Intramyocardial BNP Gene Delivery Improves Cardiac Function Through Distinct Context-Dependent Mechanisms

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Background—B-type natriuretic peptide (BNP) is an endogenous peptide produced under physiological and pathological conditions mainly by ventricular myocytes. It has natriuretic, diuretic, blood pressure–lowering, and antifibrotic actions that could mediate cardiorenal protection in cardiovascular diseases. In the present study, we used BNP gene transfer to examine functional and structural effects of BNP on left ventricular (LV) remodeling.

Methods and Results—Human BNP was overexpressed by using adenovirus-mediated gene delivery in normal rat hearts and in hearts during the remodeling process after infarction and in an experimental model of angiotensin II–mediated hypertension. In healthy hearts, BNP gene delivery into the anterior wall of the LV decreased myocardial fibrosis ($P<0.01$, $n=7$ to 8) and increased capillary density ($P<0.05$, $n=7$ to 8) associated with a 7.3-fold increase in LV BNP peptide levels. Overexpression of BNP improved LV fractional shortening by 22% ($P<0.05$, $n=6$ to 7) and ejection fraction by 19% ($P<0.05$, $n=6$ to 7) after infarction. The favorable effect of BNP gene delivery on cardiac function after infarction was associated with normalization of cardiac sarcoplasmic reticulum Ca$^{2+}$-ATPase expression and phospholamban Thr17-phosphorylation. BNP gene delivery also improved fractional shortening and ejection fraction in angiotensin II–mediated hypertension as well as decreased myocardial fibrosis and LV collagen III mRNA levels but had no effect on angiogenesis or Ca$^{2+}$-ATPase expression and phospholamban phosphorylation.

Conclusions—Local intramyocardial BNP gene delivery improves cardiac function and attenuates adverse postinfarction and angiotensin II–induced remodeling. These results also indicate that myocardial BNP has pleiotropic, context-dependent, favorable actions on cardiac function and suggest that BNP acts locally as a key mechanical load–activated regulator of angiogenesis and fibrosis. (Circ Heart Fail. 2011;4:483-495.)

Key Words: B-type natriuretic peptide  gene therapy  heart failure  myocardial infarction  angiogenesis  fibrosis

Heart failure (HF) is one of the most common causes of cardiovascular morbidity and mortality, and its prevalence is rapidly increasing as the mean age of the population advances. The major cause of systolic HF is coronary artery disease, whereas diastolic HF (HF with preserved ejection fraction [EF]) is more common in patients with hypertension. Worsening of chronic systolic or diastolic dysfunction is the most common form of acute HF, accounting for a substantial number of hospitalizations with a poor prognosis. A number of drugs, particularly β-blockers and drugs acting on the renin-angiotensin-aldosterone system, have been shown to improve survival in patients who have left ventricular (LV) systolic dysfunction. However, identifying appropriate treatments for patients who have HF with preserved EF or acute HF has been a daunting task.

Clinical Perspective on p 495

Atrial and B-type natriuretic peptides (ANP and BNP, respectively) are cardiac hormones, secretions of which are markedly upregulated during HF. Both exert potent diuretic, natriuretic, vasorelaxant, aldosterone-inhibiting, antifibrotic, and antihypertrophic effects that are mediated through their common receptor, guanylyl cyclase (GC)-A. In 2001, human recombinant BNP (nesiritide) was approved by the United States Food and Drug Administration for the treatment of acutely decompensated HF. However, the effect of nesiritide on mortality is uncertain, and controversy remains regarding the safety, efficacy, and dosing of BNP in therapy of acute HF. Intravenous infusion of nesiritide reduces LV filling pressure but has variable effects on cardiac output, urinary output, sodium excretion, and blood pressure. Ad-
verse renal consequences with nesiritide have been suggested, and careful monitoring of renal function is mandatory. Thus, novel therapeutic approaches are needed, and an attractive one is to increase BNP levels locally to avoid the systemic hemodynamic effects, excessive hypotension, and neurohumoral activation provoked by intravenous BNP.

In the present study, we examined the direct myocardial effects of BNP on cardiac function. Human BNP (hBNP) was overexpressed by using adenovirus-mediated gene delivery in normal rat hearts and in hearts during the remodeling process after myocardial infarction (MI) and in angiotensin II (Ang II)-induced hypertension. Because these experiments revealed that local BNP gene delivery improves LV systolic function during the remodeling process, we evaluated numerous potential mechanisms triggering the improvement of cardiac function and structure by BNP gene overexpression. The rationale for human BNP gene delivery into rat hearts was the unique opportunity to separate the exogenous BNP effects of BNP on cardiac function. Human BNP (hBNP) was injected with a catheter in the left carotid artery coupled with a sensor and transmitter (TA11PA-C40; Data Sciences, St Paul, MN). Twelve days after implantation, adenovirus-mediated local intramyocardial human BNP gene transfer into the LV was performed as described above.

Extraction of Cytoplasmic Protein and Western Blot Analyses

Proteins were extracted as described previously. For Western blot analysis, 30 μg of protein was subjected to SDS-PAGE, and separated proteins were electrically transferred to nitrocellulose membranes. After blocking the nonspecific background in 5% nonfat milk, nitrocellulose membranes were incubated with antiphospho-p38-MAPK, antiphospho-p44/42-MAPK, antisarcoplasmic reticulum Ca2+-ATPase (SERCA2), antiphospholamban-Ser16, antiphospholamban-Thr17, antip38-MAPK, antip44/42-MAPK, or antiphospholamban antibody. The protein amounts were detected by enhanced chemiluminescence.

Isolation and Analysis of RNA

Total RNA from whole LV tissue was isolated by the guanidine thiocyanate–CsCl method. RNA was analyzed by quantitative realtime polymerase chain reaction (RT-PCR) with TaqMan chemistry on an ABI 7300 sequence detection system (Applied Biosystems) as previously described. Sequences and probes for RNA detection are provided in online-only Data Supplement Table 2.

Radioimmunoassay and High-Performance Liquid Chromatography

Rat BNP, rat ANP, and human BNP peptide levels from plasma and tissue samples were measured by radioimmunoassay. The plasma and tissue samples for human immunoreactive amino-terminal-proBNP49–92, human ir-BNP32–38, rat ir-BNP22–42, and rat ir-NT-proANP79–98 were extracted with SepPak C8 cartridges before measurements. Analysis of the molecular form of human BNP peptides in plasma and in LV samples was performed by gel-filtration high-performance liquid chromatography (HPLC).

Cyclic GMP Assay

Cyclic GMP was detected from tissue samples using a cGMP EIA Kit (Cayman Chemical Company, Ann Arbor, MI) according to the manufacturer’s directions.

Histology, Immunohistochemistry, and Image Analysis

For histological analysis, the LVs were fixed in 10% buffered formalin solution. Transverse sections of the LV were embedded in paraffin, and 5-μm-thick sections were cut from the midsection of the heart, at the level of the papillary muscles. Sections were stained with hematoxylin and eosin or Masson trichrome to examine the fibrotic area. As shown in online-only Data Supplement Figure 1, the degree of fibrosis did not differ between PBS-based buffer–injected hearts and LacZ–injected hearts but tended to be higher in these groups than in noninjected hearts. The cross-sectional area of cardiomyocytes and the infarcted area (measured from LV circumference) were evaluated in Masson trichrome–stained sections with a digital image analysis system. To verify the expression of the transgene, the sections were incubated with specific polyclonal anti-human NT-proBNP antibody. To detect apoptotic cells, a terminal deoxynucleotidyl-transferase-mediated dUTP nick end-labeling (TUNEL) assay was performed as previously described.

Primary antibodies for lectin, Ki-67, c-kit, and CD43 were used to stain endothelial cells, proliferating cells, cardiac stem cells, and inflammatory cells, respectively. All measurements were performed by persons blinded to the treatments.

