Long-Term Cardiac pro-B-Type Natriuretic Peptide Gene Delivery Prevents the Development of Hypertensive Heart Disease in Spontaneously Hypertensive Rats

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Background—Diastolic dysfunction associated with high blood pressure (BP) leads to cardiac remodeling and fibrosis and progression to congestive heart failure. B-type natriuretic peptide (BNP) has BP-lowering, antifibrotic, and antihypertrophic properties, which makes BNP an attractive agent for attenuating the adverse cardiac remodeling associated with hypertension. In the current study, we tested the effects of sustained cardiac proBNP gene delivery on BP, cardiac function, and remodeling in spontaneously hypertensive rats (SHR).

Methods and Results—We used the myocardium-tropic adeno-associated virus serotype 9 (AAV9) vector to achieve continuously enhanced cardiac rat proBNP expression. In SHR, a single systemic administration of AAV9 vector allowed long-term cardiac BNP overexpression, resulting in reductions in systolic and diastolic BP for 9 months after injection. Left ventricular (LV) thickness, LV end-systolic dimensions, and LV mass were reduced, whereas ejection fraction was significantly increased, in BNP-treated compared with untreated SHR. Circumferential systolic strain and strain rate of the early phase of diastole were improved in BNP-treated compared with untreated SHR. Noncardiac overexpression of BNP via AAV2 vector was not associated with changes in BP and plasma BNP in SHR. Furthermore, normal Wistar rats injected with AAV9 proBNP vector showed significantly reduced heart weights 4 weeks after injection without BP reduction.

Conclusions—AAV9 vector facilitates sustained cardiac proBNP overexpression and improves LV function in hypertensive heart disease. Long-term proBNP delivery improved both systolic and diastolic function. The effects on cardiac structure and function occurred independently of BP-lowering effects in normal Wistar rats. (***Circulation.*** 2011;123:1297-1305.)

Key Words: cardiac remodeling ∎ natriuretic peptide, brain ∎ hypertension

Hypertension is a common condition that, if not controlled, progresses toward more severe cardiovascular and renal morbidity. Its major clinical phenotype is hypertensive heart disease (HHD), which is characterized by diastolic dysfunction, cardiac remodeling, and fibrosis. Over time, diastolic dysfunction evolves into systolic impairment, which leads to the worsening of overall cardiac function and to increased morbidity and mortality.[1,2] New evidence indicates that levels of the circulating cardiac B-type natriuretic peptide (BNP) are reduced in early stages of hypertension.[3] Importantly, BNP plays a critical role in cardiorenal homeostasis and, through binding to the guanylyl cyclase-A receptor, it increases sodium excretion, lowers blood pressure (BP), suppresses the renin-angiotensin-aldosterone system, inhibits cardiomyocyte hypertrophy and proliferation of cardiac fibroblasts, and has potent prolusitropic properties.[4–6] Therefore, a reduced production and/or release of this cardiovascular and renal protective hormone may expose an individual to higher risk of worsening HHD.

Clinical Perspective on p 1305

Importantly, several experimental models, as well as genetic studies in humans, have also demonstrated a key role of BNP in the control of normal cardiac function and structure and of BP. Indeed, the genetic murine BNP knockout model is characterized primarily by cardiac fibrosis,[4] whereas genetic disruption of natriuretic peptide receptor A results, in murine models of hypertension, in impaired sodium excretion, cardiac hypertrophy and fibrosis, and increased mortal-

ity,[5] underscoring the cardioprotective roles of BNP. In line with these observations, the recent seminal study by Newton-Cheh et al established in a cohort of 48 939 subjects that basal BP and risk for hypertension is associated with common genetic variants of the atrial natriuretic peptide and BNP genes that affect their circulating levels.[6] Reduced atrial natriuretic peptide and BNP levels were characterized by elevated BP and increased risk for hypertension. In contrast, the presence of the single-nucleotide polymorphism rs5068, characterized by a significantly higher level of circulating atrial natriuretic peptide and BNP, was associated with a 15% reduction in odds of hypertension. Therefore, the inherent biological properties of BNP, with its proven role in the control of cardiac structure and function, BP-lowering, and rennin-angiotensin-aldosterone system–inhibiting properties, make this hormone an attractive therapeutic tool for disease states such as hypertension and HHD, which are characterized by elevated BP, cardiovascular remodeling, and reduced levels of circulating BNP. The recent reports of an impaired endogenous production of this cardiovascular and renal protective hormone in early stages of hypertension make sustained BNP delivery strategies highly attractive and rational.[3,7]

The goal of the current study was to investigate the potential benefit of prolonged BNP production by innovative technologies in preventing the worsening of HHD in a model of spontaneous progressive hypertension. Here, in order to develop a continuous BNP-based therapy for HHD, we employed the myocardium-tropic adeno-associated virus (AAV) 9–based vector and examined the influence of long-term (up to 9 months) rat proBNP expression in spontaneously hypertensive rats (SHR) after a single intravenous injection. We hypothesized that long-term cardiac proBNP delivery would improve global cardiac structure and performance in SHR. We further hypothesized that the beneficial cardiac effects are, at least in part, independent of BP reduction in normal Wistar rats.

Methods

SHR and Wistar Rats
Four-week-old SHR and five-week-old Wistar rats were purchased from Charles River. SHR served as a model of progressive HHD. Wistar rats were used to assess the effects of sustained proBNP in normotensive rats. Strains of rats, number of animals, and treatment and duration of treatment in each experiment was summarized in Table 1. All animal studies were approved by the institutional animal care and use committee.

Plasmids
The codon-optimized rat preproBNP was synthesized by GenScript and cloned into a lentiviral vector, pSIN-CSGWdlNotI. The BamHI-XhoI short fragment, which contains rat preproBNP and WPRE posttranscriptional regulatory element, was then cloned into the mammalian expression plasmid pAAV-MCS (Stratagene), resulting in pAAV-rat-preproBNP.

