Fibronectin signaling stimulates BNP gene transcription by inhibiting neuron-restrictive silencer element-dependent repression

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Abstract

Objective: Brain natriuretic peptide (BNP) is a cardiac hormone mainly synthesized in ventricles and its expression is markedly increased in ventricular hypertrophy that involves the accumulation of extracellular matrix proteins, such as fibronectin (Fn). We recently reported that Fn signaling stimulated BNP secretion accompanied by hypertrophic responses in vitro. Methods: To elucidate the regulatory mechanism for BNP gene transcription, we examined cis-acting elements downstream of Fn signaling in rat ventricular myocytes transfected with either the −1812 human BNP-luciferase reporter gene (−1812hBNP/Luc) or one of several truncated forms. Results: A strong cis-repressor element was identified between −552 and −522 in myocytes plated on uncoated dishes. This region contains a neuron-restrictive silencer element (NRSE)-like element (NRSE\textsuperscript{BnP}) that is 90% homologous with the NRSE consensus sequence. Neuron-restrictive silencer factor (NRSF) is known to bind to NRSE and to silence transcription of genes containing NRSE. Deletion of NRSE\textsuperscript{BnP} and dominant negative NRSF markedly increased the reporter activity in transfected cells, suggesting that the NRSE/NRSF system silences basal BNP gene transcription. When myocytes were cultured on Fn-coated dishes, the reporter activity of −1812hBNP/Luc was increased by ∼600% compared with that on uncoated dishes. Interestingly, truncation from −552 to −522 reduced the Fn-inducible reporter activity. Moreover, deletion of NRSE\textsuperscript{BnP} and dominant negative NRSF also inhibited the Fn-inducible reporter activity. Electrophoretic mobility shift assays showed that Fn signaling inhibited the binding activity of NRSF to NRSE\textsuperscript{BnP}. Conclusion: These results suggest that Fn-induced BNP up-regulation in rat ventricular myocytes is due to inhibition of NRSE\textsuperscript{BnP}-dependent repression of BNP gene transcription. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Extracellular matrix; Gene expression; Hypertrophy; Myocytes; Natriuretic peptide

1. Introduction

Atrial and brain natriuretic peptide (ANP and BNP, respectively) are cardiac hormones mainly synthesized in the atria and ventricles, respectively [1], and like other embryonic genes [2,3], their expression is up-regulated in both failing and hypertrophied ventricles [4,5]. Because ANP and BNP possess diuretic, natriuretic, vasodilating properties and inhibit aldosterone and renin secretion, their augmented expression in failing ventricles would be expected to act as an intrinsic compensatory mechanism during heart failure [6,7]. This notion is supported by the
observation that mice lacking the gene encoding guaneryl cyclase-A, a common receptor for ANP and BNP, show marked ventricular hypertrophy accompanied by interstitial fibrosis [8,9]. From a clinical point of view, plasma BNP levels are now widely accepted as a more sensitive and specific molecular marker of heart failure than any other humoral factors, including ANP [10–12]. Furthermore, to better understand the clinical implications of elevated plasma BNP itself and its underlying pathology in the heart, it will be essential to elucidate the molecular mechanisms responsible for up-regulation of BNP gene transcription in ventricular myocytes (VMCs).

Earlier studies showed that BNP gene transcription is stimulated by mechanical stress [13–15] and by a number of autocrine/paracrine regulators, including G-protein-coupled receptor agonists [13] and ligands stimulating gp130 signaling [16–18]. Although the downstream intracellular signaling pathways activated by such ligands or by mechanical stress have been extensively studied, cis-acting elements regulating BNP expression are still poorly understood, an exception being the M-CAT or GATA element [19–21]. Recent evidence suggests that in addition to autocrine/paracrine factors, extracellular matrix proteins also function in the regulation of ANP and BNP gene transcription via integrin-mediated pathways. For example, expression of natriuretic peptide is augmented in regions surrounded by fibrosis [22], and β1 integrin, one of the cell surface Fn receptors and vinculin, participate in the hypertrophic response of rat VMCs [23]. In addition, we recently found that outside-in Fn signaling stimulated secretion of both ANP and BNP that was accompanied by hypertrophic protocols were carried out separately using the