Statistics

Results are expressed as mean±SEM. For statistical analyses, the data were first tested by using the Shapiro-Wilk test for normality. Because all variables were normally distributed, statistical signifi-
cance was evaluated by 1-way ANOVA followed by a least significant difference post hoc test for multiple comparisons. The hemodynamic variables (mean arterial pressure and heart rate) were analyzed with 2-way repeated-measures ANOVA. The Student \( t \) test was used for comparison between 2 groups. A probability value of \(<0.05\) was considered statistically significant.

Results

Augmentation of LV BNP Levels by Adenoviral Gene Delivery

To study the direct myocardial effects of BNP, we established an in vivo gene transfer protocol to locally increase BNP levels in the adult rat LV. A clear activation of gene expression, evaluated by RT-PCR, as well as an increase in peptide concentrations, measured by radioimmunoassay, was noted when hBNP expressing adenoviral constructs were injected into the LV free wall at \(1 \times 10^9\) infectious units. Both hBNP mRNA (Figure 1A) and peptide (Figure 1B) levels were highest at day 3 after injections and decreased significantly thereafter during the follow-up period. Human BNP was not detectable by RT-PCR and radioimmunoassay in hearts of control animals injected with virus expressing LacZ at \(1 \times 10^9\) infectious units. As shown in Figure 1C, the LV hBNP peptide levels increased to 19.6 fmol/mg at day 3 by gene transfer, that is, total (exogenous plus endogenous) BNP
peptide concentration was about 9-fold higher than endogenous rat BNP peptide concentration. These levels are quantitatively equal to the BNP levels (17.6 fmol/mg) observed in an experimental model of Ang II–induced hypertension in rats at day 3.13 In agreement with the activation of GCs by increased BNP levels, there was a 2-fold increase in LV cGMP levels at day 3 that was significantly elevated also at 1 week after hBNP gene delivery (Figure 1D). The efficiency and localization of the BNP gene delivery was further confirmed by immunohistochemistry. Immunohistochemical analysis showed local and segmental hBNP staining in the anterior wall of the LV of hBNP-Ad5–injected hearts, whereas LacZ-treated hearts were virtually negative. BNP localized subcellularly to granules, nearly all being in the perinuclear zone confirming the specificity of the antibody (Figure 1E).

We also performed HPLC analyses of LV extracts and plasma to characterize the molecular forms of BNP produced by gene transfer (Figure 2A through 2D). The gel-filtration HPLC fractions of myocardial cell lysates contained high-molecular-weight hBNP and hNT-proBNP immunoreactive material, with the size consistent with human proBNP,1–108 (Figure 2A). In plasma, there was immunoreactive material corresponding exactly to the size of the native circulating form human BNP-32 (Figure 2D), indicating that human proBNP can be processed correctly in rat cardiomyocytes. In plasma, hNT-proBNP levels were highest at day 3 after injections (Figure 2E), and the levels of active rat BNP and human BNP peptides were comparable after BNP gene delivery (Figure 2F). Because hBNP was released into the circulation, we performed telemetric monitoring of hemodynamics to characterize systemic effects of intramyocardial hBNP gene delivery. As shown in online-only Data Supplement Figure 2, hBNP gene transfer had no statistically significant effect on mean arterial pressure or heart rate, although there was a tendency for mean arterial pressure to decrease at day 2 after gene delivery. Mean arterial pressure and heart rate were similar in LacZ-injected rats and hBNP-injected rats before gene transfer.

Because by design we injected hBNP, we also tested whether locally increased BNP could influence endogenous rat natriuretic peptide levels. Indeed, as shown in online-only Data Supplement Figure 3, LV rat BNP peptide levels were significantly lower at 1 and 2 weeks after hBNP gene transfer compared with LacZ-injected hearts. Moreover,
ANP mRNA levels decreased significantly at 3 days. On the other hand, plasma levels of rat BNP and rat NT-proANP did not differ between LacZ- and hBNP-injected rats (online-only Data Supplement Figure 3).

**BNP is Antifibrotic and Angiogenic in Normal Adult Rat Heart**

The effect of BNP gene delivery on myocardial fibrosis was examined by staining histological sections with Masson trichrome. Fibrosis decreased significantly by BNP gene delivery at 2 weeks (Figure 3A through 3C). Consistent with this, BNP overexpression decreased collagen IIIα1 (Col IIIα1) mRNA levels at 2 weeks (Figure 3D). On the other hand, no changes or nonsignificant differences in other fibrosis-related genes such as collagen Iα1 (Col Iα1) (Figure 3E), transforming growth factor-β1 and transforming growth factor-β2, and fibronectin-1 gene expressions were detected (online-only Data Supplement Figure 4).

To investigate the effect of BNP gene delivery on coronary angiogenesis, histological sections were immunohistochemically stained against lectin. Local BNP gene delivery resulted in a statistically significant increase in capillary density, whereas BNP gene transfer had no effect on capillary area (Figure 4A through 4C). As shown in Figure 4D, enhanced angiogenesis was associated with the increased gene expression of fibroblast growth factor-2 at 3 days and 1 week after gene transfer. To study the potential cardiac infiltration of inflammatory cells after adenoviral BNP gene transfer, histological sections were stained against CD43. No differences between LacZ- and hBNP-treated groups were noted (online-only Data Supplement Figure 5).

To evaluate the effect of BNP gene delivery on cardiac function, we performed echocardiography. LVEF and fractional shortening (FS) of the BNP-treated animals were similar to those of LacZ-treated group (online-only Data Supplement Table 3), indicating that BNP overexpression had no effect on LV function in normal rat hearts during the 2-week follow-up period. Also, LV dimensions did not significantly differ between groups.

**Local Myocardial BNP Gene Delivery Improves Systolic Function After Infarction**

We next examined the effects of BNP gene transfer on LV function and structure in an experimental MI model in rats. In these experiments, hBNP mRNA levels in the LV at 1 and 2 weeks after infarction were quantitatively equal to the hBNP levels in normal adult rat hearts at 1 and 2 weeks after hBNP gene transfer (online-only Data Supplement Figure 6). Ligation of the LAD caused a marked decrease in EF and FS within 2 weeks, as assessed by echocardiography (Figure 5A...
through 5C). BNP gene delivery significantly improved LVEF (19%, \( P < 0.05, n = 6 \) to 7) and FS (22%, \( P < 0.05, n = 6 \) to 7) at 2 weeks after infarction (Figure 5B and 5C), whereas LV dimensions did not differ between BNP-treated hearts and LacZ-treated hearts (Figure 5D and 5E). The effects of BNP gene delivery on systolic function were not statistically significant at 1 week after infarction (online-only Data Supplement Figure 7).

To assess whether the functional improvement after MI by BNP gene transfer was related to the reduction of myocardial fibrosis, histological sections were stained with Masson trichrome. The percentage of fibrotic area measured from the infarct border zone was 3.1-fold higher in the infarcted animals than in sham-operated animals (data not shown). In contrast to the healthy heart, fibrotic area did not differ between the infarcted BNP-treated hearts and LacZ-treated hearts at 2 weeks after infarction (online-only Data Supplement Figure 8A). Also, the size of the infarcted area remained unchanged by BNP gene delivery (online-only Data Supplement Figure 8B). Collagen gene expressions were similarly upregulated in the BNP-treated hearts and LacZ-treated hearts after MI (online-only Data Supplement Figure 8C and 8D). Furthermore, there was no significant difference between tumor necrosis factor-\( \alpha \) and endothelin-1 mRNA levels between groups (online-only Data Supplement Figure 8E and 8F), together indicating that BNP gene delivery had no effect of myocardial fibrosis after infarction. When cardiomyocyte hypertrophy after hBNP gene transfer was assessed by measuring cardiomyocyte cross-sectional area, no differences between LacZ-treated groups and hBNP-treated groups were noted (online-only Data Supplement Figure 9). Moreover, histological sections stained against CD43 showed no effect of hBNP gene transfer on inflammatory cells in the LV (data not shown).