AAV9 and AAV2 Vectors
The AAV9 vector stocks were produced in human 293T cells using the helper-free transfection method according to the manufacturer's protocol (Stratagene). For AAV9 vector production, we used AAV9 capsid-expressing plasmid pRep2Cap9[8] (kindly provided by Dr James M. Wilson), whereas AAV2 vector was made with the AAV2 capsid-expressing plasmid, pAAV-RC (Stratagene). Firefly lu-

Table 1.   Summary of Rats Used

Study and Strain	Treatment	No.	Duration, wks
Cardiac delivery			
SHR	AAV9-Luc	2	3
Toxicology			
SHR	Untreated	3	3
SHR	AAV9-Luc	3	3
SHR	AAV9-BNP	3	3
Pharmacokinetics/dynamics			
SHR	Untreated	8	40
SHR	AAV9-BNP	8	40
Noncardiac delivery			
SHR	Untreated	5	8
SHR	AAV2-BNP	5	8
SHR	AAV9-BNP	3	8
Normotensive rat study			
Wistar	AAV9-GFP	6	4
Wistar	AAV9-BNP	6	4

SHR indicates spontaneously hypertensive rats; AAV, adeno-associated virus; Luc, luciferase; GFP, green fluorescent protein; and BNP, B-type natriuretic peptide.

ciferase–, humanized recombinant green fluorescent protein (GFP)–, or rat proBNP–encoding AAV genome constructs were packaged. Three days after transfection, AAV9 vector–producing 293T cells were harvested for vector purification. The cells were lysed by freeze-and-thaw cycling, followed by ultracentrifuge concentration (62 500 rpm for 2 hours) through Optiprep Density Gradient Medium (Sigma). The resulting AAV9 vectors were desalted and further concentrated using Amicon Ultra-15 100k filtration (Amicon). The titers (genomic copy numbers/mL) of concentrated AAV9 vector stocks were determined by quantitative polymerase chain reaction using plasmid DNA standards and AAV genomic sequence-specific primers and fluorescent probe.

Noninvasive Tail BP Measurement
BP of conscious rats were measured by the CODA High-Throughput Non-Iinvasive Tail BP System (Kent Scientific).

Echocardiography for Noninvasive Assessment of Ventricular Function and Structure
To evaluate cardiac function and structure we performed both standard echocardiography and 2-dimensional speckle-derived strain echocardiography examinations at 4 and 9 months post injections in the BNP-treated and the untreated SHR. We also performed standard echocardiography and 2-dimensional speckle-derived strain echocardiography in normal Wistar rats 4 weeks after AAV9 injections. All echocardiography examinations were performed by a skilled sonographer (E.A.O.) blinded to the treatment. Detailed protocols for echocardiography examinations were described in Materials in the online-only Data Supplement.

Surgical Procedure
Rats were anesthetized with isoflurane (1.5% in oxygen). Placement of PE-50 tubing into the carotid artery for BP monitoring and blood sampling was performed. A portion of the neck skin was removed, and the carotid artery was isolated and cleared. A cut was made with microsciscors and a PE-50 tubing was introduced into the vessel for direct BP monitoring. Blood was drawn to evaluate toxicological reactions in AAV9-BNP transduced rats and to measure BNP and cyclic GMP (cGMP). At the end of the experiments, we harvested rat organs for further analysis.
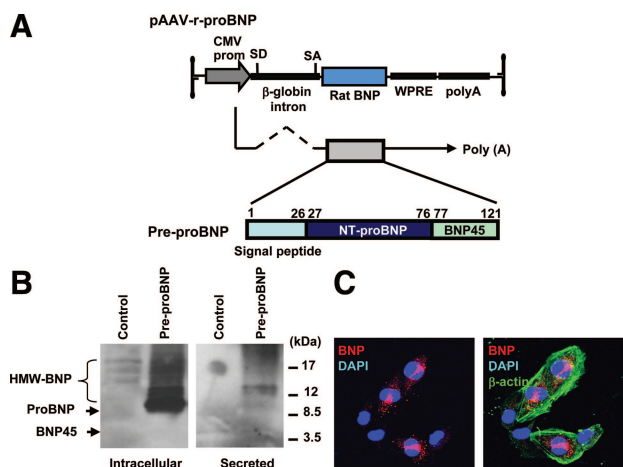
Figure 1. Generation of preproBNP-expressing AAV9 vector. **A,** Schematic representation of the AAV9 vector–encoding rat preproBNP. pAAV-r-proBNP indicates plasmid adeno-associated virus serotype 9 pro B-type natriuretic peptide; SD, splice donor; SA, splice acceptor; WPRE, woodchuck hepatitis virus posttranscriptional regulatory elements; CMV, cytomegalovirus; and NT, N-terminal. **B,** Verification of proBNP expression in 293T cells transfected with pAAV-r-proBNP. Immunoreactive BNPs in cell lysates and culture supernatants were detected by antirat BNP45 antibody. HMW indicates high molecular weight. **C,** Immunostaining of rat BNP in pAAV-r-proBNP–transfected mouse cardiomyocytes (HL1 cells). When the plasmid encoding for the full length of preproBNP was introduced in HL1 cells, immunoreactive BNP signals were detected supranuclearly and in cytoplasmic secretory vesicles. Cells were counterstained with anti-β-actin antibody (green).

Masson Trichrome Staining

The sections of frozen cardiac samples were assessed by Trichrome staining for collagen contents. Percentage of blue signals was analyzed by KS400 Image Analysis Software (version 3.0, Zeiss).
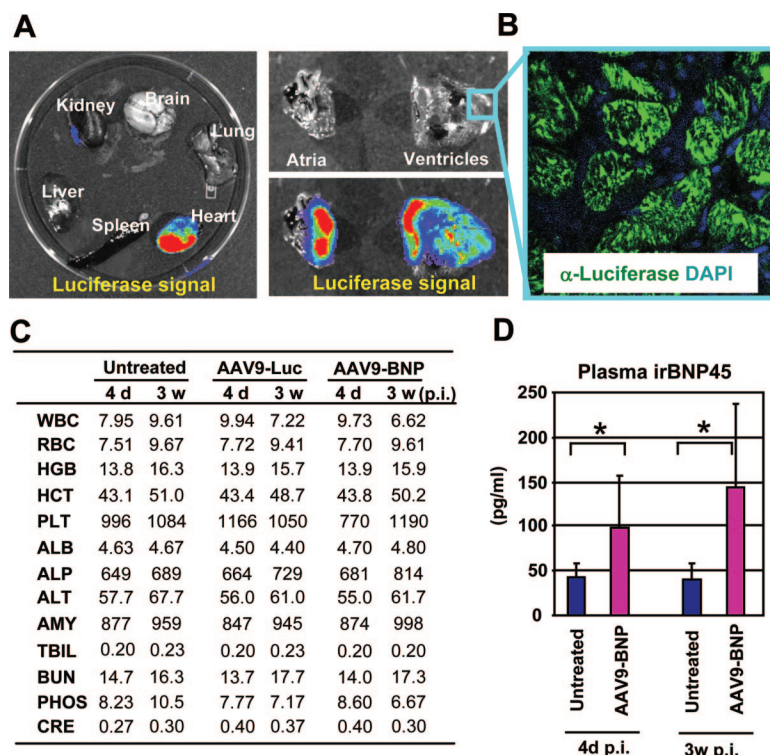
Sample Size and Statistical Analysis

Groups were compared with unpaired t test; changes within groups were assessed by paired t test. Comparisons of BP values between groups were performed by 2-way ANOVA for repeated measurements. Data were expressed as mean±SD. Significance was accepted for $P<0.05$. Detailed protocols for cell culture, immunostaining, immunoblotting, IVIS luciferase imaging, and toxicological testing are described in the online-only Data Supplement Materials.