2. Methods

2.1. Plasmid constructs

The original clone pUCHBNP was digested by HindIII/BamHI [25], and the 5′-FS of the human BNP gene (hBNP) from −1812 to the initiator ATG was cloned into the luciferase reporter (−1812hBNP/Luc) [17]. Truncated versions of hBNP/Luc were created as follows: −1812hBNP/Luc was digested by Thnl11I creating −828hBNP/Luc; by Sac II creating −423hBNP/Luc; or by ApaI creating −112hBNP/Luc. Using a deletion kit (Takara, Japan), −601, −570 and −444hBNP/Luc were created by deletion. An additional five constructs were prepared by polymerase chain reaction (PCR). We used −1812 hBNP/Luc as a PCR template with a set of four upper primers (5′-GATAG GTACC GGTTA TCAGC ACCAC GGA-3′, 5′-GATAG GTACC CGCCC CCGAG GACC GCA-3′, 5′-GATAG GTACC GCACA GCAGC GAGCA GGT-3′, and 5′-ATAGG TACCT AGCTG CGGGC CAGGG AA-3′) and one lower primer, D4 (5′-AAATG TCCAG GTGTC CTAGG TTGG-3′) to amplify −552, −522, −499 and −477 hBNP/Luc, respectively. Each of the amplified products was designed so that it could be exchanged at the KpnI and SacII restriction sites of −1812hBNP/Luc. To make the NRSE deletion construct, we prepared two sets of PCR primers. Briefly, one upper primer, D1 (5′-AAATA GGCTG TCCCC AGTGC A-3′), was set at the KpnI restriction site of the plasmid multi-cloning site, and a lower primer, D2 (5′-CGCCC TGGCA CCCCG AGGGA ACTT-3′), was set in front of NRSE. The other upper primer, D3 (5′-CAGGG CGCCC CCGAG ACCCG CA-3′), was set just behind the NRSE, and the other lower primer, D4 (5′-AAAGAC CCCGC CACCC GCAGC G-3′), was set at the SacII restriction site in the hBNP 5′-FS. The first two PCR protocols were carried out separately using the −1812hBNP/Luc construct, after which the final protocol was carried using the D1 and D4 primers with a mixture of the first two PCR products serving as a template. The resultant PCR product was exchanged at KpnI/ApaI site of −1812hBNP/Luc. All the constructs were verified by sequencing.

A dominant negative NRSF construct [26] was a kind gift from Dr Anderson (California Institute of Technology, Pasadena, CA).

2.2. Cardiac myocyte preparation

VMCs were prepared as described previously [24,27,28]. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.3. Luciferase gene reporter assay and β-gal assay

Freshly prepared VMCs (2.7×10^5 cells) were transiently co-transfected with 10μg of the indicated reporter construct and a control vector (pCMV-β-gal or pRL-TK; Toyo Ink, Tokyo) by electroporation at 280 V and 300 μF in
0.2-cm cuvettes [29]. The cells were then plated in either uncoated or Fn-coated six-well culture dishes and allowed to attach for 24 h in Dulbecco’s modified Eagle’s medium (DMEM; Flow Laboratories, Irvine, Scotland) supplemented with 10% fetal calf serum (FCS). Thereafter, cells were incubated for 30 h in serum-free DMEM containing 1 mg/ml bovine serum albumin (DMEM/BSA). The number of the cells attached to the dishes was not significantly different between uncoated dishes and Fn-coated dishes. Luciferase activity in the cell lysates was then measured with a luminometer according to the manufacturer’s protocol (Toyo Ink, Tokyo). In experiments evaluating the effects of dominant negative NRSF, relative luciferase activity for each hBNP/Luc construct was normalized to pRL-TK activity; in all other experiments, luciferase activity was normalized to pCMV-β-gal activity. The β-gal assay was performed as follows. Cell lysates were mixed with 2× assay buffer (120 mM Na₂HPO₄, 80 mM NaH₂PO₄, 2 mM MgCl₂, 100 mM β-mercaptoethanol, 1.33 mg/ml o-nitrophenyl-β-d-galactopyranoside) and incubated at 37°C for 30 min, and then the reactions were stopped by adding Na₂CO₃. Absorbance of the samples was read at 405 nm. Fn inducibility in each experiment was defined as the fold increase of the respective average relative luciferase activities measured in lysates from VMCs cultured on Fn-coated wells (n=3) over those measured in lysates from cells plated on uncoated wells (n=3).