In addition to pathological fibrosis, apoptotic cell death, cell proliferation, cardiac stem cell recruitment, and angiogenesis contribute to the LV remodeling after infarction.\(^{15,16} \) Interestingly, no differences in the capillary density and the capillary area as well as in the number of c-kit\(^{+} \) stemlike, proliferative, or apoptotic cells in the anterior wall of the LV between BNP-treated groups and LacZ-treated groups were detected (online-only Data Supplement Figure 10A through 10E).

**Figure 4.** Local BNP gene delivery increases coronary angiogenesis in normal heart. Myocardial angiogenesis was studied by immunohistochemical staining against lectin 2 weeks after hBNP gene transfer. A. Representative images. B. Capillary density, and C. capillary area were counted in 5 representative fields in the LV. D. Upregulation of LV fibroblast growth factor-2 (FGF-2) gene expression by BNP gene delivery. Results are expressed as mean \( \pm \) SEM (n = 7 to 8). *\( P < 0.05 \) versus LacZ (Student t test).

**Normalization of SERCA2 and Phospholamban Levels by BNP Gene Transfer After Infarction**

To evaluate further the potential mechanisms triggering the improvement of systolic function after infarction by local BNP gene delivery, changes in the cardiac sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2) mRNA and protein levels were assessed by RT-PCR and Western blotting, respectively. As shown in Figure 6A and 6B, BNP gene delivery normalized SERCA2 mRNA and protein levels at 1 week after infarction. Because SERCA activity is regulated in the heart by interaction with phospholamban,\(^{17} \) phosphoserine (Ser16)-specific and phosphothreonine (Thr17)-specific antibodies were used to examine the phosphorylated residues. Interestingly, BNP gene transfer increased phospholamban phosphorylation at Thr17 but not at Ser16 (Figure 6C and 6D). In agreement with changes in contractile function, \( \beta \)-myosin heavy chain and skeletal \( \alpha \)-actin mRNA levels increased significantly at 1 week after infarction by BNP gene transfer (Figure 6E and 6F).

Because it has been suggested that BNP inhibits fibrotic response through extracellular signal–related kinase signal-
ing and that p38 MAPK may limit increases in contractility through dephosphorylation of phospholamban in the myocardium, we investigated their role in mediating the effects of BNP gene transfer. However, the protein levels of p38 MAPK as well as p44/42 remained unchanged in BNP-treated hearts after infarction (online-only Data Supplement Figure 11), suggesting that BNP gene transfer did not influence on cardiac function via MAPK pathways.

**BNP Inhibits Fibrosis and Improves Systolic Function in Angiotensin II–Induced Hypertension**

The renin-angiotensin-aldosterone system has been identified as a crucial regulator of excessive myocardial fibrosis and cardiomyocyte hypertrophy. Therefore, we tested the effect of myocardial BNP gene delivery on cardiac structure and function in an experimental model of Ang II–mediated hypertension. Ang II was infused for up to 2 weeks to the hBNP-Ad5 and LacZ–injected rats, and hBNP mRNA levels were similar to the hBNP levels in normal rat hearts at 2 weeks after hBNP gene transfer (online-only Data Supplement Figure 6). Ang II–induced fibrosis, assessed from Masson trichrome–stained sections, was significantly attenuated by BNP gene transfer (Figure 7A; representative images are shown in online-only Data Supplement Figure 12). Furthermore, a significant decrease in collagen III and collagen I mRNA levels, upregulated by Ang II, was observed by BNP gene transfer (Figure 7B and 7C). The LV weight–to–body weight ratio remained unchanged (Figure 7D), indicating that BNP gene delivery did not in general modify the effects of Ang II. As after infarction, BNP gene delivery improved EF (10%, \( P < 0.01 \), \( n=6 \) to 11) and FS (20%, \( P < 0.05 \), \( n=6 \) to 11) also in Ang II–mediated hypertension, whereas no significant differences in LV dimensions between BNP-treated groups and LacZ-treated groups were noted (Figure 8A through 8E). BNP gene delivery had no effect on coronary angiogenesis in Ang II–mediated hypertension (online-only Data Supplement Figure 13), and p38 MAPK, p44/42-MAPK, SERCA2, and phospholamban levels, measured by Western blot analyses, did not differ between BNP- and LacZ-treated hearts (online-only Data Supplement Figure 14A through 14F).

**Discussion**

Natriuretic peptides (NPs) have emerged as important candidates for development of therapeutic agents in cardiovascular disease. Infusion of a recombinant form of mature BNP (nesiritide) has been used clinically in HF for its vasodilatory properties, but its use has been limited by hypotension and concerns regarding worsening of renal function. In addition to direct administration of native peptides, several other approaches alone or in combination with other pharmacological therapies have been shown to enhance function of the NP system: administration of designer NPs, inhibition of degradation of NPs and their second messenger (cGMP), and
stimulation of cGMP generation. Recently, the feasibility of an orally delivered, conjugated form of BNP in an experimental model of Ang II–mediated hypertension was demonstrated. In the present study, we describe a novel therapeutic strategy to augment the biological actions of NP system by locally increasing BNP levels in the heart using adenovirus-mediated gene delivery. BNP gene transfer decreased fibrosis and increased coronary angiogenesis in normal adult rat heart and improved LV systolic function after infarction and in Ang II–induced hypertension. Remarkably, BNP gene delivery provoked favorable responses through specific context-dependent mechanisms.

NPs have important autocrine, paracrine, and endocrine actions that are mediated through the GC-A receptor and activation of cGMP in target cells, including inhibition of cardiac hypertrophy and fibrosis. In cultured fibroblasts, BNP decreases collagen synthesis and increases matrix metalloproteinases (MMPs). Accordingly, overexpression of BNP in mice, targeted to the liver, has been shown to increase MMP-9 expression in the infarcted region after MI. Targeted deletion of the GC-A gene results in marked cardiac hypertrophy and fibrosis, whereas mice lacking the BNP gene show normal heart size but increased ventricular fibrosis. Thus, genetic studies in mice collectively support a local role for BNP in regulation of cardiac fibrosis. However, transgenic models exhibit alterations in blood pressure and cardiac function, suggesting that, in part, cardiac effects may be mediated indirectly through systemic hemody-

Figure 6. Upregulation of SERCA2 expression and phospholamban (PLB) phosphorylation by intramyocardial hBNP gene transfer in infarcted hearts. **A**, SERCA2 mRNA levels measured by RT-PCR. **B**, SERCA2 protein levels assessed by Western blot analyses. **C**, PLB phosphorylation of phospho-threonine17 (p-Thr17). **D**, PLB phosphorylation of phospho-serine16 (p-Ser16). **E**, β-myosin heavy chain (β-MHC) mRNA. **F**, Skeletal α-actin mRNA. Results are expressed as mean±SEM (n=5 to 7). *P<0.05, **P<0.01 versus sham; †P<0.05, ††P<0.01 versus LacZ with AMI (1-way ANOVA followed by least significant difference post hoc test).
namic actions. In the present study, we increased myocardial BNP peptide levels precisely to no more than those observed in experimental models of cardiac overload. These studies demonstrate that BNP exerts locally an antifibrotic effect within the heart in vivo. Lack of significant changes in blood pressure, increase in cGMP production, and decrease in the synthesis of endogenous NPs in LV, and only a 2-fold increase in LV BNP levels, are compatible with the local antifibrotic effect of BNP in normal adult rat heart. GC-A is expressed in cardiac myocytes, fibroblasts, and endothelial cells in the heart. Although marked species differences exist in the potency for cGMP production among BNPs and the potency of BNPs for cGMP production varies from cell to cell, cGMP production has been reported to be activated by human BNP in rats cells, in agreement with our present results. For example, human BNP-32 added to purified rat ventricular myocytes resulted in a marked accumulation of cGMP, similar to rat ANP. It is also notable that in bovine endothelial cells, hBNP is an even more potent activator of cGMP production than rat BNP. Further, in primary cultures of neonatal rat cardiac fibroblasts, in which both GG-A and GC-B are expressed, porcine BNP-32 (structure very similar to hBNP-32) inhibited growth factor–dependent-3H-thymidine incorporation, this effect being enhanced by phosphodiesterase inhibition. Interestingly, some BNP-containing granules were noted in ventricles after overexpression of BNP, whereas staining was diffuse in LacZ-injected hearts, suggesting that if tissue BNP concentration is high, as in patients with dilated cardiomyopathy, BNP is stored to some extent in granules.

