Brain Natriuretic Peptide Is Produced in Cardiac Fibroblasts and Induces Matrix Metalloproteinases


Abstract—Cardiac fibroblasts (CFs) produce extracellular matrix proteins and participate in the remodeling of the heart. It is unknown if brain natriuretic peptide (BNP) is synthesized by CFs and if BNP participates in the regulation of extracellular matrix turnover. In this study, we examined the production of BNP in adult canine CFs and the role of BNP and its signaling system on collagen synthesis and on the activation of matrix metalloproteinases (MMPs). BNP mRNA was detected in CFs, and a specific radioimmunoassay demonstrated that BNP was secreted into the media at a rate of 11.2 ± 1.0 pg/10⁵ cells per 48 hours (mean ± SEM). The amount of BNP secretion was significantly (P < 0.01) augmented by 10⁻⁷ mol/L tumor necrosis factor-α in a time-dependent manner. BNP significantly (P < 0.01) inhibited de novo collagen synthesis as assessed by [³H]proline incorporation, whereas zymographic MMP-2 (gelatinase) abundance was significantly (P < 0.05) stimulated by BNP between 10⁻⁷ and 10⁻⁶ mol/L. In addition, protein expression of MMP-1, -2, and -3 and membranous type-1 MMP was significantly increased by 10⁻⁷ mol/L BNP. The cGMP analogue 8-bromo-cGMP (10⁻⁴ mol/L) mimicked the BNP effect, whereas inhibition of protein kinase G by KT5823 (10⁻⁶ mol/L) significantly (P < 0.05) attenuated BNP-induced zymographic MMP-2 abundance. In summary, this study reports that BNP is present in cultured CFs and that BNP decreases collagen synthesis and increases MMPs via cGMP–protein kinase G signaling. These in vitro findings suggest a role for BNP as a regulator of myocardial structure via control of cardiac fibroblast function. (Circ Res. 2002;91:1127-1134.)

Key Words: cardiac fibroblasts ■ extracellular matrix ■ remodeling ■ cGMP ■ protein kinase G

The cardiac interstitium is a dynamic structure, as reflected by continuous synthesis and degradation of matrix proteins. The family of matrix metalloproteinases (MMPs) consists of more than 20 different zinc-containing, Ca²⁺-dependent endopeptidases. They degrade matrix proteins and therefore play an important role in the physiological regulation of the interstitium. The interstitial collagenases (MMP-1 and MMP-13), the stromelysin (MMP-3), the gelatinases (MMP-2 and MMP-9), and membranous-type 1 MMPs (MMP-14; MT1-MMP) have been demonstrated within the mammalian myocardium. Furthermore, dysregulation of MMP proteins and their endogenous inhibitor, tissue inhibitors of MMP (TIMP), has been observed in the hypertensive and the failing heart, suggesting an important role of MMP in the process of ventricular remodeling.

Cardiac fibroblasts (CFs) play a crucial role in the regulation of the extracellular matrix (ECM) of the heart by synthesizing collagen and other matrix proteins as well as promoting its degradation by secreting MMP proteins. In response to myocardial injury, activation of CFs occurs. These activated CFs (myofibroblasts) have special morphological and functional characteristics.

The natriuretic peptides (NPs) atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) play important roles in maintaining cardiorenal homeostasis under physiological and pathological conditions. ANP and BNP are synthesized by cardiomyocytes, and their production is stimulated in pathologic conditions such as myocardial infarction (MI), cardiac hypertrophy, and heart failure (HF). ANP and BNP have natriuretic, vasodilating, and lusitropic properties, and they inhibit the sympathetic and renin-angiotensin-aldosterone system. These actions are primarily mediated by the second messenger cGMP.

Cameron et al have recently reported that ANP is produced in CFs after MI, indicating that fibroblasts, like cardiomyocytes, can be a source of NPs. However, it remains unknown if BNP is produced by CFs.