2.4. Electromobility shift assay (EMSA)

After incubating cells plated on either uncoated or Fn-coated dishes for 30 h in DMEM/BSA, nuclear proteins were extracted as described previously [30]. A double-stranded probe containing the NRSE<sub>hBNP</sub> (5′-ATCAG CACCA CGGAC AGCGG C-3′) was labeled with γ<sup>32</sup>P ATP using T4 polynucleotide kinase, and EMSA was performed as described previously [31,32]. To confirm NRSE-specific binding, an unlabeled NRSE consensus or mutant NRSE (NRSE<sub>mutant</sub>, 5′-ATCAG CACCA CTTCAG AGCGG C-3′) was used as cold competitor and antibody to NRSF was used in supershift assay. The antibody to NRSF is a guinea pig polyclonal antibody against NRSF and was generated by Nozomu Mori at the National Institute for Longevity Science in Aichi, Japan (Naruse et al., details will be published elsewhere). Densitometrical analysis was performed by measuring the density of bands on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

2.5. Reverse transcriptase PCR (RT-PCR) for NRSF

VMCs were plated on either uncoated or Fn-coated dishes, grown to a density of 3×10⁴ cells/cm², and sequentially incubated in DMEM/FCS for 36 h, in DMEM/BSA for 12 h and in then DMEM/BSA for an additional 48 h. Thereafter, total RNA was isolated using TRIzol Reagent (Gibco BRL), and RT-PCR was performed as described previously [32].

2.6. Statistical analysis

Values are expressed as means±S.E.M. from three to five independent experiments. Analysis of variance (ANOVA) with post hoc Fisher’s test was used for the comparison among groups, and Student’s t-test was used for the comparison between two groups. Values of P<0.05 were considered statistically significant.

3. Results

3.1. Identification of a hBNP gene cis-element responsive to outside-in Fn signaling

To identify cis-elements in the 5´-FS of the hBNP gene responsive to outside-in Fn signaling, we enzymatically prepared four reporter constructs, designated −1812, −828, −423 and −112 hBNP/Luc (Fig. 1A). We initially evaluated the basal reporter activities of the truncated constructs in VMCs cultured on uncoated dishes. As shown in Fig. 1B, there were at least two strong positive cis-enhancer elements located between −1812 and −828 and between −423 and −112, respectively, the latter most likely being M-CAT [33,34]. There was also one cis-repressor element located between −828 and −423.

A similar pattern of transcription regulation was seen in VMCs cultured on Fn-coated dishes (Fig. 1C). Focusing on the Fn inducibility of the relative luciferase activity of each reporter construct (Fig. 1D), deletion of a segment spanning −828 to −423 substantially inhibited Fn-inducible reporter activity and, though deletion of segments spanning −1812 to −823 or −423 to −112 had no apparent effect on Fn inducibility, which suggested the presence of an element responsible for regulating Fn-inducible BNP gene expression between −823 and −423. Furthermore, Fn activated −112 construct −2.5-fold, suggesting the proximal region of BNP gene promoter is also responsible for the Fn inducibility.