Very recent studies suggest that the NP/GC-A system could be involved also in the stimulation of angiogenesis. In vitro, BNP/GC-A stimulates proliferation and migration of cultured microvascular endothelial cells by activating cGMP-dependent protein kinase I. An increase in circulating BNP levels resulting from targeted overexpression of the BNP gene in the liver of mice accelerated vascular regeneration in a limb ischemia model. Furthermore, in a pressure overload-induced hypertrophy model, selective disruption of the endothelial GC-A shows diminished angiogenesis. Our present results extend these studies by identifying BNP for the first time as a coronary angiogenic factor. BNP is among the earliest cardiac factors induced in response to hemodynamic load, and this mechanical stretch–induced activation of BNP gene expression is mediated by GATA. Thus, we hypothesize that BNP acts locally as a major mechanical load–activated and GATA-4–activated regulator of angiogenesis. Notably, also GATA-4 has been identified as a regulator of coronary angiogenesis. Angiogenesis by the NP/GC-A system in vivo could be achieved by endothelial release of factors that act on other cell types or by other indirect mechanisms. In the present study, BNP-induced angiogenesis was associated with the increased gene expression of fibroblast growth factor-2. Fibroblast growth factor-2 is involved in regulation of cell survival, migration, fibrosis, and matrix production/degradation but is also a strong cardioprotective and angiogenic mediator. The effects of NPs are not mediated by the endothelial release of VEGF-A and autocrine/paracrine activation of the VEGF receptor in vitro in endothelial cell cultures.

Our results demonstrate that BNP gene delivery significantly improved LV systolic function after infarction, suggesting that the enhancement of BNP function selectively in the heart may be a potential new therapy after MI. Because growing evidence indicates that the loss of functional capil-
laries and microvessels is a critical determinant of myocardial remodeling, we investigated whether BNP has an impact on myocardial angiogenesis. However, the capillary density and capillary area as well as cardiac fibrosis, apoptotic cell death, cell proliferation, and cardiac stem cell recruitment, all probably contributing to the structural and functional remodeling of infarcted myocardium, remained unchanged by BNP gene delivery. Therefore, we evaluated numerous other potential mechanisms triggering the improvement of LV function by BNP gene transfer after MI. These studies revealed that BNP overexpression did not influence MAPK pathways after MI but was associated with normalization of SERCA2 level, preceding the improvement of contractile function. SERCA2 is responsible for calcium reuptake from the cytosol into the lumen of the sarcoplasmic reticulum, and reduced SERCA2 expression, observed consistently in HF, impairs the calcium-handling and contractile functions of the heart. Because long-term SERCA2 gene transfer restores contractile function in the rat ischemic HF model, the improvement of cardiac function by BNP gene delivery may be related to normalization of SERCA2 levels.

The activity of SERCA is regulated by phospholamban. In its unphosphorylated form, phospholamban inhibits SERCA2 Ca\(^{2+}\) affinity, and phosphorylation by protein kinase A and Ca\(^{2+}\)/calmodulin kinase (CaMKII) relieves this inhibition and results in increased contractility through enhanced Ca\(^{2+}\) reuptake into the sarcoplasmic reticulum. In failing hearts, the phosphorylation levels of phospholamban at Ser16 and Thr17 are decreased. In the present study, BNP overexpression increased phospholamban phosphorylation of Thr17 but not Ser16 at 1 week after infarction. This effect of BNP gene transfer on phosphorylation of Thr17 residue of phospholamban may be indirect, because phosphorylation of phospholamban by GMP-dependent protein kinase occurs at Ser16. Consistent with the finding that BNP gene delivery influenced on phospholamban at Thr17 by CaMKII, a previous study showed that in intact cardiac myocytes, Thr17 phosphorylation by CaMKII occurs in the absence of Ser16 phosphorylation. More recently, inhibition of phospholamban with gene transfer in a model of HF resulted in improved contractility, reversal of adverse remodeling, and a decrease in fibrosis. Taken together, the favorable effect of BNP gene delivery on cardiac function after MI may be mediated through normalization of phosphorylation of phospholamban and SERCA2 expression, restoring cardiomyocyte function.
by improving Ca2+ uptake into the sarcoplasmic reticulum. In agreement with the changes in contractile function, β-myosin heavy chain and skeletal α-actin mRNA levels by BNP gene transfer were increased after infarction. Although the reported effects at 2 weeks may represent long-term functional and structural effects, the longer-term effects were not evaluated; therefore, for example, it is not known if survival of the animals after infarction is improved by intramyocardial BNP gene delivery.

Angiotensin type 1 receptors mediate Ang II–stimulated collagen synthesis and induce proliferation of cardiac fibroblasts, thus enhancing cardiac remodeling.20 Thus, we examined the structural and functional effects of BNP gene delivery in an experimental model of Ang II–mediated hypertension. BNP gene transfer significantly improved systolic function as well as reduced myocardial fibrosis and collagen gene expression. Interestingly, no differences in the capillary density and capillary area were observed between LacZ-treated hearts and BNP-treated hearts. Thus, antifibrosis appears to mediate the favorable effect of local BNP gene delivery on cardiac function in Ang II–mediated hypertension. We also did not observe any difference of the signaling pathways (SERCA2 expression, phospholamban phosphorylation, MAPKs). Recently, BNP has been reported to provoke angiogenesis in vitro cultured microvascular endothelia a mild but significant increase in the levels of phosphorylated p38 MAPK within 15 minutes.34 In addition, ANP increases p38 MAPK activity in vivo within 20 minutes in the liver,49 whereas in human umbilical vein endothelial cells, ANP induces inhibition of p38 MAPK through activation of its upstream regulator, the MAPK phosphatase-1.50 The reason for these discrepant findings may be the differences in experimental settings, and clearly more studies are necessary to better understand the role of p38 MAPK in mediating the effects of the NPs on cardiac function.