Results

In Vitro Expression and Localization of proBNP

After successfully engineering AAV9 encoding for the rat preproBNP, which comprises the signal peptide, N-terminal proBNP, and BNP1–45 domains (Figure 1A), we verified the protein expression of BNP in 293T cells. Nonglycosylated proBNP (10 kDa) and high–molecular-weight (HMW, 12 to 24 kDa) glycosylated proBNP were detected in the cell lysates by Western blotting analysis. Of note, HMW forms of BNP were predominantly secreted (Figure 1B). When the preproBNP was expressed in mouse cardiomyocytes (HL1 cells,[9] kindly provided by Dr William C. Claycomb) and analyzed by immunostaining with an antirat BNP1–45 antibody, clear supranuclear localization of immunoreactive BNP (red), as well as discrete cytoplasmic body signals (red), were detected (Figure 1C).

In Vivo Cardiac-Specific Tropism of AAV9 Vector–Mediated Gene Transfer

AAV9-based vectors have been shown to be cardiotropic. We therefore packaged the rat preproBNP– or firefly luciferase–expressing vectors in AAV9 capsid and examined the influence of AAV9 vector-mediated gene delivery in SHR. Four-week-old SHR (n=2) were used for this study. Three weeks after tail intravenous injection of AAV9-carrying luciferase (10^{12} genome copy/animal), we determined the tissue specificity of the AAV9
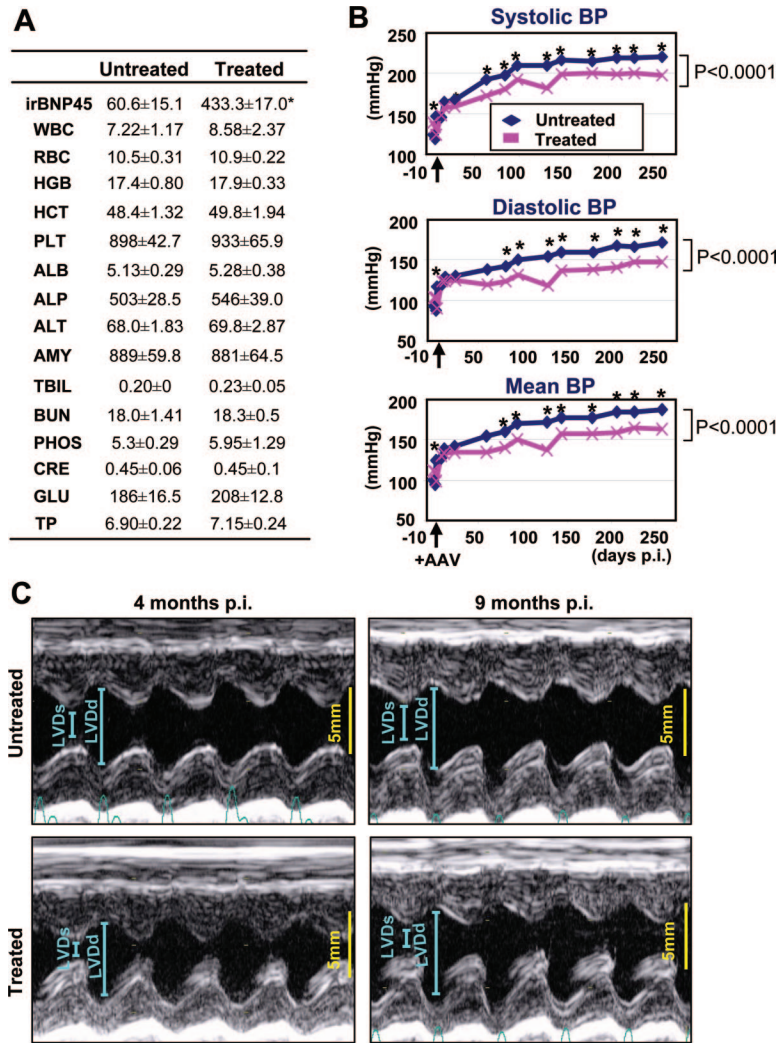

	Untreated		AAV9-Luc		AAV9-BNP	
	4 d	**3 w**	**4 d**	**3 w**	**4 d**	**3 w (p.i.)**
WBC	7.95	9.61	9.94	7.22	9.73	6.62
RBC	7.51	9.67	7.72	9.41	7.70	9.61
HGB	13.8	16.3	13.9	15.7	13.9	15.9
HCT	43.1	51.0	43.4	48.7	43.8	50.2
PLT	996	1084	1166	1050	770	1190
ALB	4.63	4.67	4.50	4.40	4.70	4.80
ALP	649	689	664	729	681	814
ALT	57.7	67.7	56.0	61.0	55.0	61.7
AMY	877	959	847	945	874	998
TBIL	0.20	0.23	0.20	0.23	0.20	0.20
BUN	14.7	16.3	13.7	17.7	14.0	17.3
PHOS	8.23	10.5	7.77	7.17	8.60	6.67
CRE	0.27	0.30	0.40	0.37	0.40	0.30

Figure 2. AAV9 vector facilitates efficient cardiac gene delivery in spontaneously hypertensive rats (SHR). **A,** Distribution of luciferase activities in firefly luciferase–expressing AAV9 vector–administered SHR organs was monitored by Xenogen IVIS Living Image. Strong luciferase expression in heart demonstrated efficient cardiac gene delivery by AAV9 in SHR (n=2) (left panel). Higher magnifications of luciferase signals were found in both atria and ventricles (right panels). **B,** Detection of luciferase by immunostaining. Luciferase in the sections of heart ventricles were detected by anti-firefly luciferase antibody, confirming the efficient cardiac luciferase gene expression on AAV9 vector–mediated gene transfer. **C,** No apparent toxicity observed in AAV9 vector–administered SHR. Toxicological and pharmacological parameters in vector-injected SHR (n=3) were measured at 4 days and 3 weeks after vector administration. Averages of 3 rats were shown. *$P<0.05$ versus respective untreated controls. WBC indicates white blood cells; RBC, red blood cells; HGB, hemoglobin; HCT, hematocrit; PLT, platelets; ALB, albumin; ALP, alkaline phosphatase; ALT, alanine transferase; AMY, amylase; TBIL, total bilirubin; BUN, blood urea nitrogen; PHOS, phosphorus; and CRE, creatinine. **D,** Sustained BNP expression in the proBNP-expressing vector–administered SHR (n=3). The levels of plasma immunoreactive BNP were measured at 4 days and 3 weeks after vector administration by the rat BNP45 ELISA. Error bars indicate ±SD. *$P<0.05$ versus respective untreated controls. irBNP indicates immunoreactive B-type natriuretic peptide; AAV9-BNP, adeno-associated virus serotype 9 B-type natriuretic peptide; and p.i., post injection.

