate the role of each of these candidate sites in signaling either p38- or strain-dependent *hBNP* promoter activity, we introduced site-directed mutations (Figure 5) into each candidate sequence, alone or in combination, and examined the effect of these mutations on both basal and strain-activated promoter activity. As shown in Figure 6a, mutation of each of the SSREs individually led to a near-equivalent reduction in the response to strain. Mutation of the upstream SSREs

(sites 2 and 3) effected a further reduction, whereas mutation of all 3 sites led to the maximal level of inhibition observed (~60% of the wild-type response). Wild-type p38 stimulated the –904 hBNPLuc reporter almost 3-fold (Figure 6b). As with the –1,595 reporter (see Figure 4), this stimulation was blocked by treatment with SB203580 or cotransfection with the IκB 2N mutant. Introduction of mutations at each of the SSREs identified in Figure 5 resulted in complete abrogation of the p38 effect. Similarly, the induction by mechanical strain (~3.5-fold) was inhibited to an equivalent degree (~60%) by treatment with SB230580, cotransfection with the IκB mutant (2N), and introduction of the triple mutation (sites 1–3) into the hBNP promoter (Figure 6c). Of note, none of the 3 perturbations proved to be additive with the others, implying use of a shared signaling pathway.

As noted above, NF-κB is typically concentrated in the cytoplasmic compartment in the inactivated state. Activation is followed by translocation to the nucleus and association with cognate DNA recognition elements (50). If our model is correct, we would predict that application of mechanical strain should promote interaction of NF-κB with the SSREs present in the proximal hBNP gene promoter. As shown in Figure 7a, application of strain led to a time-dependent increase in the association of myocyte nuclear protein with the SSRE direct repeat (sites 2 and 3) in the electrophoretic mobility shift assay. This interaction was blocked by increasing concentrations of unlabeled wild-type SSRE sequence, but not by mutant SSRE sequence (Figure 7b). In addition, inclusion of antibody directed against the p50 component or against the RelA component p65 resulted in a supershift of the strain-induced complex. Collectively, these data indicate that mechanical strain promotes a time-dependent association of NF-κB with specific SSRE-like sequences in the hBNP promoter, and that this association results in increased promoter activity.

Discussion

We have documented previously that mechanical strain is a potent activator of hBNP gene transcription in neonatal rat ventricular myocytes (4, 39). The present work extends those findings in demonstrating that (a) mechanical strain, like other hypertrophic agonists (34, 38), increases p38 activity in these cells; (b) activation of p38 plays an important role in mediating strain-dependent hBNP promoter activity; and (c) p38 induction of hBNP promoter activity operates through an NF-κB–dependent pathway that targets 3 SSRE-like structures in the 5′ flanking sequence of the hBNP gene.

Considerable recent effort has been devoted to the identification of signaling pathways linking various mechanical and biochemical stimuli (e.g., phenylephrine, ET, AII, or mechanical strain) to the phenotypic changes (e.g., increased cell size and sarcomeric assembly) and changes in gene expression (e.g., increased ANP, BNP, skeletal α-actin, and β-myosin heavy chain expression) that accompany hypertrophy. Intracellular calcium (11), protein kinase C (10), nonreceptor protein tyrosine kinases (14, 15), calcineurin (12,