Overall, our results using different experimental models indicate that increased myocardial BNP modulates cardiac function through unique context-dependent and/or disease-dependent mechanisms, involving antifibrosis, angiogenesis, or normalization of SERCA2 expression and phosphorylation of phospholamban. The mechanism(s) for these distinct actions of BNP remain to be established, but the context-dependent effects may be dependent on distinct pathophysiological processes in MI and hypertension-induced heart disease; for example, MI fibrosis is more resistant than angiotensin II–induced fibrosis to antifibrotic effect of BNP. In agreement with this hypothesis, we reported previously that in the adult normal rat heart, the physiological consequence of p38 MAPK overexpression is cardiac cell proliferation and myocardial inflammation associated with fibrosis,51 whereas normalization of decreased p38 MAPK activity prevented adverse postinfarction remodeling through a distinct angiogenic and antiapoptotic mechanism.13 Accordingly, we hypothesize that activation or inhibition of specific substrates of cGMP-dependent protein kinases (cGKs), stimulated by BNP through GC-A,24 may be context-dependent. cGMP-dependent protein kinases are essential in various cells and tissues for the regulation of diverse functions comprising tissue contractility, cell motility, cell contact, cellular secretion, cell proliferation, and cell differentiation.21,24 Nonetheless, these context-dependent favorable effects appear to be unique for BNP. For example, adrenomedullin gene delivery under identical experimental conditions attenuated angiotensin II–induced LV hypertrophy without compromising cardiac function but enhanced LV dilatation and anterior wall thinning and augmented the deterioration of LV function after infarction.52

Despite optimal treatment with existing drugs, the prognosis of HF remains poor, and thus therapeutic strategies designed to specifically attenuate myocardial remodeling process would represent a promising new approach to treat patients with HF. Very recently, in a proof-of-concept pilot human study, low-dose intravenous infusion of recombinant BNP for 72 hours preserved ventricular function and structure after MI.53 In the present study, we report that the BNP gene delivery may be a potential new therapy after infarction. Our studies also indicate that BNP gene delivery has pleiotropic, context-dependent, favorable actions on cardiac function. Finally, myocardial BNP may act locally as a key mechanical load–activated regulator of angiogenesis and fibrosis. It would thus be attractive to develop novel pharmacological therapies for the enhancement of BNP function selectively in the heart and to investigate whether phosphodiesterase inhibitors or direct guanylate cyclase activators modify the favorable antifibrotic and angiogenic effects of BNP gene delivery on cardiac function.

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Disclosures

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References


Heart failure is one of the most common causes of cardiovascular morbidity and mortality, and its prevalence is rapidly increasing as the mean age of the population advances. Despite optimal treatment with existing drugs, the prognosis of heart failure remains poor, and thus therapeutic strategies designed to specifically attenuate the myocardial remodeling process would represent a promising new approach to treat patients with heart failure. In the present study, we report that B-type natriuretic peptide (BNP) gene delivery may be a potential new therapy after infarction. Our studies indicate that BNP gene delivery has unique pleiotropic, context-dependent, favorable actions on cardiac function because (1) local BNP gene delivery into the rat adult heart improved left ventricular contractility during the remodeling process both after infarction and in response to pressure overload; (2) in healthy heart, local increase in LV BNP peptide levels by gene delivery was antifibrotic and angiogenic without affecting systolic function; and (3) the favorable effect of BNP on cardiac function after infarction but not in Ang II–induced hypertension was associated with normalization of SERCA2 expression and phosphorylation of phospholamban. Finally, our results suggest that myocardial BNP acts locally as a key mechanical load–activated regulator of angiogenesis and fibrosis. It would be attractive to develop novel pharmacological therapies for the enhancement of BNP function selectively in the heart and to investigate whether phosphodiesterase inhibitors or novel direct guanylate cyclase activators modify the favorable antifibrotic and angiogenic effects of BNP gene delivery on cardiac function.
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SUPPLEMENTAL MATERIAL

Intramyocardial BNP gene delivery improves cardiac function through distinct context-dependent mechanisms

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SUPPLEMENTAL METHODS

Recombinant Adenoviral Vectors

For constructions of human BNP (hBNP), a full-length coding region of hBNP cDNA was cloned into the SalI and HindIII sites of the pShuttle-CMV vector (Qbiogene Inc., Illkirch, Cedex-France). The sequences for the cloning primers used were as follows; hBNP forward 5'-GCG TCG ACT CCA GAG ACA TGG ATC CCC AG-3' and reverse 5'-CCC AAG CTT TTA ATG CCG CCT CAG CAC-3'. The pShuttle-CMV-LacZ was a commercial plasmid (Stratagene, La Jolla, CA, USA). Adenoviruses (serotype 5) were prepared by standard protocols (Qbiogene Inc., Illkirch, Cedex-France) and purified by centrifugation on iodixanol (OptiPrep, Axis-Shield PoC AS, Oslo, Norway). The adenoviral titers (infectious unit, ifu) were determined by AdEasy Viral Titer Kit (Stratagene). Infectious unit is biologically equivalent to plaque forming unit (PFU). β-galactosidase concentration was determined by luminescent β-gal Kit (Clontech Laboratories Inc., Palo Alto, CA, USA).

Intramyocardial Gene Transfer

We and others have previously shown that local injection of adenoviral constructs into the left ventricular (LV) free wall is an efficient site-specific method of gene delivery that targets high expression of the transgene in the left ventricle without affecting other organs or other regions of the heart.1,2 Male Sprague-Dawley rats weighing 250-300 g were anesthetized with medetomidine hydrochloride (Domitor, 250 μg/kg i.p.) and ketamine hydrochloride (Ketamine, 50 mg/kg i.p). Rats were connected to the respirator through a tracheotomy. A left thoracotomy and pericardial incision was performed. Adenovirus-mediated gene transfer into the LV free wall was performed as previously described.2,3 Different doses of adenoviral constructs were first tested to increase LV BNP peptide levels closely to those observed in experimental models of cardiac overload. Then, recombinant adenovirus (1x10⁹ infectious units, ifu), in a 100 μl volume, was injected using a Hamilton precision syringe directly into the anterior wall of the left ventricle. The syringe was inserted in one site of the LV free wall (apex to base), and then slowly the solution was injected while withdrawing the syringe. After the operation, anaesthesia was partially antagonized with atipamezole hydrochloride (Antisedan, 1.5 mg/kg i.p.) and rats were hydrated with physiological saline solution (5 ml s.c.). For postoperative analgesia, buprenorphine hydrochloride (Temgesic, 0.05-0.2 mg/kg s.c.) was administered.
All experimental protocols were approved by the Animal Use and Care Committee of the University of Oulu and conforms to the Guide for the Care and Use of Laboratory Animals Published by the US National Institutes of Health. The total number of animals used was 327. Number of rats in each experiment is summarized in supplemental Table 1.

**Acute Myocardial Infarction**

Acute myocardial infarction (AMI) was produced by ligation of the left anterior descending coronary artery (LAD) as previously described. The sham-operated rats underwent the same surgical procedure without the ligation of LAD. Recombinant adenovirus was injected into the anterior wall of the left ventricle before the ligation of LAD. The adenoviral gene delivery to the sham-operated hearts was performed using the same technique without the ligation of LAD.

**Angiotensin II-mediated Hypertension**

Angiotensin II (Ang II, 33.3 μg/kg/h) was administered via subcutaneously implanted osmotic minipumps (Alzet model 2002; Scanbur BK AB, Sollentuna, Sweden), as described previously. Minipumps were implanted subcutaneously before the gene delivery. Ang II–mediated hypertension has been used extensively in key studies in the development of antihypertensive agents. Using this experimental model of hypertension, mean arterial pressure increases rapidly (within 3 hours) and remains significantly elevated throughout the 2-weeks period. Under our experimental conditions, Ang II type 1 receptor (AT1-R) blockade by losartan completely abolished Ang II–induced changes in the cardiac gene expression, left ventricular weight to body weight ratio and hemodynamics.