A

	Untreated	Treated
irBNP45	60.6±15.1	433.3±17.0*
WBC	7.22±1.17	8.58±2.37
RBC	10.5±0.31	10.9±0.22
HGB	17.4±0.80	17.9±0.33
HCT	48.4±1.32	49.8±1.94
PLT	898±42.7	933±65.9
ALB	5.13±0.29	5.28±0.38
ALP	503±28.5	546±39.0
ALT	68.0±1.83	69.8±2.87
AMY	889±59.8	881±64.5
TBIL	0.20±0	0.23±0.05
BUN	18.0±1.41	18.3±0.5
PHOS	5.3±0.29	5.95±1.29
CRE	0.45±0.06	0.45±0.1
GLU	186±16.5	208±12.8
TP	6.90±0.22	7.15±0.24

B

Figure 3. Effects of long-term BNP overexpression in spontaneously hypertensive rats (SHR). **A,** No apparent toxicity observed in SHR at 4 months after proBNP-expressing AAV9 vector administration. Toxicological and pharmacological parameters of the treated and untreated SHR were shown (n=4, each group). No toxicity was observed in the rats whereas plasma immunoreactive BNP45 was significantly elevated in the AAV9 vector-treated group. irBNP indicates immunoreactive B-type natriuretic peptide; WBC, white blood cells; RBC, red blood cells; HGB, hemoglobin; HCT, hematocrit; PLT, platelets; ALB, albumin; ALP, alkaline phosphatase; ALT, alanine transferase; AMY, amylase; TBIL, total bilirubin; BUN, blood urea nitrogen; PHOS, phosphorus; CRE, creatinine; GLU, glucose; and TP, total protein. **B,** BP measurements of proBNP-expressing AAV9 vector–administered SHR. BP in treated and untreated SHR were measured by tail-cuff method (n=8). *$P<0.05$ versus respective untreated controls. BP indicates blood pressure; AAV, adeno-associated virus; and p.i., post injection. **C,** M-mode echocardiography of untreated and AAV9-proBNP–treated SHR at 4 and 9 months post AAV9 vector injection. AAV9 vector–treated SHR had significantly improved diastolic functions at 4 months and both diastolic and systolic functions at 9 months compared with untreated SHR. LVDs indicates left ventricular end-systolic dimension; LVDd, left ventricular end-diastolic dimension; and p.i., post injection.

vector by luciferase expression in the SHR, which demonstrated high levels of luciferase expression in myocardium (Figure 2A through 2B). To confirm luciferase expression in the heart, we stained the heart section with antiluciferase antibody, and the signals were detected predominately in the cardiomyocytes (Figure 2B). When we injected AAV9-luciferase (n=3) and AAV9-preproBNP (n=3) and compared short-term (4 days) and long-term (3 weeks) toxicological responses with those of the untreated SHR (n=3), no notable toxicity was observed among these 3 groups of rats (Figure 2C). However, plasma BNP, by rat BNP1-45 ELISA, was significantly higher in the AAV9-preproBNP–treated SHR compared with untreated SHR both at 4 days and 3 weeks after injections (Figure 2D), thus confirming the sustained BNP expression on AAV9 vector-mediated gene delivery.

Effects of Sustained proBNP Expression in SHR

Next, we monitored the effects of sustained proBNP expression in SHR through cardiac proBNP delivery by AAV9 vector. Four months after AAV9-preproBNP injections in SHR, there was no toxicological reaction compared to untreated SHR. Importantly, plasma immune reactive BNP was significantly higher in the AAV9-preproBNP–treated group compared with the untreated

SHR (Figure 3A). Tail cuff BP measurements indicate significant reduction in systolic BP (SBP), diastolic BP (DBP), and mean arterial pressure (MAP) in the AAV9-preproBNP–treated SHR as compared with untreated SHR. Indeed, in the AAV9-preproBNP, SBP was significantly reduced 1 month after injection and was followed by a reduction in both DBP and MAP at 2 months postinjection as compared with the untreated SHR. These reductions in SBP, DBP, and MAP in conscious rats continued throughout the 9-month study (Figure 3B).

Echocardiographic parameters in untreated SHR and in AAV9 preproBNP–treated SHR are summarized in Table 2. Although no difference was detected in heart rate between AAV9 preproBNP–treated and untreated SHR both at 4 and 9 months postinjection, echo analysis indicated a significant improvement of diastolic function at 4 and 9 months, as well as systolic function at 9 months postinjection in AAV9 preproBNP–treated SHR as compared with untreated SHR (Figure 3C). Of note, ejection fraction, diastolic posterior wall thickness, left ventricular end-diastolic dimension (LVDd), and diastolic strain rate early circumferential were improved and LV mass index was lower at 9 months in the AAV9 preproBNP–treated SHR even when compared with 4 months untreated SHR (Table 2).

At 9 months postinjection, 4 rats per group were euthanized for intra-arterial BP measurement. Direct intracarotid SBP and

Table 2.   Echocardiographic Parameters in Untreated and BNP-Treated SHR

	4 Months p.i.		9 Months p.i.	
	Untreated	BNP Treated	Untreated	BNP Treated
HR	403±27.3	392±22.3	381±25.2	393±13
SWTd	2.09±0.1	1.86±0.1*	2.71±0.1†	2.17±0.2*†
PWTd	2.03±0.1	1.86±0.2*	2.16±0.3	1.87±0.1*‡
LVDd	6.77±0.2	6.71±0.1	7.56±0.6†	7.57±0.4†‡
LVDs	3.83±0.4	3.47±0.1*	4.66±0.6†	3.96±0.3*†
Ejection fraction	80±4.1	85±1.8*	74±4.5†	83±2.1*†‡
LV mass index	0.44±0.01	0.4±0.02*	0.49±0.01	0.4±0.01*†‡
sSR circumferential	−4.6±0.7	−4.75±0.6	−3.74±0.4†	−5.04±0.4*
dSR-E circumferential	2.41±0.8	4.07±1.5*	2.09±0.8	3.25±0.9*‡
sSR-radial	7.17±1.1	6.77±0.8	6.34±1.6	8.13±1.9*†
dSR-E radial	−3.29±1.2	−5.72±2.5*	−2.41±1.3	−4.57±2.1*†

n=8 for both treated and untreated SHR.

*$P<0.05$ vs respective untreated; †$P<0.05$ vs 4 months within group; ‡$P<0.05$ between 9 months BNP-treated and 4 months untreated.

p.i. indicates postinjection; HR, heart rate; SWTd, septal wall thickness at end diastole; PWTd, posterior wall thickness at end diastole; LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; sSR, systolic strain rate; and dSR, diastolic strain rate.