Thus, the −828 to −423 segment contains an unique element that is responsible for both the repression of basal hBNP gene transcription and enhancement of Fn-inducible hBNP gene transcription. To identify exactly the unique cis-element in the segment, we examined the −828 to −423 segment in more detail using seven additional truncated mutants (Fig. 2A). As shown in Fig. 2B,C, relative luciferase activity of the −522hBNP/Luc construct was 44- and 21-fold higher than that of −552hBNP/Luc in cells grown on uncoated and Fn-coated dishes, respectively, which indicated the presence of a strong negative
Fig. 1. Schematic representation of enzymatically-prepared BNP reporter genes and their luciferase activities in VMCs. (A) The -1812 hBNP/Luc construct was enzymatically digested with Tth111I, SacII or ApaI, creating three truncated forms. (B, C) Relative luciferase activities of the hBNP/Luc constructs transfected into VMCs grown on uncoated (B) or Fn-coated (C) dishes. VMCs were co-transfected with the respective constructs (10 µg) and a control vector (pCMV-β-gal), and then cultured on uncoated or Fn-coated dishes. Luciferase activity measured in the cell lysates was normalized to control vector activity and is expressed as relative luciferase activity. (D) Fn inducibility was defined as the fold increase in the average relative luciferase activity measured in lysates of VMCs cultured on Fn-coated dishes (n=3) over that measured in lysates of cells grown in uncoated dishes (n=3). All data are expressed as means±S.E.M. of three to five independent experiments.

Fig. 2. Reporter gene activity of various truncated forms of BNP reporter genes in VMCs. (A) Seven constructs were prepared by deleting selected segments between -828 and -423 and amplifying the products using PCR. (B, C) Relative luciferase activities of hBNP/Luc constructs transfected into VMCs grown in uncoated (B) or Fn-coated (C) dishes. VMCs were co-transfected with the respective constructs (10 µg) and pCMV-β-gal and then cultured on uncoated or Fn-coated dishes, after which luciferase activity in the lysates was normalized to control vector activity. (D) Fn-induced luciferase activity expressed as the fold increase in luminescence measured in lysates of VMCs cultured in Fn-coated wells (n=3) over that from lysates of cells grown in uncoated wells (n=3). All data are expressed as means±S.E.M. of three to five independent experiments.
inducibility (Fig. 2D) from -423 construct. Thus, it appears that the segment between -522 and -522 contains a repressor element that negatively regulates basal BNP gene transcription and is responsive to outside-in Fn signaling.

3.2. NRSE<sup>BNP</sup> was involved in Fn-induced up-regulation of hBNP gene transcription

A search for known cis-acting negative elements between -552 and -522 of the 5'-FS of the BNP gene yielded the 21-bp NRSE<sup>BNP</sup>, which is 90% homologous with the NRSE consensus sequence (Fig. 3A). NRSE, also known as repressor element-1 (RE1), has been reported to be a negative-acting DNA regulatory element that prevents expression of NRSE containing genes in non-neuronal cells [35,36]. To assess the efficiency of NRSE<sup>BNP</sup> regulation of BNP gene expression, we made a construct in which only NRSE<sup>BNP</sup> was deleted from -1812hBNP/Luc (−1812ΔNRSEhBNP/Luc) (Fig. 3B). As shown in Fig. 3C,D, the relative luciferase activity of -1812ΔNRSEhBNP/Luc was much greater than that of -1812hBNP/Luc and somewhat greater than that of -522 hBNP/Luc. Furthermore, deletion of NRSE<sup>BNP</sup> significantly reduced Fn inducibility (Fig. 3E) from ~8-fold to 3.5-fold. It appears, therefore, that the repressor element regulating basal BNP gene transcription and involved in Fn-induced up-regulation of BNP gene transcription is NRSE<sup>BNP</sup>

