Gene Expression of Brain Natriuretic Peptide in Isolated Atrial and Ventricular Human Myocardium
Influence of Angiotensin II and Diastolic Fiber Length

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Background—We studied the effects of angiotensin II (Ang II) and diastolic overstretch on the induction of cardiac growth in isometrically contracting muscle preparations from human right atria and left ventricles. We used the gene expression of brain natriuretic peptide (BNP) as a molecular marker of cardiac hypertrophy.

Methods and Results—Northern blot analysis was performed in human atrial muscle preparations, which were either incubated in $10^{-6}$ mol/L Ang II for 45 minutes or diastolically stretched to 120% of optimum muscle length. Similar experiments were performed with human left ventricular muscle preparations. Results were as follows: (1) BNP gene expression increased in human atrial myocardium 4-fold when stimulated by Ang II ($n=7$, $P<0.001$). (2) Diastolic overstretch increased BNP expression in a time-dependent manner. The linear regression equations for the BNP/GAPDH ratio as a function of time (hours) were $y=1.21+0.62x$ ($P<0.001$) for overstretched preparations and $y=1.07-0.01x$ ($P=NS$) for atrial preparations kept at physiological muscle length. (3) In left ventricular human muscle preparations, diastolic overstretch and Ang II increased BNP gene expression as well. (4) In addition, the Ang II subtype 1 receptor blocker losartan was able to block the effects of Ang II and diastolic overstretch.

Conclusions—Cardiac hypertrophy can be induced in isolated human atrial and left ventricular intact myocardium by Ang II and diastolic overstretch but not by isometric afterload. The fact that the induction of cardiac growth is inhibited by the blockade of Ang II subtype 1 receptors is of scientific and clinical importance. (Circulation. 2000;102:3074-3079.)

Key Words: genes ■ hypertrophy ■ angiotensin ■ peptides ■ myocardium

To prevent cardiac hypertrophy and thereby the hypertrophy-associated reprogramming of cardiac gene expression,1 the hypertrophic stimuli2 and their underlying molecular mechanisms must be investigated.3,4 From studies in isolated neonatal rat myocytes, it is known that mechanical overstretch5–8 as well as neuroendocrine mechanisms, especially angiotensin, may initiate the cardiac growth process.9–12

Angiotensin II (Ang II) has been demonstrated to exert positive inotropic effects in the cardiac muscle of many species.13–19 We have previously shown that in human myocardium, Ang II has an inotropic action in atrial but not in ventricular preparations because of the lack or decoupling of angiotensin receptors.20 This finding leads to the question of whether angiotensin is a direct growth factor in human ventricular myocardium as it is in neonatal rat myocytes. Because of the lack of cultured human cardiomyocytes, we studied the influence of Ang II and diastolic overstretch in small muscle strips prepared from human right and left ventricles obtained from patients that underwent orthotopic heart transplantation because of end-stage heart failure classified as New York Heart Association (NYHA) grade III to IV or from patients that underwent surgery because of valvular heart disease (aortic or mitral valve replacement). Furthermore, in isometrically contracting human muscle preparations, the influence of systolic stress on cardiac growth can be tested in addition to and independent of diastolic stretch and neuroendocrine mechanisms. Therefore, by the use of human cardiac isolated but intact muscle tissues for the study of induction of cardiac growth, differential effects of systolic and diastolic stress and hormonal influences can be separated.

Recently, Nakagawa et al10 described the brain natriuretic peptide (BNP) as an early responsive ‘emergency’ gene to stress in the myocardium, because in their experiments BNP was expressed earlier and to a greater amount than was atrial
natriuretic peptide when a number of different stimuli were applied (see also Molkentin et al4). Therefore, we used mRNA of BNP as a molecular marker of induction of cardiac hypertrophy. In the present study, it is shown for the first time that BNP is expressed in isolated isometrically contracting preparations from right atrial and left ventricular human myocardium as a result of neuroendocrine stimulation (Ang II) and mechanical factors (continuous diastolic overstretch but not systolic afterload). The present study underlines the importance of the renin-angiotensin system and mechanical factors in the genesis of cardiac hypertrophy in human myocardium and has implications for optimal medical treatment.

Methods

Patients

Right atrial appendices were received from 26 patients who underwent routine aortocoronary bypass surgery. In none of these patients was the ejection fraction <40%. Preoperative medication consisted of calcium channel blockers, nitrates, β-adrenoceptor blockers, and cholesteryl-synthetase blockers. Only 2 of the patients were taking ACE inhibitors, and no patient was taking an angiotensin receptor blocker.