DBP was reduced in the AAV9 preproBNP–treated anesthetized SHR (Figure 4A) whereas no significant differences were found in body weights and heart rates between treated and untreated rats. The heart weight corrected for the body weight was significantly reduced in the BNP-treated as compared with the control SHR (0.37 ± 0.01 versus 0.43 ± 0.02, respectively, $P<0.05$). Plasma BNP was higher in the BNP-treated as compared with the control SHR (Figure 4B). Although plasma cGMP was not different, urinary cGMP was greater in the BNP-treated as compared with the control SHR (Figure 4B). Connective tissue (assessed by Mason's trichrome staining) tended to increase in heart sections of untreated SHR as compared with AAV9-preproBNP–treated SHR (Figure 4C through 4D).

Noncardiac BNP Transduction by AAV2 Vector

Next, we assessed the effects of noncardiac proBNP gene delivery on BP, plasma BNP levels, and heart weight in SHR. For noncardiac gene delivery, we administered conventional AAV2 vectors through intraperitoneal injection. One month after administration of AAV2 vector carrying luciferase, we found high levels of luciferase expression in peritoneum, but not in heart (Figure 5A), confirming efficient, but noncardiac, gene delivery by AAV2 vector. To assess the influence of noncardiac proBNP gene delivery, SHR were injected with preproBNP-carrying AAV2 vector and compared with untreated (n=5) and AAV9-preproBNP vector-administered rats (n=3). Tail cuff BP measurements indicate that AAV2-preproBNP administration had no significant effects on SBP and DBP. In contrast, SBP was significantly reduced 5 and 8 weeks after injection of the AAV9-preproBNP vector (Figure 5B). The preproBNP gene delivery by AAV9, but not AAV2, showed significantly higher plasma level of BNP (Figure 5C) and significant reduction in the heart weight/body weight ratio (Figure 5C), suggesting the requirement of cardiac preproBNP delivery for efficient BNP release and the anti-cardiac hypertrophic effects of BNP in SHR.

Effects of Sustained proBNP Expression in Normotensive Rats

To investigate whether the beneficial effects on both cardiac structure and function observed in the BNP-treated SHR were mainly due to the sustained BP lowering effects, we injected AAV9 encoding for GFP (n=6) or preproBNP (n=6) in normal Wistar rats. All rats underwent echocardiographic examination 4 weeks after injections, and were euthanized for acute experiments thereafter. Normal rats treated with AAV9 carrying GFP showed wide spread GFP expression in cardiomyocytes 4 weeks after injection, further confirming the cardiac transduction of the AAV9 vector (Figure 6A). Echocardiographic examination by strain analysis demonstrated that at 4 weeks post injection, AAV9-preproBNP–treated normal rats had a significantly improved systolic function compared with AAV9-GFP rats as indicated by a thinner septal wall thickness at end diastole and higher systolic strain rate circumferential, whereas LVMi was only slightly reduced ($P=0.07$, NS) (Table 3). AAV9-preproBNP–treated normal rats had significantly higher plasma level of BNP compared with the GFP-control rats (Figure 6B). Although direct intracarotid BP measurement found similar SBP, DBP and MAP between the 2 groups, the heart weight corrected for the body weight was significantly reduced in the BNP-treated as compared with the GFP-control rats. (Figure 6B and 6C).

Discussion

Due to the low toxicity and efficient and long-term transduction in vivo, AAV vectors are currently evaluated in many clinical studies.[10–12] Intriguingly, AAV vector genome packaged with the capsid protein from AAV9 was shown to efficiently deliver marker genes into cardiac tissue in neonatal and adult mice, as well as newborn rhesus monkeys.[13,14] The current study demonstrates successful in vivo cardiomyocyte transduction via AAV9 vector which facilitated sustained cardiac proBNP overexpression. Long-term
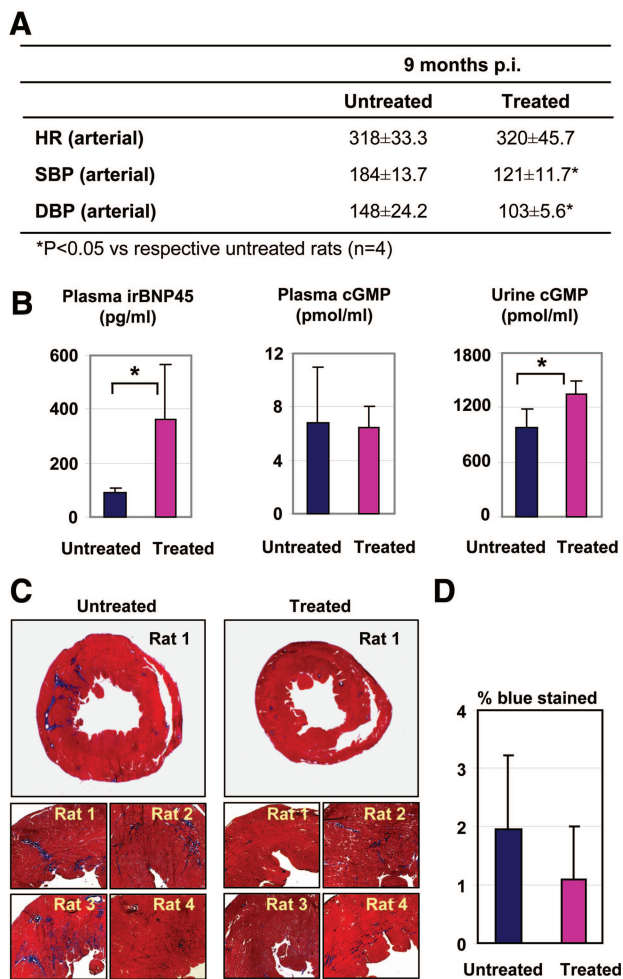
A

	9 months p.i.	
	Untreated	Treated
HR (arterial)	318±33.3	320±45.7
SBP (arterial)	184±13.7	121±11.7*
DBP (arterial)	148±24.2	103±5.6*

*P<0.05 vs respective untreated rats (n=4)

B

C

D

Figure 4. Effects of long-term BNP overexpression on cardiac remodeling. **A**, Intra-arterial measurements of heart rate (HR), systolic BP (SBP), and diastolic BP (DBP) in anesthetized, treated (n=4), and untreated (n=9) spontaneously hypertensive rats (SHR) are indicated. Values are mean±SD. BP indicates blood pressure; p.i., post injection. **B**, Plasma immunoreactive BNP45 (irBNP45), plasma cyclic GMP (cGMP), and urinary cGMP are shown. Error bars indicate ±SD. *P<0.05 versus respective untreated controls. **C**, Cross sections were stained by Masson trichrome staining for muscle fibers (red) and collagen/fibrosis (blue). Representative images of whole section of treated and untreated SHR (upper panels) and higher magnifications of heart images of rats (lower panels) are shown. **D**, Connective tissue deposition was evaluated as percentage of blue signals/red signals by KS400 image analysis software. The averages of 4 heart samples in treated and untreated SHR groups are shown.

proBNP delivery led to reduced BP and improved LV function and structure in an HHD rat model without any short- or long-term toxicological adverse effects or development of tolerance. Although long-term proBNP delivery improved both systolic and diastolic function, the effect on diastolic performance was more remarkable and preceded the improvement in systolic function in this HHD model. Importantly, the effects on cardiac structure and function occurred independently of BP-lowering effects in normal Wistar rats.