3.3. Co-transfection of dominant negative NRSF released repression of BNP gene transcription

Earlier reports have shown that NRSF is a novel member of the zinc finger transcription factor family that binds to NRSE and acts as a strong suppressor protein [35,36]. To assess the involvement of NRSF in NRSE<sup>BNP</sup>-mediated repression of the BNP gene, a dominant negative NRSF construct was co-transfected with -1812hBNP/Luc or -1812ΔNRSEhBNP/Luc, and reporter activities were evaluated. Dominant negative NRSF significantly increased the luciferase activity of -1812hBNP/Luc to a level comparable to that of -1812ΔNRSEhBNP/Luc in cells grown on both uncoated and Fn-coated dishes (Fig. 4B,C). Expectedly, the Fn inducibility of luciferase activity previously observed in -1812hBNP/Luc was significantly decreased by ~50% in the presence of dominant negative NRSF (Fig. 4D). On the other hand, the NRSE-deleted construct was significantly repressed by dominant negative NRSF, although the mechanism is not clear at present.

3.4. NRSF could bind to NRSE<sup>BNP</sup>

To confirm that NRSF binds to NRSE<sup>BNP</sup>, EMSA was carried out using nuclear extracts from undifferentiated P19 cells that are known to contain abundant NRSF [37]. As shown in Fig. 5, the NRSE<sup>BNP</sup> probe formed an EMSA-detectable complex that was completely disrupted by an unlabeled probe but not by unlabeled NRSE<sup>mutant</sup>. Furthermore, the band was supershifted by the antibody to

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**Fig. 3.** Sequence of NRSE<sup>BNP</sup> and the reporter gene activity of an NRSE<sup>BNP</sup> deletion mutant. (A) Comparison of NRSE<sup>BNP</sup> and the consensus NRSE sequence in the mouse SCG-10 gene. (B) Schematic drawing of a -1812hBNP/Luc deletion mutant (−1812ΔNRSEhBNP/Luc). (C, D) -1812ΔNRSEhBNP/Luc was prepared as described in Methods, after which VMCs grown on uncoated (C) and Fn-coated (D) dishes were co-transfected with 10 μg of the respective construct and pCMV-β-gal. Luciferase activities were normalized to control vector activities. (E) Fn-induced luciferase activity expressed as the fold increase in luminescence measured in lysates of VMCs cultured on Fn-coated wells (n=3) over that from lysates of cells grown on uncoated wells (n=3). All data were expressed as mean±S.E.M. of three independent experiments.
Fig. 4. Effect of NRSF on the regulation of hBNP gene expression. VMCs were co-transfected with 10 μg of the appropriate construct, control vector pRL-TK and 5 μg of a dominant negative NRSF construct, after which they were cultured on uncoated or Fn-coated dishes. Luciferase activities were normalized to control vector activities. (A) Schematic drawing of vectors. (B, C) Relative luciferase activity measured in lysates of cells transfected with hBNP/Luc constructs and grown on uncoated dishes (B) and Fn-coated dishes (C) (n=3). (D) Fold increase in the relative luciferase activity measured in lysates from cells cultured on Fn-coated dishes (n=3). All data are expressed as means±S.E.M. of three independent experiments. *P<0.001 vs. without dominant negative NRSF.

NRSF. These results confirmed that NRSF could bind to NRSE<sup>BNP</sup>.

3.5. Fn inhibited binding of NRSF to NRSE<sup>BNP</sup>

RT-PCR analyses confirmed that NRSF mRNA was in fact expressed in VMCs cultured on both uncoated and Fn-coated dishes, and that the levels of expression were similar in the two cell groups (data not shown).

To determine whether nuclear proteins isolated from VMCs could bind to NRSE<sup>BNP</sup>, EMSA was carried out using γ<sup>32</sup>P-labeled NRSE<sup>BNP</sup> as a probe. In the absence of any competitors, ventricular nuclear extracts and the NRSE<sup>BNP</sup> probe formed an EMSA-detectable complex (Fig. 6A, lane 3) that moved in a manner similar to the complex formed with nuclear extracts from undifferentiated P19 cells (Fig. 6A, lane 1). Complex formation using nuclear extract from VMCs was completely disrupted when either unlabeled probe or NRSE consensus sequence was used as a competitor (Fig. 6A, lanes 5 and 6), which makes it likely that the nuclear protein bound to NRSE<sup>BNP</sup> was NRSF in VMC.