Pieces of left ventricular papillary muscles were received from patients who required operative mitral valve replacement because of mitral valve stenosis (n=5) or mitral valve incompetence (n=5). Myocardium was also obtained from the free left ventricular wall from 1 patient with severe aortic stenosis undergoing aortic valve replacement. Left ventricular free wall myocardium was obtained from explanted hearts (n=6) from patients suffering from end-stage heart failure because of dilated cardiomyopathy. These patients were treated before surgery with digitalis (digoxin or digitoxin), ACE inhibitors (captopril, enalapril, or benazepril), and loop diuretics treated before surgery with digitalis (digoxin or digitoxin), ACE inhibitors, and no patient was taking an angiotensin receptor blocker.

Transport of Myocardium and Preparations

All myocardial tissues used in the present study were freshly obtained from the operation room of the Department of Cardiac Surgery, which is near the Department of Cardiology and Angiology of the Medizinische Universitätsklinik Freiburg. As soon as the surgeon had cut the atrial appendix, a piece from the left ventricle, and (in the case of heart transplantation) the whole heart, tissues were immediately submerged into Krebs-Ringer solution (see below), which contained 30 mmol/L butanediol monoxime.21,22 At that time, small pieces of myocardium were immediately frozen in liquid nitrogen for control experiments. The transportation time from the operation room to the laboratory where muscle strips were prepared and experiments were performed was <15 minutes under all circumstances.

Trabeculae or muscle strips were prepared and mounted as described in detail previously.23

Study Protocols

Atrial Preparations

In the first set of experiments, physiologically contracting muscle preparations were exposed to Ang II (10^{-6} mol/L) for a period of 45 minutes. Control preparations were superfused by standard Krebs-Ringer solution, which was composed as follows (mmol/L): Na^+ 152, K^+ 3.6, Cl^- 135, HCO_3^- 25, Mg^2+ 0.6, H_2PO_4^- 1.3, SO_4^{2-} 0.6, Ca^2+ 2.5, and glucose 11.2, along with insulin (10 IU/L). This solution was constantly bubbled with a gas mixture of 5% CO_2 and 95% O_2. In the second set of experiments, physiologically contracting muscle preparations were overstretched for different periods of time to study the influence of duration of diastolic overstretch on BNP gene expression. For this purpose, optimum muscle length (L_{max}) was measured microscopically. Thereafter, the muscle length was increased in 0.1-mm steps by means of a micrometer screw to the final muscle length of 120% L_{max}. By this procedure, developed tension decreased by ~50%, whereas resting tension exponentially increased (see Table 1). Control experiments were performed in which the muscle length was kept constant at L_{max}.

In addition, 3 muscle preparations were continuously incubated with losartan (10^{-6} mol/L) 10 minutes before and during the whole period of diastolic overstretch. To determine whether the overstretch effect is (at least in part) reversible regarding active force development, additional experiments (n=6) were performed in which muscle length was gradually reduced in 0.1-mm steps from 120% L_{max} to 100% L_{max}. These experiments revealed that at least 30% of the decrease of active force development is acutely reversible (see Discussion).

Furthermore, to study the influence of hypoxia, 6 preparations were made hypoxic by lowering the PO_2 of the bathing solution from >500 mm Hg to <10 mm Hg. Although hypoxia severely decreased peak force development, the ratio between BNP mRNA and GAPDH mRNA was not significantly altered, either at 30 or at 60 minutes of exposure to hypoxia (see below).

Left Ventricular Preparations

In the first set of experiments, isometrically contracting muscle preparations were incubated for 1 hour with Ang II (10^{-6} mol/L). In the second set of experiments, physiologically contracting muscle preparations were incubated for 4 hours with losartan (10^{-6} mol/L). In the third set of experiments, muscles were preincubated with losartan (10^{-6} mol/L) for 10 minutes, and Ang II was added (10^{-6} mol/L) for a period of 1 hour. Losartan remained present subsequent to the preincubation period.

In the fourth set of experiments, muscles were overstretched to 120% L_{max} for a period of 1, 2, 3, and 4 hours to study the time dependence of BNP expression during overstretch. Control experiments were simultaneously performed in which the muscles were kept at L_{max} and contracted isometrically (constant afterload).

Molecular Biology

After each experiment, muscle preparations were immediately frozen in liquid nitrogen. Muscle tissues were stored at ~80°C.

The amount of mRNA signal intensity was measured by using a specific ^32P radioactive scanner (Phospholmager, Fuji BAS 2400) within the linear range of detection. To compare relative BNP mRNA expression, the ratio between BNP mRNA and GAPDH mRNA expression signals was calculated to correct for differences in RNA loading.