HMW proBNP Secretion

We and others have shown that the proBNP glycosylation process is necessary for the release of proBNP and that
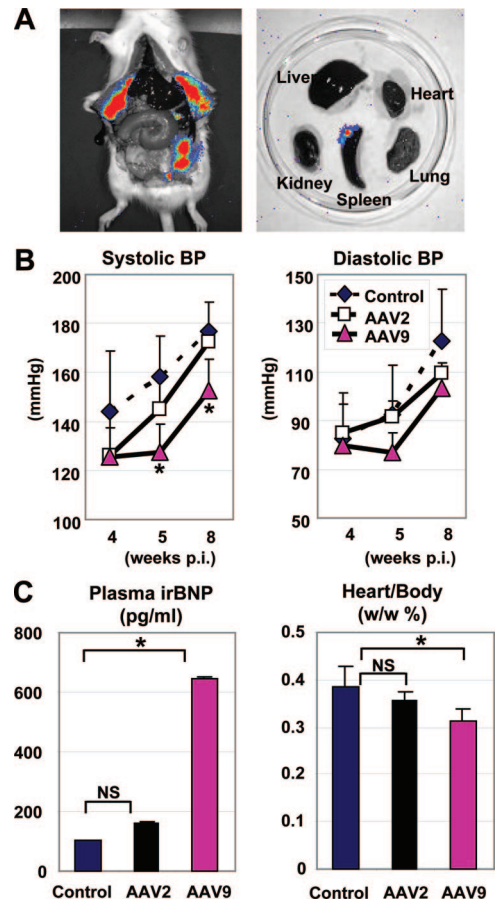
A

B

C

Figure 5. Effects of noncardiac BNP overexpression in spontaneously hypertensive rats (SHR). **A**, Distribution of luciferase activities in firefly luciferase–expressing AAV2 vector–administered SHR organs was monitored by Xenogen Living Image. Strong luciferase expression was evident in peritoneum by AAV2 in SHR (left panel), whereas no detectable luciferase expression was observed in heart (right panels). **B**, BP measurements of proBNP-expressing AAV2 and AAV9 vector–administered SHR. BP in AAV2-treated (n=5), AAV9-treated (n=3), and untreated SHR (n=5) were measured by tail-cuff method. Error bars indicate ±SD. *P<0.05 versus respective untreated controls. BP indicates blood pressure; AAV2, adeno-associated virus serotype 2; AAV9, adeno-associated virus serotype 9; and p.i., post injection. **C**, Plasma immunoreactive BNP45 (irBNP45) and the heart weight/body weight ratios are shown. Error bars indicate ±SD *P<0.05 versus respective untreated controls.

glycosylated proBNP is circulating in humans.[15] We speculate that once glycosylated proBNP is released into the bloodstream, a progressive deglycosylation occurs and proBNP is processed to mature BNP and N-terminal proBNP at the tissue level. In the current study, we further demonstrated that rat BNP is released from preproBNP-expressing 293T cells as a HMW form.

Effects on BP, Plasma BNP, cGMP, and Urinary cGMP

Rat proBNP overexpression was associated with significant and sustained BP reduction in SHR. Indeed, SBP, DBP, and MAP were lower in the BNP-treated as compared with the control SHR from 2 months up to 9 months post-AAV9 injection. This reduction of BP was rather modest and occurred without
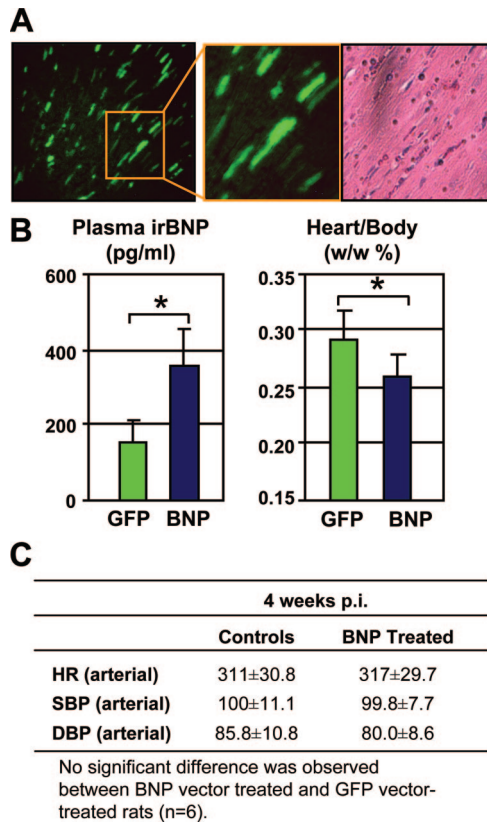
Figure 6. Effects of AAV9 vector–mediated long-term BNP expression in normal Wistar rats. **A,** Efficient cardiac transgene expression on systemic AAV9 vector administration. Normal rats were injected by green fluorescent protein (GFP)–carrying AAV9 vector. Four weeks after injection, heart sections were analyzed for GFP expression. **B,** Plasma immunoreactive B-type natriuretic peptide (irBNP) and the heart weight/body weight ratios are shown. Error bars indicate ±SD. *$P<0.05$ versus respective untreated controls. **C,** Intra-arterial measurements of heart rate (HR), systolic blood pressure (SBP), and diastolic blood pressure (DBP) in anesthetized, treated, and untreated normotensive Wistar rats are indicated. p.i. indicates post injection. Values shown are mean±SD.

Table 3. Echocardiographic Parameters in Control (n=6) and BNP-Treated (n=6) Normal Rats

	4 Weeks p.i.	
	Controls	BNP Treated
HR	407±27.2	408±24.5
SWTd	2.03±0.15	1.80±0.11*
PWTd	1.66±0.09	1.77±0.19
LVDd	7.0±0.67	7.1±0.53
LVDs	3.84±0.67	3.94±0.37
Ejection fraction	83±1.72	83±4.17
LV mass index	0.35±0.01	0.33±0.01
sSR circumferential	−5.37±0.5	−6.54±0.8*
dSR-E circumferential	5.09±0.7	5.98±1.0
sSR-radial	7.63±1.5	9.08±1.7
dSR-E radial	−5.59±1.3	−7.84±2.7

Values are mean±SD.

*$P<0.05$ vs respective Controls.

p.i. indicates postinjection; BNP, B-type natriuretic protein; HR, heart rate; SWTd, septal wall thickness at end diastole; PWTd, posterior wall thickness at end diastole; LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; sSR, systolic strain rate; and dSR, diastolic strain rate.