Although it is well established that BNP has growth-inhibiting properties in the heart, the role of BNP on the regulation of the cardiac interstitium remains undefined. Given the widespread cross-talk of the NPs with other systems that are activated in cardiorenal disorders, we aimed to investigate whether CFs are a source of BNP and whether BNP and its signaling system contribute to the regulation of...
collagen synthesis and to the activation of MMPs. In addition, we previously demonstrated a cross-talk between cardiotrophin-1 and endothelin-1 (ET-1) in the activation of CFs.\(^{22}\) We continued to address the cross-talk between BNP and proinflammatory cytokine, tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), and profibrotic factor ET-1 in CFs.

### Materials and Methods

The present study was performed in accordance with the Animal Welfare Act and with approval of the Mayo Clinic Institutional Animal Care and Use Committee.

#### Cell Culture

Normal adult canine ventricular fibroblasts were prepared as previously described.\(^{22}\) After digesting the minced left ventricular free wall with trypsin and collagenase, collected cells were preincubated in Medium 199 (M199) (BioWhittaker Inc) with 10% FBS for 2 hours to separate CFs from myocytes. The cells were then cultured with fresh M199 supplemented with 10% FBS in 5% CO\(_2\) and 95% humidified air at 37°C. After the cells achieved confluence, the medium was changed to serum-free M199, containing 5 μg/mL insulin, 5 μg/mL transferrin, and 5 μg/mL sodium selenite. After a 24-hour incubation, the medium was changed to new fresh serum-free medium described above in the presence or absence of TNF-\(\alpha\), ET-1, BNP, and 8-bromo-cGMP (8-Br-cGMP). In an additional experiment, an inhibitor for specific cell-permeable protein kinase G (PKG), KT5823, was added to the culture medium 30 minutes before adding synthetic canine BNP.\(^{22}\) We used CFs with three to five passages in this experiment. The cells were mitogenic and noncontractile on microscopic observation.

#### Immunohistochemistry

Immunohistochemical analysis was performed by indirect immunoperoxidase labeling as previously described.\(^{22}\) Briefly, cultured CFs were fixed with 10% formaldehyde. After blocking the endogenous peroxidase with 0.6% hydrogen peroxide in methanol and reducing the nonspecific background with 5% normal goat serum, slides were incubated with primary antibodies at room temperature for 24 hours. Cells were then incubated with a second antibody–horseradish peroxidase conjugate. Visualization was achieved by incubating the cells with freshly prepared reagent containing 3-amino-9-ethylcarbazole (Sigma) dissolved in N,N-dimethylformamide, hydrogen peroxide, and sodium acetate. Cells were counterstained with hematoxylin. Specificity of the antibody was confirmed by substitution with non-immune goat serum or absorption test.

#### Radioimmunoassay

Conditioned media, as well as cells homogenized in 1N acetic acid, were extracted with C-8 Bond-Elute cartridges. BNP-like immunoreactivity (BNP-LI) in the extract was measured with a specific radioimmunoassay (canine BNP-32, Phoenix Pharmaceuticals, Inc, Belmont, Calif). Briefly, standards or samples were incubated with antibody for canine BNP for 20 hours at 4°C. 125I-BNP (10 000 cpm) was then added, followed by additional incubation for 18 hours at 4°C. After 18 hours of incubation with a secondary antibody, free and bound fractions were separated, and the bound fraction of the samples was measured by gamma counter (Wallac). The recovery was 73%, and interassay and intra-assay variability were 11% and 7%, respectively. There was no cross-reactivity with ANP or C-type NP.

### BNP mRNA Expression

Total RNA 500 ng was extracted using the TRIzol method (Invitrogen). The total RNA was then reverse-transcribed into cDNA using the Superscript I RT-PCR Kit (Invitrogen). Polymerase chain reaction (PCR) was carried out with BNP-specific primers (forward: 5'-TATCGGTCCAGCTACAGG-3'; reverse: 5'-ACAGGGCAATCTGTTT-3'), resulting in a 445-bp fragment. As a reference gene, we used human GAPDH (primers: forward: 5'-TACAGGTCGCAGGCTACAGGCC-3'; reverse: 5'-TGAGAGCCGGAAGTTTGGG-3'), resulting in a 160-bp fragment. PCR was performed with an initial step of denaturation at 94°C for 1 minute, 30 cycles of 30 seconds at 94°C, 1 minute at 55°C, and 1 minute at 72°C, followed by a final extension at 72°C for 7 minutes. The entire reaction products were electrophoresed onto 0.8% agarose gels, and the intensity was determined densitometrically (Multilimer, Bio-Rad). We repeated this experiment three times, and consistent results were obtained.