<table>
<thead>
<tr>
<th>TABLE 1. Muscle Weight, Dimensions, Peak Developed Tension, and Resting Tension of Muscle Preparations Used for Overstretch Experiments</th>
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</thead>
<tbody>
<tr>
<td>Muscle Weight, mg</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>Atrial preparations (n=21)</td>
</tr>
<tr>
<td>Ventricular preparations (n=14)</td>
</tr>
</tbody>
</table>

Values are mean±SD. CSA indicates cross-sectional area; PDT, peak developed tension; and RT, resting tension.

*P<0.01 vs data at L_{max}.
RNA Isolation and RNA Blot Analysis

Total RNA was obtained from cardiac tissue either according to the manual of RNAzol B (Tel-Test), which was based on the method of Chomczynski and Sacchi,24 or according to a different protocol provided by Qiagen. Briefly, tissues were homogenized in lysis buffer by use of a mechanical homogenizer and then digested with protein kinase K (225 μg/mL) for 10 minutes at 55°C to facilitate optimal extractability of cellular RNAs. The protease-digested lysates were then passed through Qashredder columns (Qiagen) to shear high molecular weight genomic DNA. The subsequent isolation of total RNA was performed by the use of RNeasy Mini Spin columns (Qiagen) according to the manufacturer’s protocol. Total RNA (2 to 5 μg per lane) was fractionated on a 1.2% agarose gel containing 3.7% formaldehyde and 10 mmol/L sodium phosphate, pH 7.4, and subsequently transferred to Hybond-N nylon membranes (Amersham) in 10× SSC. Hybridization was performed with 32P-labeled cDNA probes in ULTRAhyb hybridization buffer (Amersham) at 42°C overnight. Processed blots were exposed to Hyperfilm MP (Amersham) or directly analyzed by PhosphoImager (Fuji BAS 2400). Autoradiograms were quantified by computer-assisted densitometry with the use of the Alphalager analysis system (Alpha Innotech Corp). The ratio between the measured signal intensity of the BNP mRNA bands and the intensity of the corresponding GAPDH mRNA signals is shown in arbitrary units.

cDNA Probes

Total RNA from cardiac tissue was isolated as described above. First-strand cDNAs were synthesized with oligo(dT)-primed RNA with the use of Superscript II (GIBCO-BRL) according to the supplied protocol. cDNA fragments specific for GAPDH and BNP were obtained by polymerase chain reaction with the use of previously reported primers (see Seilhamer et al25). Losartan26,27 was kindly provided by Merck, Sharp & Dohme (Munich, Germany).

Statistical Analysis

Linear regression analysis and calculation of correlation coefficients were performed according to Sachs.28 The unpaired t test was also used.28

Results

Human Atrial Myocardium and Ang II

Incubation of isometrically contracting preparations from right human atria with Ang II at a concentration of 10−6 mol/L for a period of 45 minutes significantly (P<0.01) increased the ratio between BNP and GAPDH mRNA levels by ≈4-fold (7 preparations; see Figure 1A and Figure 2). The 7 control preparations, which were perfused with a standard Krebs-Ringer solution without Ang II for the same period of time, showed only very little (nonsignificant) increase in BNP gene expression (Figure 2) despite isometric contraction at 60 bpm. Those preparations exposed to Ang II incubation showed a mild positive inotropic effect, as we described previously29: increase in force development of 32±10%.

Human Atrial Myocardium and Diastolic Overstretch

In 21 preparations, muscle length was adjusted to 120% lmax for 30 and 60 minutes and 2, 3, 4, and 8 hours. Thereby, the ratio between BNP and GAPDH mRNA gradually increased, as shown in Figure 1B for the 4-hour experiment.

To analyze the time dependence of BNP gene expression as induced by continuous diastolic stretch, data were subjected to linear regression analysis. For the function between increase in BNP mRNA (ordinate in Figure 3, arbitrary units) and the duration of diastolic stretch (abscissa in Figure 3, units given in hours), we calculated the equation y = 1.21 + 0.62x, indicating a 6-fold increase after 8 hours of diastolic stretch (Figure 3, Table 2). For the respective control preparations, which were kept at the physiological muscle length of 100% lmax, a linear equation was analyzed with a slope of approximately zero and an intercept of almost unity (y = 1.07 + 0.01x), indicating no significant alteration in BNP gene expression (Figure 3, Table 2). The 2 linear regression equations were significantly different from each other (P<0.001). Three preparations that were also adjusted to 120% lmax were preincubated with 10−6 mol/L losartan, an Ang II subtype 1 receptor blocker. By this pharmacological intervention, stretch-induced BNP gene expression was blocked by >80% (Figure 3).