**Echocardiographic Measurements**

Transthoracic echocardiography was performed using the Acuson Ultrasound System (Sequoia™ 512) and a 15-MHz linear transducer (15L8) (Acuson, MountainView, CA, USA) as previously described. Before examination, rats were sedated with ketamine (50 mg/kg i.p.) and xylazine (10 mg/kg i.p.). Using two-dimensional imaging, a short axis view of the left ventricle at the level of the papillary muscles was obtained, and a two dimensionally guided M-mode recording through the anterior and posterior walls of the LV was obtained.
LV end-systolic and end-diastolic dimensions as well as the thickness of the interventricular septum and posterior wall were measured from the M-mode tracings. LV fractional shortening (FS) and ejection fraction (EF) were calculated from the M-mode LV dimensions using the following equations:

\[
\text{FS} (\%) = \left( \frac{\text{LVEDD} - \text{LVESD}}{\text{LVEDD}} \right) \times 100
\]

\[
\text{EF} (\%) = \left( \frac{\text{LVEDD}^3 - (\text{LVESD})^3}{\text{LVEDD}^3} \right) \times 100
\]

An average of three measurements of each variable was used. All echocardiographical measurements were performed by skilled sonographers (E.M. and H.L.) blinded to the treatments. After echocardiography, the animals were sacrificed. Blood samples were collected into pre-cooled tubes containing ethylenediamine tetra-acetic acid (1.5 mg/1ml blood), and hearts were weighed and the ventricles were immersed in liquid nitrogen and stored at -70°C for later analysis.

**Telemetric Monitoring**

Rats were anesthetized with medetomidine hydrochloride and ketamine hydrochloride anesthesia combined with preoperatively administered buprenorphine (0.05 mg/kg s.c.) and carprofen (Rimadyl, 5 mg/kg s.c.). For telemetric monitoring of hemodynamics, rats were instrumented with a catheter in the left carotid artery coupled with a sensor and transmitter (TA11PA-C40; Data Sciences, St. Paul, MN, USA) implanted under the skin. After operation the anesthesia was partially antagonized with atipamezole hydrochloride (1.5 mg/kg, i.p.) and the rats were given buprenorphine hydrochloride (0.05-0.2 mg/kg s.c.) for analgesia. Twelve days after implantation, adenovirus-mediated local intramyocardial human BNP gene transfer into the left ventricle was performed as described above. Mean arterial pressure and heart rate were continuously measured through the experiment. Hemodynamics were recorded every 3 minutes and averaged for every hour. Results shown in this study represent 24-h average every day, except at day 0 due to intramyocardial gene transfer.

**Extraction of Cytoplasmic Protein and Western Blot Analyses**

To extract the cytoplasmic protein, the left ventricular tissue was broken and reduced to a powder in liquid nitrogen. The thawed powder was homogenized in a lysis buffer (20 mmol/l Tris-HCl [pH 7.5], 10 mmol/l NaCl, 0.1 mmol/l EDTA, 0.1 mmol/l EGTA, 1 mmol/l β-glycerophosphate, 1 mmol/l Na3VO4, 2 mmol/l benzamidine,
1 mmol/l phenylmethylsulfoxide, 50 mmol/l NaF, 1 mmol/l dithiothreitol and 10 µg/ml each of leupeptin, pepstatin and aprotinin). The cytosolic fraction was separated out by centrifugation at 2000 rpm in +4°C for 1 minute. To separate the cytoplasmic protein fraction, 5 x nuclear extraction buffer (NEB) (100 mM Tris-HCl [pH 7.5], 750 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5% Triton X 100, 12 mM sodium pyrophosphate, 5 mM β-glycerophosphate, 5 mM Na3VO4) was added to the tissue homogenate following by centrifugation at 12500 rpm in +4°C for 20 minutes. The supernatant was frozen in liquid nitrogen and stored in −70°C until assayed. Protein concentrations were examined by Bio-Rad Laboratories Protein Assay.

For western blot analysis, 30 µg protein was subjected to SDS-PAGE and separated proteins were electrically transferred to nitrocellulose membranes. After blocking the nonspecific background in 5% non-fat milk, nitrocellulose membranes were incubated with anti-phospho-p38-mitogen-activated protein kinase (MAPK), anti-phospho-p44/42-MAPK, anti-sarcoplasmic reticulum Ca2+-ATPase (SERCA2), anti-phospholamban-Ser16, anti-phospholamban-Thr17, anti-p38-MAPK, anti-p44/42-MAPK or anti-phospholamban antibody. After washing the filters were incubated with an HRP-conjugated anti-rabbit, anti-mouse or anti-goat secondary antibody. Antibodies were obtained from Santa Cruz Biotechnology (CA, USA) and Cell Signaling Technology (Beverly, MA, USA).

For a second western blot, the membranes were stripped in buffer containing 62.5 mmol/l tris (pH 6.8), 2% SDS, and 100 mmol/l mercaptoethanol. The protein amounts were detected by enhanced chemiluminescence. The films were scanned and analyzed with Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

**Isolation and Analysis of RNA**

The RNA was extracted from the left ventricular tissue by using the guanidine-thiocyanate-CsCl method. Human B-type natriuretic peptide (BNP), rat atrial natriuretic peptide (ANP), rat BNP, skeletal α-actin, β-myosin heavy chain (β-MHC), collagen Iα1 and IIIα1, endothelin-1, fibroblast growth factor-2 (FGF-2), tumor necrosis factor-α (TNF-α), fibronectin-1, transforming growth factor β1 (TGFβ1) and β2, and 18S mRNA levels were analyzed by the RT-PCR using TaqMan chemistry on an ABI 7300 Sequence Detection System (Applied Biosystems) as previously described.
The sequences of the forward and reverse primers and for fluorogenic probes for RNA detection are shown in the Tables section (Table 2) of the online-only Data Supplement. The results were normalized to 18S RNA quantified from the same samples.

Radioimmunoassay and HPLC

Rat BNP, rat ANP and human BNP peptide levels from plasma and tissue samples was measured by RIA. The plasma and tissue samples for human immunoreactive amino-terminal-proBNP<sub>10-29</sub> (ir-NT-proBNP<sub>10-29</sub>), human ir-BNP-32, rat ir-BNP<sub>22-42</sub> and rat ir-NT-proANP<sub>79-98</sub> were extracted with SepPak C<sub>18</sub> cartridges before measurements. The cardiac tissue peptide levels were measured from diluted aliquots of the guanidine thiocyanate extracts prepared for RNA determination. The extraction buffer did not interfere with assay at the dilutions used. Human BNP assays do not cross-react (<0.01%) with peptides derived from rat proBNP.

Analysis of the molecular form of human BNP peptides in plasma and in LV samples was performed by gel filtration HPLC. Synthetic human BNP<sub>77-108</sub> and recombinant human NT-proBNP<sub>1-76</sub> peptides were used as a calibrators. The samples were applied to a Biosuite 125 HR column [7.8 (i.d.) x 300 mm; Waters] and eluted with 10% acetonitrile in aqueous trifluoroacetic acid (1ml/l trifluoroacetic acid in water). The flow rate was 1ml/min, and 0.5 ml fractions were collected.

Cyclic GMP Assay

LV tissue samples were homogenized in 10 volumes of 5% trichloroacetic acid (TCA) at 4°C. Samples were centrifuged and TCA was extracted from the supernatant by adding 5 volumes of water-saturated ether for 3 times. Residual ether was removed from the aqueous layer by heating at 70°C. Cyclic GMP was detected in non-acetylated samples using a cyclic GMP EIA Kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to manufacturer’s directions.
Histology, Immunohistochemistry and Image analysis

For histological analysis, the left ventricles were fixed in 10% buffered formalin solution. Transversal sections of the left ventricle were embedded in paraffin, and 5-μm sections were cut. Sections were cut from the mid-section of the heart, at the level of the papillary muscles. Samples from different animals were obtained in an identical way and from the corresponding sites in order to make the samples fully comparable. Sections were stained with hematoxylin and eosin or Masson's trichrome to examine the fibrotic area of the left ventricle. To study the local response to adenovirus-mediated gene transfer, fibrotic area in the left ventricle was measured at 2 weeks after intramyocardial injection of adenoviral construct expressing LacZ and PBS-based buffer (3%-iodixanol-PBS) as well as from the hearts with needle-stick (no injection of fluid) and non-injected hearts.