### Reverse-Phase High-Performance Liquid Chromatography

The molecular forms of BNP in the culture medium were analyzed by reverse-phase high-performance liquid chromatography (HPLC). The extracts were eluted from the column with a linear gradient of 5% to 80% acetonitrile in 0.1% trifluoroacetic acid, and the BNP-LI in each fraction was measured by radioimmunoassay.

#### Infracellular cGMP

Confluent cells were incubated with Hanks’ balanced salt solution (Invitrogen) containing 20 mmol/L N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 0.1% bovine serum, and 0.5 mmol/L 3-isobutyl-1-methylxanthine (Sigma) for 30 minutes at 37°C. BNP was added at the indicated concentrations and times. The reaction was terminated by aspirating the medium and adding cold 6% trichloroacetic acid. Cells were extracted with water-saturated ethyl ether, and cGMP was measured with a radioimmunoassay kit (NESS, Life Science Products, Inc).

### Collagen Synthesis

The de novo synthesis was assessed by measuring [\(^3\)H]proline incorporation in the acid-insoluble fraction of the cells, as previously described.\(^{22}\) After incubating the cells in serum-free medium described above for 48 hours, cells were treated with or without BNP and were pulsed with 5.0 μCi/mL of [\(^3\)H]proline for 40 hours. Radioactivity was measured with a scintillation counter (LS 6000SC, Beckman Inc).

#### Zymographic MMP Abundance

Concentrated conditioned media (1.0 or 5.0 μg protein), using centrifugal filter devices (Microcon, Millipore), was mixed with Laemmli sample buffer (Bio-Rad) for 10 minutes at room temperature. These samples were then loaded onto 10% Tris-glycine gels described above for 48 hours, cells were treated with or without BNP and were pulsed with 5.0 μCi/mL of [\(^3\)H]proline for 40 hours. Radioactivity was measured with a scintillation counter (LS 6000SC, Beckman Inc).

### Western Blotting

For detection of MMP and TIMP proteins in CFs, Western blotting was performed as previously described.\(^{22}\) Briefly, denatured protein (30 μg total proteins for MMPs, 50 μg total proteins for TIMPs) was subjected to SDS-PAGE on a 10% (for MMP-1, -2, -3, -9, and -13 and MT1-MMP) or 15% (for TIMP-1 and TIMP-2) polyacrylamide gel (Bio-Rad), and separated proteins were electrically transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). After blocking the nonspecific background, PVDF membranes were incubated with the antibodies for anti-rabbit polyclonal MMP-1 (AB806;
1:1000), MMP-2 (AB809; 1:1000), MMP-3 (AB811; 1:1000), MMP-9 (AB804; 1:1000), MMP-13 (AB8114; 1:1000), MT1-MMP (AB8221; 1:1000), TIMP-1 (AB8116; 1:1000), and TIMP-2 (AB8107; 1:1000) for 1 hour at room temperature. After washing the membrane with Tween-20 buffer, the membranes were incubated with goat anti-rabbit horseradish peroxidase (1:1000) for 1 hour at room temperature. The immunoreactive bands were then visualized by ECL Plus detection (Amersham), and the intensities of the band were analyzed densitometrically (Fluor-S MultiImager, Bio-Rad). Recombinant polypeptides and extract of U937, a lymphoma cell line (provided by Dr Leibson, Immunology, Mayo Clinic Rochester, Minn), were used as positive controls for MMP and TIMP.

Protein Content
Protein concentrations were determined with BCA Protein Assay Reagent (Pierce).