Effects on Cardiac Function and Structure

Long-term overexpression of proBNP prevented the development of HHD, which began at 4 weeks of age in the SHR. Indeed, AAV9-induced proBNP production resulted in a sustained and significant reduction (up to 9 months) of SBP and DBP. Echo analysis demonstrated a significant improvement of diastolic function at 4 months posttransfection in the BNP-treated group as compared with the untreated SHR. Importantly, at 9 months, untreated SHR also developed signs of impaired systolic function, which was prevented in the BNP-treated SHR. Of note, global cardiac function and remodeling were not only improved in the BNP-treated SHR compared with untreated SHR of the same age, but also, BNP-treated SHR at 9 months showed improved diastolic function and reduced cardiac hypertrophy even when compared with the untreated SHR at 4 months of age. This finding further supports the beneficial role of BNP in preventing cardiac dysfunction and remodeling. All these favorable actions occurred without signs of any short- or long-term toxicological side effects, and BNP maintained its biological actions up to 9 months postinjection without developing tolerance. It should be noted that, although sustained, the BP reduction was minimal. Thus, further studies are required to address the pathogenic role of BNP in hypertension.

In this study, we used a comparable vector dose for both AAV9 and AAV2 to determine differences between cardiac and noncardiac BNP overexpression. In SHR transduced with noncardiac AAV2 vector, no increase in plasma immunoreactive BNP was observed. This could be due to less efficient intracellular processing and/or release of rat BNP in noncardiac cells. Furthermore, in these AAV2-transduced SHR we did not observe changes in heart weight compared with untreated controls. Of note, however, a transgenic mouse model by Ogawa et al with liver-specific BNP overexpression, characterized by 10-fold increases in BNP messenger RNA, showed 10- to 100-fold increases in circulating BNP with concomitant BP reduction compared with their nontransgenic

changes in heart rate. Of note, although BP was reduced at the vector dose used in the current study, it did not completely normalize BP, which remained elevated throughout the period of observation. However, it is possible that a higher vector dose would result in a more profound BP reduction. Although the use of telemetry would have helped in better assessing BP changes throughout the study, we confirmed a significant BP-lowering effect of BNP in unconscious BNP-treated SHR compared with the untreated SHR via direct intra-arterial BP measurements at the time of the surgical procedures (9 months).

Plasma immunoreactive rat BNP45 was elevated in the BNP-treated as compared with the control SHR at 4 days, 3 weeks, and 4 and 9 months postinjection, confirming a sustained overexpression of BNP in the heart. At 9 months postinjection, plasma cGMP was not different between the BNP-treated and the control SHR. In contrast, urinary cGMP was increased in the BNP-treated as compared with the control SHR. Thus, the lack of elevation of plasma cGMP may be explained by the increased urinary cGMP excretion.

littermates.[16] This, perhaps, indicates that noncardiac cells are also able to release BNP but only if a higher vector dose is used.

Effects in Normal Rats

We also extended our studies to normal rats to investigate the antihypertrophic actions of BNP overexpression in the absence of hypertension. In this model, age-induced systolic impairment was significantly ameliorated in the BNP-treated rats by echo strain analysis at 4 weeks. Also, cardiac mass was reduced in the BNP-treated rats as compared with the controls, and heart weight/body weight was significantly lower in the BNP-treated rats as compared with the controls. Importantly, the improved cardiac function and the reduced cardiac mass were observed 4 weeks after injections of the AAV9 vector and occurred despite no difference in BP (measured directly intracarotid) between the BNP-treated and the control group.

Long-Term BNP Delivery

On the basis of the current studies, the possible use of long-term supplementation of the cardiorenal protective hormone BNP could be employed in hypertension to prevent progression toward more severe stages of HHD and the onset of heart and renal failure. Today, the use of long-term peptide delivery is far from being implemented in clinical practice, although recent studies in experimental hypertension have reported the successful conjugation of BNP, which elicited 1-week delivery of oral BNP in normal dogs and resulted in sustained BP reduction, suppression of the rennin-angiotensin-aldosterone system, and increased natriuresis and diuresis in a canine hypertensive model.[17,18] In the current study, instead of oral delivery we used a gene transfection strategy, which facilitated a 9-month delivery of bioactive BNP with single intravenous injection of the AAV9 vector. Clearly, sustained overexpression of BNP in SHR reduced BP, decreased left ventricular hypertrophy, tended to reduce fibrosis, and improved systolic and diastolic function. To date, there is a large unmet need for novel therapies for diastolic dysfunction. Studies have reported the positive lusitropic actions of BNP in animal models of heart failure complementing studies in isolated cardiomyocytes. Currently, the mechanism(s) for the antihypertrophic and positive lusitropic actions of BNP overexpression in the SHR are not clear. These mechanism(s) may include: (1) a direct effect on the cardiomyocyte and the extracellular matrix which enhance cardiomyocyte relaxation, (2) reduced cardiac fibrosis, and (3) the BP-lowering effect observed throughout the study.

Discordant results have been reported relative to the use of this hormone in congestive heart failure patients treated with intravenous BNP and suffering from severe renal dysfunction. Moreover, an increased risk of mortality has also been reported secondary to the intravenous administration of BNP in acutely decompensated heart failure.[19–21] In depth analysis of these data suggests that high doses of BNP are responsible for the manifestation of these side effects, given that BP reduction leads, in turn, to decreased renal perfusion and function. Therefore, these studies suggest using great caution when BNP is used in humans. The latest results from the Acute Study of Clinical Effectiveness of Nesiritide in De-

compensated Heart Failure Trial (ASCEND-HF), however, indicate that the use of BNP in patients with acute heart failure is safe even while showing only a modest but not significant improvement of both symptoms and mortality.[22] Altogether, these results may suggest that the optimal therapeutic approach for BNP could be achieved through a long-term low-dose delivery strategy. Here, we do not suggest the use of genetic therapy for the treatment of human HHD. However, the use of regulatory promoters that can activate BNP production only under increased need, such as elevated BP, can be used to minimize possible side effects of BNP on BP. We are currently evaluating the possible use of BNP promoter as a stress-inducible promoter to drive BNP expression in models of CHF and impaired renal perfusion characterized by low BP.

In conclusion, the current findings demonstrate the successful cardiac delivery of the AAV9 vector, which mediated sustained cardiac proBNP overexpression without any short- or long-term toxicological effects and any signs of tolerance. Importantly, sustained cardiac BNP overexpression reduced BP and improved LV function in a model of progressive HHD after a single intravenous injection. Although long-term proBNP delivery improved both systolic and diastolic function, the effect on diastolic performance was more remarkable and appeared earlier during the development of HHD. Ultimately, sustained overexpression of BNP in SHR prevented the development of HHD, as 9-month-old BNP-treated SHR had a significantly improved cardiac function and structure even when compared with 4-month-old untreated SHR. Noncardiac BNP overexpression was not associated with increase in plasma BNP, changes in BP, and reduced heart weight. The direct cardiac effects of overexpressed BNP seem to be, at least in part, independent of BP-lowering action as indicated by the improved systolic function and reduced heart weight in the normotensive rats that was observed despite no changes in BP.