13), the small G proteins (18–21), and various members of the extended MAPK family (22–38) have been suggested as candidate mediators of various aspects of the hypertrophic phenotype. In the case of the MAPKs, the data have to some degree been inconclusive.
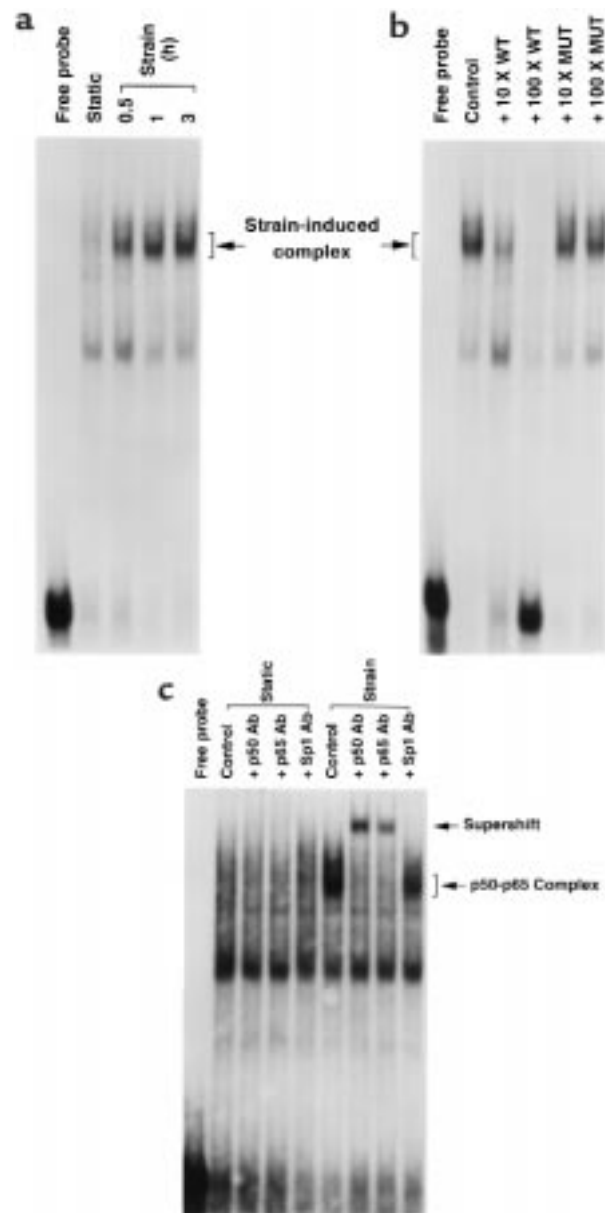

Figure 7
Nuclear extracts from myocytes subjected to mechanical strain interact with SSRE-like sites in hBNP promoter. (**a**) Cells were subjected to strain stimulus for different periods of time. Cells were harvested and nuclear extracts were prepared. Ten micrograms of nuclear extract was incubated with ^{32}P-labeled oligonucleotide encoding SSRE-like sites (see Methods) and was subjected to electrophoretic mobility shift assay. (**b**) Ten micrograms of extract from cells subjected to 1 hour of mechanical strain was incubated with labeled oligonucleotide in the absence or presence of increasing concentrations of unlabeled oligonucleotide (10- to 100-fold excess) encoding wild-type or mutant SSRE sequence. (**c**) Ten micrograms of extract from myocytes subjected to strain for 1 hour was preincubated on ice for 1 hour with 1 μg of polyclonal antibody directed against p50, p65, or Sp1 before addition of labeled probe. Position of p50-p65 complex and supershift complex are indicated.

Evidence has been advanced supporting (25–27) or refuting (29, 32) a role for the Ras/Raf/ERK pathway in signaling hypertrophy-dependent phenotypic changes and gene expression in neonatal rat myocytes. Gillespie-Brown et al. (25) showed that dominant-negative MEK1, an activating kinase positioned immediately upstream from ERK, blocked phenylephrine-induced activation of a cotransfected rat *ANP* promoter. The inhibition was amplified further by cotransfection with dominant-negative ERK, whereas transfection of constitutively active MEK1 or wild-type ERK resulted in increased *ANP* promoter activity. Thorburn et al. confirmed the dependence of the phenylephrine induction of *ANP* promoter activity on ERK (27) and Raf (54), but noted that the cytoskeletal organization that typifies hypertrophy in this model did not appear to traffic through either. A subsequent study from Post et al. (29) seemingly dissociated activation of the ERK pathway from stimulation of *ANP* promoter activity, a finding supported by the later studies of Thorburn et al. (55), who found that activation of the *ANP* promoter by transfected MEKK1, a MAPK kinase kinase capable of activating either ERK or JNK signaled through the latter and not the former. Activation of ERK in this study resulted in, if anything, a reduction in *ANP* promoter activity.

The evidence in favor of JNK involvement in the hypertrophic process is somewhat stronger. In addition to the study of Thorburn et al. (55) alluded to above, the work of Ramirez et al. (30), Wang et al. (31), and Choukroun et al. (32) has established a close link between hypertrophic stimuli (i.e., either phenylephrine or endothelin), JNK activation, and different parameters associated with the hypertrophic phenotype. However, at least 1 study (37) has provided evidence that assigns a relatively minor role to the JNK pathway in signaling hypertrophy. Thus, as with the ERKs, the role of JNKs in signaling the hypertrophic process remains controversial.