Reagents
Peptides for BNP, ET-1, and TNF-α and rabbit polyclonal antibody for BNP were purchased from Phoenix Pharmaceuticals, Inc. Monoclonal antibodies for vimentin, desmin, smooth muscle factor, α-smooth muscle actin, and myosin heavy chain for smooth muscle were from DAKO. Recombinant polypeptides (MMP-2, MT-1 MMP, and TIMP-2), positive control for MMP-1 and -3, and antibodies for rabbit polyclonal antibodies for MMP-1, -2, -3, -9, -13, and -14 and TIMP-1 and -2 were from Chemicon (Temecula, Calif). KT5823 was from Calbiochem, and 8-Br-cGMP and insulin-transferrin-sodium selenite media supplement were from Sigma.

Statistical Analysis
All data are expressed as mean±SEM. Data for zymography and Western blotting are expressed as folds compared with the respective control. Unpaired Student’s t test was used for comparison between two groups. Multiple groups were assessed with 1-way ANOVA followed by Scheffé’s test. Statistical significance was accepted at P<0.05.

Results
Characterization of CFs
Immunohistochemical studies revealed that cultured cells exhibited positive staining for vimentin and smooth muscle α-actin, whereas staining for desmin, myosin heavy chain for smooth muscle, and von Willebrand factor was negative (Figure 1). We therefore identified the cells as cardiac myofibroblasts.

Presence of BNP in CFs
Figure 2A illustrates the immunohistochemical staining for BNP in CFs. BNP-LI was diffusely present in the cytoplasm of these cells. BNP mRNA, which was detected in CFs
incubation with TNF-α/H9251 cellular BNP concentration did not significantly change after TNF-α/H9262 content. BNP (10-7mol/L) significantly increased the protein expression of MMP-2 (Figure 5A) (47 kDa at 12 hours [P<0.01], 72 kDa at 24 hours [+91%, P<0.05]). MMP-9 (54-kDa) (Figure 5D) protein expression tended to increase at 12 hours and was significantly increased by 14% at 24 hours (P<0.05). BNP did not change the protein levels for MMP-9 and MMP-13 (data not shown). Whereas TIMP-1 protein was unchanged by BNP (data not shown), TIMP-2 protein expression significantly increased by 12% after 24 hours (P<0.05) of BNP stimulation (Figure 5E).

Effect of BNP on Zymographic MMP-2 Abundance
Figure 4A illustrates the effect of 10-6 mol/L BNP on the zymographic MMP abundance in the conditioned medium. Zymographic MMP abundance appeared at the molecular weight corresponding to MMP-2 (72 and 68 kDa). Zymographic MMP-2 abundance gradually increased with serum depletion. In addition, BNP significantly enhanced the stimulation of zymographic total MMP-2 abundance, with the maximal effect observed at 12 hours (P<0.05, compared with controls). Figure 4B illustrates the dose-dependent effect of BNP on zymographic total MMP-2 abundance after stimulation for 12 hours. BNP significantly stimulated the zymographic total MMP-2 abundance at concentrations of 10-2 and 10-6 mol/L (P<0.05).

Effect of BNP on MMP and TIMP Protein Expression
Figure 5 illustrates the effect of BNP on the protein expression of MMPs and TIMP-2 by Western blotting. Compared with untreated cells, 10-6 mol/L of BNP significantly increased the protein expression of MMP-1 (Figure 5A) (47 kDa at 12 hours [+93%, P<0.05]), MMP-2 (Figure 5B) (68 kDa at 12 hours [+30%, P<0.01] and 24 hours [+91%, P<0.05]), 72 kDa at 24 hours [+33%, P<0.05]), and MMP-3 (Figure 5C) (57 and 59 kDa at 12 hours [+10%, P<0.01] and 45 to 48 kDa at 24 hours [+9%, P<0.05]). MT-1 MMP (54-kDa) (Figure 5D) protein expression tended to increase at 12 hours and was significantly increased by 14% at 24 hours (P<0.05). BNP did not change the protein levels for MMP-9 and MMP-13 (data not shown). Whereas TIMP-1 protein was unchanged by BNP (data not shown), TIMP-2 protein expression significantly increased by 12% after 24 hours (P<0.05) of BNP stimulation (Figure 5E).
Effects of cGMP Analogue and Protein Kinase G Inhibitor on Zymographic MMP Abundance

Figure 6A illustrates the effect of 8-Br-cGMP, a cGMP analogue, on the zymographic total MMP-2 abundance. After 12 hours of stimulation with 8-Br-cGMP (10^{-6} mol/L), zymographic total MMP-2 abundance was significantly increased by 12% (P<0.01) compared with untreated cells. Pretreatment with the specific protein kinase G inhibitor KT5823 (10^{-6} mol/L) significantly (P<0.05) reduced BNP-induced zymographic total MMP-2 abundance (Figure 6B).