Human Left Ventricular Preparations and Ang II

Four preparations obtained from human left ventricles were exposed to Ang II at a concentration of 10−6 mol/L for 1 hour. This treatment resulted in an increase of the BNP/GAPDH mRNA ratio by 2.5-fold (Figure 4), despite a lack of an inotropic effect. Control experiments were carried out in 2
Figure 2. Increase of ratio between BNP and GAPDH mRNA of human myocardial tissue obtained from right atria of 14 patients undergoing routine aortocoronary bypass surgery. Hatched bar represents control experiments without treatment (n=7 experiments). Open bar represents preparations that were exposed to Ang II (AT II, 10^{-6} mol/L) for 45 minutes (n=7 experiments). This increase in BNP gene expression was statistically significant (P<0.01).

Human Left Ventricular Preparations and Diastolic Overstretch

In 14 preparations, muscle length was adjusted to 120% \( l_{\text{max}} \) for 1 hour (n=2 preparations), 2 hours (n=1 preparation), 3 hours (n=2 preparations), and 4 hours (n=9 preparations) (Figure 4). The BNP/GAPDH mRNA ratio significantly increased in a time-dependent manner (Figure 4). In control preparations, which were kept at physiological muscle length of 100% \( l_{\text{max}} \), no change of BNP gene expression was observed except in the presence of Ang II (see above). Furthermore, when 3 preparations were incubated with losartan (10^{-6} mol/L), no BNP gene expression was obvious despite continuous diastolic overstretch (4-hour overstretch, middle bar of Figure 4).

**Discussion**

**Isometric Contraction Mode and BNP Expression**

The systolic contraction mode may have an influence on cardiac growth and thereby BNP expression. Therefore, the mode of contraction, ie, isometric shortening, afterloaded contraction, or diastolic contraction, may have a different impact on BNP mRNA expression. To study the influence of diastolic stretch or neuroendocrine mechanisms on BNP expression, it is a prerequisite to investigate the influence of systolic contraction and especially afterload on BNP expression. Neither in human atrial nor ventricular muscle preparations did the isometric contraction mode have any influence on BNP expression: The linear regression equation for the relationship between BNP expression and duration of the experiments even revealed a negative slope of \(-0.04\) with an intercept of almost unity for those atrial muscle preparations that contracted isometrically at 60 bpm and were not stretched beyond \( l_{\text{max}} \), which is believed to be within the physiological range (Figure 3). In ventricular muscle preparations, the isometric contraction mode with a stimulation frequency of 60 bpm did also not increase BNP expression significantly over a period of 4 hours, as indicated by the open bars in Figure 4 (except with Ang II stimulation). One might argue that the preparations might have a hypoxic core, which (with time) may trigger BNP expression. In a set of additional experiments (see Methods), it was shown that hypoxia (\( \text{PO}_2 < 10 \text{ mm Hg} \)) had no effect on BNP expression.

**Ang II and BNP Gene Expression**

The fact that Ang II is able to induce BNP gene expression in atrial human muscle tissues (Figures 1 and 2) was not unexpected. Compared with left ventricular human myocardium, the number of Ang II subtype 1 receptors is much higher in right atrial myocardium.\(^{29}\) Additionally, as we have shown previously, Ang II exerts positive inotropic effects in human atrial but not human ventricular myocardium.\(^{20}\)

In contrast to the lack of a positive inotropic effect of Ang II in human left ventricular myocardium,\(^{20}\) a clear 2.5-fold increase of BNP gene expression was found after a 1-hour exposure of left ventricular muscle preparations to Ang II; this increase could be completely blocked by losartan (Figure 4). Therefore, the present study demonstrates not only the existence of Ang II subtype 1 receptors but also the functional coupling of these receptors with respect to cardiac growth. In conjunction with our previous data,\(^{20}\) it can also be concluded that intracellular signal transduction must be different for the effects on contractility and hypertrophic stimulation. There is clear evidence that the hypertrophic stimulus is mediated by protein kinase C and mitogen-activated protein kinase.\(^{3,7,8,11}\)

On the other hand, the production of inositol 1,4,5-trisphosphate may be responsible for a change in systolic calcium transients via inositol 1,4,5-trisphosphate receptors in the sarcoplasmic reticulum.\(^{30}\) Therefore, in atrial and ventricular human myocardium, hypertrophic stimuli may be regulated by the same second-messenger system, whereas differences in Ang II–mediated inotropic effects may be due to differences in cardiac cell morphology and receptors.