Several researchers have identified p38 as an important signaling pathway in hypertrophy (33–38). Zechner et al. showed that overexpression of MKK6, a proximally positioned kinase that specifically activates p38 in ventricular myocytes, led to activation of ANP, BNP, and skeletal α-actin promoter activity, augmented cell size, and increased sarcomeric organization in a fashion similar to that seen with phenylephrine (34). It is noteworthy that activation of JNK with MEKK1$_{COOH}$ or of ERK with Raf-1 BXB each increased cell size and effected modest increases in natriuretic peptide and α-SkA promoter activity, but did not increase sarcomeric organization. The observed increase in *ANP* promoter activity appears to signal through a proximal serum response element, possibly through activation of an associated transcription factor, ATF6 (35). Clerk et al. (38) found that both phenylephrine and endothelin-1 increased p38 activity through a protein kinase C–dependent mechanism in neonatal myocytes; however, SB203580, the p38 antagonist, failed to prevent the morphological changes associated with myocyte hypertrophy after 4–24 hours of agonist stimulation. It did, however,

diminish cell profile and myofibrillar organization after 48 hours, leading Clerk and colleagues to conclude that p38 does not play a role in the immediate induction of the morphological changes associated with hypertrophy, but may play a role in maintaining this phenotype over a longer period of time. The effects of p38 appear to be isoform specific. Infection of cardiac myocytes with adenoviral vectors encoding constitutively active MKK6 (MKK6bE) or MKK3 (MKK3bE) led to characteristic hypertrophic changes; however, forced expression of MKK3 also led to apoptosis in the myocyte population (33). Interestingly, constitutively active MKK6 has been shown to have antiapoptotic activity in these cells (36). The hypertrophic response was enhanced by coinfection with wild-type p38β, and was blocked by a dominant-negative p38β mutant. In contrast, the proapoptotic effects of MKK3bE were amplified by wild-type p38α and were blocked by dominant-negative p38α (33).

It has been shown that p38 signals through the transcription factor NF-κB in some (48, 49), though not all (50), systems. Our data indicate that p38 signals through NF-κB in activating the *hBNP* promoter. Both strain- and p38-activated promoter activity are blocked to varying degrees by a constitutive inhibitor of NF-κB activity, the 2N mutant of IκB; this inhibition is not additive with that produced by SB203580. Interestingly, Zechner et al. (35) found that NF-κB is activated by p38 in cardiac myocytes, but that this activation is not related to the antiapoptotic activity of p38 (see above). Thus, NF-κB may be linked to some but not all trophic activity associated with this signaling cascade.

The p38/NF-κB contribution to the strain response operates, at least in part, through a series of NF-κB binding sites present in the *hBNP* promoter. It is interesting that shear stress, another mechanical stimulus of transcription in vascular endothelial cells, has been shown to signal through a specific element — SSRE — harboring an NF-κB recognition site (52, 53). Thus, activation of NF-κB appears to be a consistent theme linking mechanical stimuli to activation of gene expression in the cardiovascular system.

A number of transcription factors have been identified as effectors of hypertrophic stimuli in driving gene expression, including transcription enhancing factor-1 (56, 57), serum response factor (58, 59), Sp1 (59), activator protein 1 (60, 61), and nuclear factor of activated T cells (NF-AT) (12, 13). NF-AT, in fact, has been linked specifically to activation of the *hBNP* gene in vitro (12). It is probable that these factors act in an overlapping (and possibly, at times, a redundant) fashion to link the upstream effector cascades to the genomic activity that culminates in hypertrophy. The potential interaction of these transcription factors with NF-κB in promoting these changes remains unexplored.

In summary, the induction of *hBNP* promoter activity by mechanical strain is, at least in part, dependent on stimulation of p38 in the cardiac myocyte and the subsequent activation of NF-κB. This activation may play an important role in signaling the increased *BNP* gene expression that accompanies hemodynamic overload and cardiac hypertrophy in the intact animal.

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