Combined Effects of BNP and TNF-α/ET-1 on the Zymographic MMP-2 Abundance

Figure 7A shows the effect of BNP and TNF-α on MMP-2 abundance. In this experiment, TNF-α (10^{-7} mol/L) alone did not significantly alter zymographic total MMP-2 abundance at 24 hours. However, TNF-α additionally increased BNP-induced MMP-2 abundance (P<0.05). Figure 7B illustrates the effect of BNP and ET-1 on zymographic total MMP-2 abundance. ET-1 (10^{-6} mol/L) significantly (P<0.05) downregulated the zymographic MMP-2 abundance after 3 hours of incubation. Coincubation of BNP (10^{-6} mol/L) and ET-1 (10^{-7} mol/L) reversed the action of ET-1, resulting in increased zymographic MMP-2 abundance.

Discussion

The goal of this study was to investigate the presence of BNP in CFs and its potential role in the regulation of ECM turnover. This study demonstrates that CFs produce BNP. We also report that BNP inhibits collagen synthesis and increases MMP expression. This modulation is, at least in part, mediated by a cGMP/PKG pathway. Lastly, we demonstrate a cross-talk between the BNP/ET-1 and BNP/TNF-α on zymographic MMP-2 abundance.

CFs are activated in pathological conditions. A form of the phenotypic heterogeneic fibroblasts, termed myofibroblasts, is characterized by some features of smooth muscle differentiation.8,9 They are reported to play a major role in the inflammatory response and to secrete cytokines and growth factors. Myofibroblasts have been associated with repair processes after myocardial injury. We speculate that under pathological conditions in vivo, CFs are a source of BNP and that CF function is modulated by BNP. Consistent with our study, Cameron et al17 have recently reported that ANP is synthesized in ovine CFs after MI, suggesting an important role for ANP production by CFs in the remodeling process. Our study shows that an inflammatory cytokine TNF-α, which has been associated with ventricular remodeling,23 stimulates BNP secretion, indicating that there is some relationship between these two factors in HF. We also demonstrate that CFs secrete the bioactive form BNP1-32 into...
the medium, implying that they also synthesize an enzyme that cleaves BNP, from its precursor protein.

Another finding of our study is that BNP induces protein expression of MMP-1, -2, and -3 and MT1-MMP and increases MMP-2 release. To the best of our knowledge, this is the first report to show this antifibrotic mechanism of BNP in addition to its inhibitory effect on collagen synthesis. In addition, we report that the cGMP/PKG signaling pathway is involved in the modulation of MMP-2 release. Our finding complements reports that the nitric oxide (NO)/cGMP system stimulates MMP-2 and -9 activity in the rabbit carotid artery and MMP-13 in bovine vascular endothelium. More recently, Death et al have reported that nitroglycerin stimulates MMP expression in human macrophages. In contrast to these actions, NO also inhibits MMP-2 and -9 in rat aortic smooth muscle cells. Additional studies are warranted to define the unique actions of cGMP signaling on MMP function.