**TABLE 2. Linear Regression Analysis for Function of BNP mRNA/GAPDH mRNA Ratio and Duration (Hours) of Overstretch in Atrial Muscle Preparations**

<table>
<thead>
<tr>
<th>Muscle Length</th>
<th>100% ( l_{\text{max}} )</th>
<th>120% ( l_{\text{max}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope</td>
<td>-0.01±0.015</td>
<td>0.62±0.04</td>
</tr>
<tr>
<td>Intercept</td>
<td>1.07±0.06</td>
<td>1.21±0.15</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>-0.563</td>
<td>0.989</td>
</tr>
<tr>
<td>Significance</td>
<td>P=NS</td>
<td>P&lt;0.001</td>
</tr>
</tbody>
</table>

Values are mean±SD. See Figure 3.
to the existence of inositol 1,4,5-trisphosphate receptors of the sarcoplasmic reticulum.

**Diastolic Stretch and BNP Gene Expression**

In human right atrial and human left ventricular myocardium, BNP gene expression is significantly increased when the muscles are continuously overstretched to a muscle length of 120% L_{max} corresponding to ~2.7-μm sarcomere length (Figures 3 and 4). Interestingly, this BNP gene expression is time dependent (Figure 3). Control muscle preparations that were stretched to a muscle length of only 100% L_{max}, ie, within the physiological range, did not respond with an increase in BNP gene expression, as can be easily seen from the linear regression equation with a slope of almost zero (Figure 3).

One could argue that the process of overstretch might have damaged the muscle tissue and speculate that the increase in BNP expression is an indicator of degeneration or necrosis. There are 3 arguments against this hypothesis: (1) Peak developed force is decreased by only 55% and 40% when the muscle is stretched from 100% L_{max} to 120% L_{max} (Table 1). (2) Almost one third of the stretch-induced loss of peak developed force is acutely reversible in restretch experiments (see Methods). (3) Hypoxia (P_{O2} < 10 mm Hg) had no influence on BNP expression (see Methods). (4) Furthermore, mechanical damage due to overstretch may even occur in vivo in certain types of overload.

Regarding BNP expression, one can further ask about differences between failing and nonfailing human myocardium. Although we have not systematically investigated nonfailing left ventricular myocardium, there are at least 2 arguments that make significant differences unlikely: (1) BNP expression in atrial nonfailing myocardium is similar to that in failing ventricular myocardium regarding overstretch and Ang II exposition. (2) Left ventricular myocardium was obtained from 1 patient who suffered from mitral valve stenosis. This nonfailing left ventricular myocardium showed the same BNP expression as did the failing left ventricular myocardium.

Because this stretch-induced stimulation of BNP gene expression may be mediated, at least in part, by an autocrine/paracrine secretion of Ang II, atrial and left ventricular muscle preparations were treated with losartan before diastolic overstretch. This pharmacological intervention pre-
vented the stretch-induced stimulation of BNP gene expression by >80% and proves the neuroendocrine nature of the induction of cardiac hypertrophy. This finding in human atrial and left ventricular myocardium is in good agreement with studies in cultured neonatal rat myocytes in which Ang II subtype 1 receptor antagonists were shown to block stretch-induced mitogen-activated protein kinase activity and c-fos gene expression.\(^{31}\) The effect on BNP gene expression that could not be blocked by losartan was quite small, ie, \(\approx 20\%\) in atrial and \(\approx 10\%\) in left ventricular myocardium. From the data presented so far, it cannot be decided whether other nonsecretory factors exist or whether the Ang II subtype 1 receptors were not completely blocked.

**Clinical Implications**

The conclusions drawn from data presented in the present study are of clinical relevance: (1) Diastolic stretch beyond \(I_{\text{max}}\), ie, preload (rather than afterload), seems to be the mechanical factor for the induction of cardiac hypertrophy. (2) This stimulation is also mediated by autocrine/paracrine secretion of Ang II in left ventricular human myocardium. Therefore, proper treatment of a variety of cardiovascular diseases that will all finally end in congestive heart failure must include preload-lowering strategies, such as those including nitrates, ACE inhibitors, and diuretics, as well as appropriate renin-angiotensin blockade. (3) Again, we would like to state that ACE inhibitors or Ang II receptor blockers can be used to prevent the induction of cardiac hypertrophy without the risk of accompanying negative inotropic effects. (4) If concentrically hypertrophied hearts operate at fiber lengths greater than \(I_{\text{max}}\), BNP could be used as an clinical index of hypertrophy, and the use of diuretics in hypertensive patients could be beneficial.

**Acknowledgments**

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**References**


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