Disclosures

None.

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CLINICAL PERSPECTIVE

Today, the clinical use of B-type natriuretic peptide (BNP) is limited to the treatment of acute heart failure. Concerns have been raised about the use of this hormone in humans because of the risk of worsening renal function in patients with reduced cardiac output and low blood pressure. However, the recent results from the Acute Study of Clinical Effectiveness of Nesiritide in Decompensated Heart Failure Trial (ASCEND-HF) have shown that BNP is safe in patients with acute heart failure, although the benefit of short-term BNP administration in these patients remains questionable. Our current study has demonstrated the possible beneficial effects of long-term BNP administration in hypertension to prevent the development of hypertensive heart disease and the worsening of cardiac remodeling. Indeed, the use of a low yet prolonged dose of BNP with more stable hemodynamic conditions where blood pressure is high may be a more efficacious therapeutic option. In addition, long-term BNP delivery has also been challenging because of its short in vivo half-life and proteic structure, which limits its use as an intravenous or subcutaneously administered drug. In this regard, however, novel technologies are arising that allow oral delivery of proteins, making the long-term use of this cardiovascular hormone feasible. It is conceivable that the sustained use of oral BNP, in the presence of stable hemodynamic conditions, may prevent the progression of hypertensive heart disease and cardiac dysfunction if used early in the course of hypertension. Alternatively, novel gene delivery strategies for BNP overexpression could represent a high-risk high-impact therapeutic strategy, especially in the setting of resistant hypertension and hypertrophic cardiomyopathy, to protect the heart.

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Long-Term Cardiac pro-B-Type Natriuretic Peptide Gene Delivery Prevents the Development of Hypertensive Heart Disease in Spontaneously Hypertensive Rats
Alessandro Cataliotti, Jason M. Tonne, Diego Bellavia, Fernando L. Martin, Elise A. Oehler, Gerald E. Harders, Jarryd M. Campbell, Kaw-Whye Peng, Stephen J. Russell, Lorenzo S. Malatino, John C. Burnett, Jr and Yasuhiro Ikeda

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SUPPLEMENTAL MATERIAL

Cell culture. HEK 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 50 U/ml penicillin, and 50 µg/ml streptomycin. A murine atrial cardiomyocyte cell line, HL-1[1], was kindly provided by Dr. William C. Claycomb (Louisiana State University Medical Center, New Orleans) and cultured in Claycomb's medium with 10% FBS, 100 µM norepinephrine, and 4 mM L-glutamine on 0.02% gelatin/fibronectin-coated flasks or plates.

Transfection, Immunoblotting and Immuno-staining. Fugene6 (Roche) was used for transfection. For immunoblotting, immuno-reactive rat BNPs were detected using rabbit anti-rat BNP1-45 antibody (AssayPro) and HRP-conjugated anti-rabbit IgG antibody. Immunostaining of immuno-reactive rat BNP was performed using the same anti-rat BNP1-45 antibody and FITC-conjugated anti-rabbit IgG antibody.

IVIS imaging. The cardiac luciferase expression was monitored by Xenogen IVIS biophotonic imaging machine. Upon luciferin administration through IP, anesthetized rats were euthanized and the organs were harvested immediately. Harvested tissues were placed on the 10cm plates on the imaging chamber anda background photo of the tissues and a color overlay of the emitted photon data were obtained.

Toxicological and pharmacological tests. For toxicological and pharmacological tests, hematological parameters (VetScan HM2 Hematology

System; 50 μl blood in EDTA for WBC counts, WBC histogram, Hb, Hct, MCV, MCH, MCHC, RDW, graphic RBC histogram, PLT count, MPV, PCT, PDW and Graphic platelet histogram) and chemistry (VetScan Classic; 100μl blood in lithium heparin; ALB, ALP, ALT, AMY, BUN, CA++, CRE, GLOB, GLU, K+, Na+, PHOS, TBIL, TP) were measured.

Echocardiography (ECHO) for non-invasive assessment of ventricular function and structure. To evaluate cardiac function and structure we performed both standard ECHO and Two-Dimensional Speckle-Derived Strain ECHO (2DSE) examinations at four and nine months post injections in the BNP-treated and the untreated SHR. We also performed standard ECHO and 2DSE in normal Wistar rats at 4 weeks after AAV9 injections. All ECHO examinations were performed by skilled sonographer (E.A.O.) blinded to the treatment. Detailed protocols for ECHO examinations were described in Supplemental Materials. Standard ECHO: After removing chest hair, ultrasonic scans was performed in all rats in supine position using a Vivid 7 system (GE Healthcare, Milwaukee, WI) equipped with a 10S ultrasound probe (11.5 MHz) with ECG monitoring. M-mode images and gray scale 2D images (300-350 frames /sec) of the parasternal long-axis and mid-LV was recorded for off-line analysis. LV end-diastolic (LVDd) and end-systolic (LVDs) dimensions, septal diastolic (SWTd) and posterior wall diastolic (PWTd) and systolic (PWTs) thicknesses were measured from M-mode images. LV mass was calculated according to uncorrected cube assumptions as LV mass = $1.055 \times [(LVDd+SWTd+PWTd)^3-(LVDd)^3]$, where 1.055 is the specific gravity of myocardium. LV mass was

corrected for body weight (LVMi) for analysis. End-systolic (ESV), end-diastolic

and stroke volumes (SV), and ejection fraction (EF) was calculated using the

Teichholz formula: LV volume = 7 x [(LVDd)^3/(2.4+LVDd)]. Relative wall

thickness (RWT) was calculated as RWT = (SWTd + PWTd)/LVDd. All

parameters represented the average of three beats. 2DSE: Using EchoPAC

software (EchoPAC PC – 2D strain, BTO 6.0.0, GE Healthcare, Milwaukee, WI),

which included a high resolution speckle tracking analysis library for off-line

analysis, endocardial border was carefully manually traced at end-systole in LV

short-axis views at the middle level (i.e. at the level of papillary muscles). Ideal

width of circular region of interest was chosen in order to include the entire

myocardial wall. Speckle tracking was performed by the software and global

strain and circumferential strain rate parameters were measured computing the

mean of the six middle LV segments. The analysis included peak circumferential

systolic strains (sS) and strain rates (sSR) for evaluation of myocardial systolic

function and peak early circumferential strain rates (dSR-E) for evaluation of

myocardial diastolic function. All parameters represented the average of three

beats. Relevant to this proposal, using standard ECHO and 2DSE, we were able

to detect significant improvement in both systolic and diastolic function in a rat

model of cardiac dysfunction when compared to the untreated SHR.

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