The infarcted area was measured from LV circumference in the Masson's trichrome stained sections using a digital image analysis system (Nikon NIS_Elements BR 2.30 software). One block and section per heart taken from the same site in all samples was used for staining. To assess cardiomyocyte hypertrophy, cross sectional area of cardiomyocytes was calculated from five correspondingly located fields per sample (3 from epicardial and 2 from endocardial side of the left ventricle). Cross sectional area of ten cells per field was measured using the Nikon NIS_Elements BR 2.30 software.

Biotinylated lectin-GSL-1 (B-1205, Vector laboratories, Burlingame, CA, USA) was used to stain endothelial cells. The number of capillaries was calculated from five representative high power fields (40×) from the left ventricle of each section; 3 from epicardial and 2 from endocardial side of the left ventricle were selected. To detect apoptotic cells, in situ labeling of the 3' ends of the DNA fragments generated by apoptosis-associated endonucleases was performed using the ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD, USA), as previously described Briefly, DNA fragmentation was identified by applying terminal transferase enzyme with digoxigenin-labeled nucleotides. Anti-digoxigenin antibody was used to recognize the digoxigenin-labeled nucleotide chains attached to the 3'-ends of sample DNA. A color reaction was produced with diaminobenzidine and the sections were counterstained with hematoxylin. The apoptotic cells and bodies were counted in 5 high power fields (40× objective) choosing hot spot areas in each sample in order to make the results comparable.
To examine the efficiency and localization of the BNP gene delivery, the sections were incubated with specific polyclonal anti-human NT-proBNP antibody at the dilution of 1:2000 3 days after gene transfer. Primary antibody for c-kit (sc-168, Santa Cruz Biotechnology, CA, USA) was used to stain stem cells. The number of c-kit+ cells in the anterior wall of left ventricle was counted. The area of counted section was examined by computerized methods and a number of positively staining cells was related to the area (cells/35 mm²). To identify cells undergoing division, immunohistochemical labeling of nuclear Ki-67 antigen was performed by using monoclonal mouse anti-rat Ki-67 antigen antibody (DakoCytomation, Glostrup, Denmark). The whole left ventricle was scanned and stained cells were counted from high power fields (40×) choosing 5 hot spot areas in each sample. Primary antibody for CD43 (ab22351, Abcam, UK) was used to detect inflammatory cells in the left ventricle. The number of positively stained cells was counted from the whole left ventricle of all samples and related to area (cells/35 mm²) in order to make the samples comparable. The primary antibodies were detected by peroxidase conjugated EnVision Detection Kit system (DakoCytomation, Denmark) and the samples were counterstained with haematoxylin. All measurements were performed blinded by persons, who were not aware of the treatments.

**Statistics**

Results are expressed as mean±SEM. Statistical analyses were performed using SPSS version 16.0.1 (SPSS Inc., Chicago, IL, USA). For statistical analyses, the data were first tested by using the Shapiro-Wilk test for normality. Because all variables were normally distributed, statistical significance was evaluated by one-way ANOVA followed by a least significant difference (LSD) post hoc test for multiple comparisons. The hemodynamic variables (mean arterial pressure and heart rate) were analyzed with two-way repeated-measures ANOVA. Student's t-test was used for comparison between two groups. A P-value of <0.05 was considered statistically significant.
### Online-only Data Supplement Table 1. Summary of rats used.

<table>
<thead>
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<th>Study</th>
<th>Treatment</th>
<th>No.</th>
<th>Duration</th>
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</thead>
<tbody>
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<td>LacZ-Ad5</td>
<td>7</td>
<td>1 week</td>
</tr>
<tr>
<td></td>
<td>hBNP-Ad5</td>
<td>7</td>
<td>1 week</td>
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<tr>
<td></td>
<td>LacZ-Ad5</td>
<td>8</td>
<td>2 weeks</td>
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<tr>
<td></td>
<td>hBNP-Ad5</td>
<td>8</td>
<td>2 weeks</td>
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<tr>
<td></td>
<td>Untreated</td>
<td>6</td>
<td>1 week</td>
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<tr>
<td></td>
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<td>AMI</td>
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<tr>
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<td>2 weeks</td>
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<td></td>
<td>Untreated control</td>
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<td>2 weeks</td>
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<tr>
<td></td>
<td>hBNP-Ad5</td>
<td>7</td>
<td>2 weeks</td>
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<td></td>
<td>LacZ-Ad5</td>
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<tr>
<td></td>
<td>LacZ-Ad5</td>
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<td>2 weeks</td>
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<td>2 weeks</td>
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<tr>
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<td></td>
<td>hBNP-Ad5</td>
<td>4</td>
<td>10.5 days</td>
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LacZ-Ad5 indicates LacZ-overexpressing adenovirus (serotype 5) injection; hBNP-Ad5, human B-type natriuretic peptide Ad5-injection; AMI, acute myocardial infarction; Sham, the sham-operated rats; Ang II, angiotensin II-induced hypertension.
Online-only Data Supplement Table 2. Forward and reverse primer and fluorogenic probe sequences used for real time quantitative RT-PCR analysis.

<table>
<thead>
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<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
<th>Fluorogenic probe Sequence</th>
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hBNP, human B-type natriuretic peptide; rBNP, rat BNP; ANP, atrial natriuretic peptide; β-MHC, β-myosin heavy chain; FGF-2, fibroblast growth factor-2; TNF-α, tumor necrosis factor-α; TGFβ1, transforming growth factor β1; TGFβ2, transforming growth factor β2; RT-PCR, real time quantitative reverse transcription-PCR.
Online-only Data Supplement Table 3. Effect of intramyocardial BNP gene delivery on cardiac function in normal adult rat heart. Adenoviral gene construct expressing human BNP (hBNP) and LacZ were injected into LV free wall and echocardiographic measurements were performed at 1 week and 2 weeks after gene transfer. The results are expressed as mean±SEM (n=7-8). P=ns vs LacZ (Student's t-test).

<table>
<thead>
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<th>Variable</th>
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<th>hBNP</th>
<th>LacZ</th>
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<td>2 weeks</td>
<td>66±2</td>
<td>60±3</td>
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<td>Fractional shortening (%)</td>
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<td>Interventricular septum</td>
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<td>2.7±0.2</td>
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<td>2 weeks</td>
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<td>systole (mm)</td>
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<td>Left ventricle</td>
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<td>2 weeks</td>
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Supplemental Figure 1.
Supplemental Figure 2.
Supplemental Figure 3.
Supplemental Figure 4.
Supplemental Figure 5.

This figure shows a bar graph comparing the number of CD43+ cells/35mm² between LacZ and hBNP groups. The y-axis represents the number of CD43+ cells, and the x-axis represents the experimental conditions: LacZ and hBNP. The graph includes error bars indicating variability.
Supplemental Figure 6.
Supplemental Figure 7.
Supplemental Figure 8.
Supplemental Figure 9.
Supplemental Figure 10.
Supplemental Figure 11.
Supplemental Figure 12.
Supplemental Figure 13.
Supplemental Figure 14.
FIGURE LEGENDS

Supplemental Figure 1. Effect of adenovirus-mediated gene transfer on the fibrotic area in the left ventricle (LV). Paraffin-embedded histological sections were stained with Masson’s trichrome to define the area of fibrosis at 2 weeks after intramyocardial injection of adenoviral construct expressing LacZ and PBS-based buffer (3%-iodixanol-PBS) as well as from the hearts with needle-stick (no injection of fluid) and non-injected hearts. A, B, Representative images are shown. C, Fibrosis were examined by computerized methods. The degree of fibrosis did not differ between PBS-based buffer- and LacZ-injected hearts, but tended to be higher in these groups than in non-injected hearts. The results are expressed as mean±SEM (n=3-4). P=ns vs LacZ (one-way ANOVA followed by LSD post hoc test).