The intensity of the band shifted by nuclear extract from VMCs cultured on Fn-coated dishes (Fig. 6A, lane 4 and Fig. 6B) was weaker than that from cells cultured on uncoated dishes (Fig. 6A, lane 3 and Fig. 6B). And when consider altogether, these findings suggest a unique scenario in which outside-in Fn signaling might attenuate the binding activity of NRSF to NRSE<sup>BNP</sup>, thereby up-regulating BNP gene transcription.

4. Discussion

The present study demonstrates that in neonatal rat VMCs, outside-in Fn signaling stimulates BNP gene transcription through inhibition of NRSE<sup>BNP</sup>-mediated repression. This may provide new insight into the regulation of the BNP gene itself, into Fn-stimulated downstream signaling pathways, and into the significance of the NRSE/NRSF system in cardiomyocytes.

Most earlier investigations focused on elucidating which enhancer elements are responsible for regulating agonist-
BNP®rmed the involvement of NRSF in NRSE-mediated silencing. We also found NRSE to be involved in the mechanism responsible for up-regulation of BNP gene transcription by outside-in Fn signaling. This is the first demonstration that inhibition of transcriptional silencing is responsible for induction of BNP gene in cardiomyocytes. NRSE was initially identified as a silencer element regulating neuron-specific expression of the neuronal genes, including those encoding SCG10 and the type II sodium channel [39,40]. NRSF, which is expressed in non-neuronal cells but not in terminally differentiated neurons, silences transcription of NRSE-containing genes in the former by binding to NRSE. Consequently, expression of NRSE-containing genes has been thought to be limited to neurons of the central nervous system [41]. So the present study proves that the NRSE/NRSF system acts outside the central nervous system. NRSE-like elements have been observed within regions of other cardiac genes, including the 3'-FS of ANP, the 5'-FS of skeletal α-actin and the 5'-FS of the sodium-potassium ATPase α3 subunit; in particular, the NRSE-like element of the ANP gene is highly conserved among mammals, and we found that it mediates endothelin-induced ANP gene expression [31]. All these findings suggest that the NRSE/NRSF system could contribute to down-regulate cardiac embryonic genes as ANP and BNP mainly via β1 integrin signaling pathways, and that this effect was accompanied by phosphorylation of focal adhesion kinase (FAK) and was almost completely blocked by the tyrosine kinase inhibitor genistein [24]. And in the present study we focused on the significance of NRSE<sup>BNP</sup> element in BNP gene transcription under both basal and Fn-stimulated conditions. However, the present study also showed that outside-in Fn signaling activated ~112 construct by ~2.5-fold, suggesting the proximal region of the promoter region of the BNP gene may be also responsible for the Fn inducibility. Since in the proximal region of 5'-FS of the hBNP gene some positive enhancer elements such as GATA binding element and SP-1 element have been reported [19–21], which might be candidates for this up-regulation, further studies are necessary to understand the whole transcriptional mechanism down stream of outside-in Fn signaling in VMCs.

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or strain-induced, cardiomyocyte-specific BNP gene expression. These studies revealed that proximal M-CAT element is essential for basal and phenylephrine-inducible BNP gene transcription [34], while SSREs are also involved in the strain-induced up-regulation of BNP gene expression [38]. However, no cis-repressor element regulating BNP gene transcription has yet been precisely located, though LaPointe et al. reported there was a negative regulatory region between −906 and −500 in the 5'-FS of the hBNP gene [29]. In the present study, analyses of BNP promoter activity carried out using truncated or deleted mutants demonstrated that NRSE<sup>BNP</sup> situated between −552 and −522 acts as a repressor element for basal BNP gene transcription. Moreover, co-transfection of a dominant negative NRSF construct con-
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