A balance between synthesis and degradation of ECM determines collagen content of the heart. Excessive collagen deposition may increase stiffness, whereas excessive collagen degradation contributes to ventricular dilatation. Although hemodynamic load as well as neurohumoral factors have been reported to modulate the expression of MMP, the role of BNP in the regulation of MMP induction is unknown. MMP-1 and -3 are able to cleave several ECM proteins, including collagen types I and III. MMP-2 degrades both gelatin (denatured collagen) and interstitial collagens, and augmented MMP-2 expression has been associated with the progression of heart failure. We also report here that BNP increases the expression of MT-1 MMP. Because MT-1 MMP not only directly degrades several ECM proteins but also activates pro-MMP-2, it can be regarded as a key enzyme in ECM degradation. With all of this in mind, BNP might have an important role in the degradation of the ECM. BNP increased MMP-2 protein at 12 and 24 hours, MMP-1 only at 12 hours, and MT-1 MMP at 24 hours, suggesting a time-dependent MMP modulation by BNP in the CFs. In addition to the stimulation of MMPs, BNP also increased the expression of TIMP-2 protein. This may counter excessive MMP-2 and MT1-MMP activation; however, the biological significance of TIMP-2 remains to be defined. Although previous studies support that TIMP-2 inhibits MMP-2 activity, Hernandez-Barrantes et al reported that the TIMP-2/MT1-MMP complex stimulates the activation of MMP-2, thus implying that TIMP-2 may also play a role in the degradation of ECM proteins. NPs, including BNP, have been reported to inhibit CFs proliferation and collagen synthesis. Furthermore, BNP gene-deficient mice show marked ventricular fibrosis with pressure overload. Our data support and extend these previous studies in that BNP acts as an antifibrotic agent not only by inhibiting ECM protein synthesis but also by stimulating the degradation of ECM proteins via a cGMP-PKG pathway.

The precise role of BNP as an antifibrotic factor in the ventricle remains to be elucidated. Cardiomyocytes synthesize BNP in response to stretch to reduce preload and afterload. The systemic effects of BNP, like natriuresis and vasodilation, can be regarded as beneficial cardiac unloading mechanisms that help to limit additional remodeling. Several neurohumoral systems that promote fibrosis are activated in HF, such as the renin-angiotensin-aldosterone and endothelin system. Guarda et al reported that ET-1 attenuated collagenase activity in CFs. Our data, as shown in Figure 7B, support this observation and demonstrate that BNP can reverse the action of ET-1, suggesting that stimulation of MMPs by BNP may be a compensatory response to prevent excessive collagen deposition induced by profibrotic factors. This antifibrotic action of BNP may be beneficial for the heart. However, in certain circumstances, it could be possible that BNP, by increasing MMP activity and ECM degradation, could promote ventricular remodeling leading to dilatation. In this study, coincubation of TNF-α and BNP had a synergistic effect on increasing MMP-2 release. Whether the actions of...
BNP are beneficial or deleterious very likely depends on the balance of fibrosis-promoting and fibrosis-inhibiting factors. The concentration of BNP used to induce MMP stimulation in the present study is higher than the concentration observed in the plasma of patients with severe HF. However, it is possible that similar high BNP concentrations can be achieved in vivo at the receptor level in pathological conditions, primarily attributable to cardiomyocyte production of BNP and partly attributable to CF production of BNP. Although we used high concentration of BNP, the increases in MMP were rather small. In addition, endogenous BNP secreted by CFs was not enough to augment MMP release on the basal level (Figure 6B) and on the stimulation with TNF-α (data not shown). However, there are several explanations for this phenomenon. First, because MMP-2 release even in controls gradually increased time-dependently, the culturing conditions may partly offset the difference in MMP-2 abundance between BNP-treated cell cultures and controls. Supporting this hypothesis, a study by Leicht et al showed that serum depletion increased the MMP activity in rat CFs. Second, high concentrations of BNP were needed in this cell culture system to increase intracellular cGMP. In contrast, degradation of bioactive BNP only seems to play a minor role, because unlabeled synthetic BNP in the medium of the fibroblast cell culture decreased by only 9% after 24 hours (data not shown).

In summary, this study demonstrates that cultured canine CFs produce BNP. In addition, BNP and its signaling system are involved in the induction of MMPs. Furthermore, cross-talk of BNP with ET-1 and TNF-α occurs on modulating MMP-2 abundance. Although it is difficult to trace a biological or a clinical implication of these in vitro findings, these results provide new insight into the antifibrotic properties of the cardiac hormone BNP and its signaling system that may be of importance in the progression of HF. Additional studies are clearly warranted to clarify the role of BNP in the remodeling of the heart in vivo.

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