Supplemental Figure 2. Effect of adenovirus-mediated human BNP (hBNP) gene transfer into the left ventricle on mean arterial pressure and heart rate in conscious rats measured by telemetric monitoring. Local intramyocardial hBNP gene delivery was performed 12 days after implantation of transmitter at day 0. A, hBNP gene transfer had no statistically significant effect on mean arterial pressure (F=1.876 vs LacZ, repeated-measures ANOVA, P=ns), although there was a tendency for mean arterial pressure to decrease at 2 day after gene delivery. B, Heart rate. F=0.907 vs LacZ (repeated-measures ANOVA), P=ns. The results are expressed as mean±SEM (n=4).

Supplemental Figure 3. Effect of intramyocardial human BNP gene delivery on endogenous rat natriuretic peptides in normal adult rats. A, LV rat BNP mRNA, B, LV rat BNP peptide, C, Plasma rat BNP peptide, D, LV rat ANP mRNA, E, LV rat NT-proANP peptide and F, Plasma rat NT-proANP levels after gene transfer. LacZ, open bars; human BNP-Ad5, solid bars. The results are expressed as mean±SEM (n=7-8). *P<0.05 vs LacZ (Student’s t-test).

Supplemental Figure 4. Effect of intramyocardial human BNP gene delivery on expression of fibrosis-related genes in normal heart. A, Transforming growth factor β1 (TGFβ1), B, TGFβ2, and C, Fibronectin-1 mRNA levels in the left ventricle after gene transfer. LacZ, open bars; human BNP-Ad5, solid bars. The results are expressed as mean±SEM (n=7-8). P=ns vs LacZ (Student’s t-test).
Supplemental Figure 5. The number of inflammatory cells were studied by immunohistochemical staining against CD43 at 2 weeks after human BNP (hBNP) gene delivery in normal adult rats. The number of positively stained cells was counted from the whole left ventricle of all samples and related to area (cells/35 mm²) in order to make the samples comparable. The results are expressed mean±SEM (n=7-8). P=ns vs LacZ (Student’s t-test).

Supplemental Figure 6. Cardiac specific activation of BNP by adenoviral gene delivery into the left ventricle. Human BNP mRNA levels were measured by RT-PCR 1 and 2 weeks after gene transfer into normal rat heart and after acute myocardial infarction (AMI) or 2 weeks after angiotensin II (Ang II)-induced hypertension. The results are expressed as mean±SEM (n=5-7). ***P<0.001 vs at 1 week normal heart (Student’s t-test).

Supplemental Figure 7. Effect of intramyocardial BNP gene delivery on cardiac function after AMI. Adenoviral gene construct expressing human BNP (hBNP) and LacZ were injected into LV free wall and LAD was ligated immediately thereafter. Echocardiographic measurements (A through D) were performed at 1 week post-infarction and after gene transfer. A, Ejection fraction. B, Fractional shortening. C, The diastolic diameter of the interventricular septum. D, The diastolic diameter of left ventricle. The results are expressed as mean±SEM (n=6-7). *P<0.05, **P<0.01, ***P<0.001 vs sham (one-way ANOVA followed by LSD post hoc test).

Supplemental Figure 8. Myocardial fibrosis after local BNP gene delivery post-infarction. A, Paraffin-embedded histological sections were stained with Masson’s trichrome to define the area of fibrosis by computerized methods at 2 weeks after AMI and human BNP (hBNP) gene delivery. B, Infarct size was assessed as a percentage of LV circumference from paraffin-embedded sections stained with Masson’s trichrome 2 weeks after MI and gene transfer. C, Collagen Iα1 (Col Iα1) mRNA, D, Collagen IIIα1 (Col IIIα1) mRNA, E, Tumor necrosis factor-α (TNF-α) mRNA, and F, Endothelin-1 mRNA levels were similarly up-regulated at 1 week and 2 weeks post-infarction in hBNP- and LacZ-treated hearts. The results are expressed as mean±SEM (n=5-7). *P<0.05, **P<0.01, ***P<0.001 vs sham (one-way ANOVA followed by LSD post hoc test).
**Supplemental Figure 9.** Cardiomyocyte cross sectional area in the left ventricle after local human BNP (hBNP) gene delivery at 2 weeks post-infarction. Paraffin-embedded histological sections were stained with Masson’s trichrome to define the cross sectional area of cardiomyocytes. The results are expressed mean±SEM (n=5-8). ***P<0.001 vs LacZ; †††P<0.001 vs hBNP (one-way ANOVA followed by LSD post hoc test).

**Supplemental Figure 10.** Myocardial angiogenesis, cardiac stem cell recruitment, cell proliferation and apoptotic cell death in the left ventricle after local human BNP (hBNP) gene delivery at 2 weeks post-infarction. A through B, Angiogenesis was studied by immunohistochemical staining against lectin. A, Capillary density, and B, Capillary area/field were counted in 5 representative fields from the peri-infarct area of each section. C, The number of c-kit+ cells in the anterior wall of left ventricle was counted. The area of counted section was examined by computerized methods and a number of positively staining cells was related to the area (cells/35 mm²). D, Immunohistochemical staining against Ki-67 was performed to study the effect of BNP gene transfer on cellular proliferation. The whole left ventricle was scanned and stained cells were counted from high power fields (40×) choosing 5 hot spot areas in each sample. E, The rate of apoptosis was assessed by TUNEL. The apoptotic cells and bodies were counted in 5 high power fields (40× objective) from the peri-infarct regions choosing hot spot areas in each sample in order to make the results comparable. The results are expressed as mean±SEM (n=5). P=ns vs LacZ with AMI (Student’s t-test).

**Supplemental Figure 11.** Effect of intramyocardial human BNP (hBNP) gene delivery on p38-MAPK and p44/42-MAPK in the left ventricle 1 week and 2 weeks post-infarction. MAPK levels were examined by western blot analyses. A, p38-MAPK levels was similarly reduced in hBNP- and LacZ-treated hearts. B, p44/42-MAPK levels were similarly increased in hBNP- and LacZ-treated hearts. The results are expressed as mean±SEM (n=5-7). *P<0.05 vs sham (one-way ANOVA followed by LSD post hoc test).

**Supplemental Figure 12.** Intramyocardial human BNP (hBNP) gene delivery decreases myocardial fibrosis in angiotensin II-induced (Ang II) hypertension at 2 weeks. Paraffin-embedded histological sections were stained with Masson’s trichrome to define the area of fibrosis. Representative images are shown.
**Supplemental Figure 13.** Effect of intramyocardial BNP gene delivery on coronary angiogenesis in Ang II-induced hypertension. Ang II was infused for 2 weeks to the human BNP (hBNP)-Ad5 and LacZ-Ad5-injected rats. A, Capillary density, and B, Capillary area/field were counted in 5 representative fields in the left ventricle. The results are expressed as mean±SEM (n=7-8). P=ns vs LacZ with Ang II (Student’s t-test).

**Supplemental Figure 14.** Effect of intramyocardial BNP gene delivery on LV p38 MAPK and p44/42 levels, SERCA2 expression and phospholamban (PLB) phosphorylation in Ang II-induced hypertension. Ang II was infused for 2 weeks to the human BNP(hBNP)-Ad5 and LacZ-Ad5-injected rats. A, p38 MAPK, and B, p44/42 levels were examined by western blot analyses. C, SERCA2 mRNA levels measured by RT-PCR. D, SERCA2 protein levels assessed by western blot analyses. E, PLB phosphorylation of phospho-threonine17 (p-Thr17). F, PLB phosphorylation of phospho-serine16 (p-Ser16). The results are expressed mean±SEM (n=6-11). *P<0.05 vs LacZ (one-way ANOVA followed by LSD post hoc test).
SUPPLEMENTAL REFERENCES


