Relevance of Brain Natriuretic Peptide in Preload-Dependent Regulation of Cardiac Sarcoplasmic Reticulum Ca\textsuperscript{2+} ATPase Expression

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Background—In heart failure (HF), ventricular myocardium expresses brain natriuretic peptide (BNP). Despite the association of elevated serum levels with poor prognosis, BNP release is considered beneficial because of its antihypertrophic, vasodilating, and diuretic properties. However, there is evidence that BNP-mediated signaling may adversely influence cardiac remodeling, with further impairment of calcium homeostasis.

Methods and Results—We studied the effects of BNP on preload-dependent myocardial sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA2a) expression. In rabbit isolated muscle strips stretched to high preload and shortening isotonically over 6 hours, the SERCA/glyceraldehyde phosphate dehydrogenase mRNA ratio was enhanced by 168\% (n=8) compared with unloaded preparations (n=8; P<0.001). Recombinant human BNP at a concentration typically found in end-stage HF patients (350 pg/mL) abolished SERCA upregulation by stretch (n=9; P<0.0001 versus BNP free). Inhibition of cyclic guanosine 3\textsuperscript{\prime},5\textsuperscript{\prime} monophosphate (cGMP)–phosphodiesterase-5 mimicked this effect, whereas inhibition of cGMP-dependent protein kinase restored preload-dependent SERCA upregulation in the presence of recombinant human BNP. Furthermore, in myocardium from human end-stage HF patients undergoing cardiac transplantation (n=15), BNP expression was inversely correlated with SERCA levels. Moreover, among 23 patients treated with left ventricular assist devices, significant SERCA2a recovery occurred in those downregulating BNP.

Conclusions—Our data indicate that preload stimulates SERCA expression. BNP antagonizes this mechanism via guanylyl cyclase-A, cGMP, and cGMP-dependent protein kinase. This novel action of BNP to uncouple preload-dependent SERCA expression may adversely affect contractility in patients with HF. (Circulation. 2006;113:2724-2732.)

Key Words: calcium ■ heart failure ■ mechanics ■ natriuretic peptides ■ sarcoplasmic reticulum

Brain natriuretic peptide (BNP) is expressed in ventricular myocardium of heart failure (HF) patients and is clinically used as a surrogate indicating disease severity and prognosis.\textsuperscript{1,2} Because of its vasodilating and diuretic properties, BNP is being used therapeutically in acute states of HF.\textsuperscript{3,4} Recently, direct myocardial effects of natriuretic peptides, mediated through their membrane receptor guanylyl cyclase-A, were described. These effects resulted in decreased nuclear translocation of transcription factors, which may influence gene expression.\textsuperscript{5,6} One of those genes may be the sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA2a). Because decreased SERCA2a expression is of significant relevance for the pathophysiology of HF,\textsuperscript{7,8} we tested the hypothesis that BNP may adversely affect myocardial SERCA2a expression and Ca\textsuperscript{2+} homeostasis. We studied the effects of BNP on preload-dependent SERCA2a expression and the signaling pathways involved in rabbit isolated muscle strips. Furthermore, we examined the relationship between BNP and SERCA2a expression in end-stage HF patients and how it is affected by mechanical support in HF patients treated with left ventricular assist devices (LVADs).

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Methods

Muscle Preparation and Mechanical Stretch

The investigation conforms to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85–23, revised 1996).
Female chinchilla bastard rabbits (1.5 to 2 kg, Charles River, Kisslegg, Germany) were heparinized and anesthetized with thiopental sodium (50 mg/kg IV). Hearts were excised and retrogradely perfused with modified Krebs-Henseleit solution as described.9 Right ventricular trabeculae or thin papillary muscles were dissected and mounted in culture chambers (Scientific Instruments, Heidelberg, Germany) between a force transducer and a hook connected to a micrometer drive allowing for length adjustment. The system is equipped with a servomotor with force-feedback function and allows cultivation of functionally intact multicellular muscle preparations for up to 48 hours at 37°C with physiological protein turnover maintained.10 After [Ca2+]i was raised to 1.0 mmol/L, the Krebs-Henseleit solution was replaced with tissue culture medium at 1.25 mmol/L [Ca2+]i (M-199, Invitrogen, Karlsruhe, Germany) supplemented with 20 μUL human insulin, 0.2% (ω/vol) BSA, 70 μmol/L streptomycin, and 100 IU/mL penicillin and equilibrated with 100% O2. Preparations were allowed to stabilize for 1 hour under continuous electrical stimulation (1 Hz, 3 to 5 V) and subsequently assigned to 2 experimental groups. In the first group, the stretch group, preparations were stretched progressively over 30 minutes to 3-nm/mm2 resting tension, corresponding to Lmax at which isometric tension would be maximum, and allowed to shorten isotonically from this level of resting tension. Isotonic shortening means that afterload is zero. Group 2 was unloaded; ie, preparations remained at slack under otherwise identical conditions. This protocol was selected because it results in significant preload-dependent induction of SERCA expression. Isotonic shortening was recorded continuously over 24 hours at the designated loading conditions. At the end of incubation, muscle preparations were harvested from the culture chamber, rapidly frozen in liquid N2 in RNAlater (Qiagen, Hilden, Germany) solution for RNA assays or homogenization buffer (see below) for protein analysis, and stored at −80°C. Freshly isolated RV papillary muscles not used for in vitro experiments were immediately frozen and served as a control group. In a subset of preparations, after the 6-hour incubation period, the frequency dependence of force development was assessed by recording isometric force at stimulation rates of 1 to 4 Hz. Preparations in the slack group were stretched over 30 minutes as described above, and force development was allowed to reach steady state before the frequency protocol was initiated. In isotonic preparations, the force-feedback motor unit was switched off, and force development was allowed to reach steady state before the frequency protocol was initiated.

**Pharmacological Interventions**

Calcineurin activity was inhibited by incubation in medium containing 1 μmol/L cyclosporine-A (CsA; Sigma, Taufkirchen, Germany). This concentration of CsA significantly reduced calcineurin phosphatase activity in vitro in a protein extract from rabbit myocardium by 24±5% (P<0.05), whereas a lower concentration (100 nmol/L) did not significantly suppress calcineurin activity (data not shown). The calcineurin inhibitory action of CsA is limited by the concentration of its binding partner, cyclophilin.11 Because the protein extract (and thus endogenous cyclophilin present in the extract) must be diluted for phosphatase activity measurements, the degree of inhibition measured in vitro probably reflects an underestimation of the inhibition of calcineurin activity provided by CsA in intact myocardium. CsA was dissolved at 10 mmol/L in ethanol (final concentration, 0.01%). Recombinant human BNP (rhBNP; Calbiochem, Schwabach, Germany) was dissolved in 5% acetic acid (final concentration, 0.0005%) and added to the medium at 10 or 100 pmol/L. Cyclic guanosine 3′,5′ monophosphate (cGMP)–phosphodiesterase-5 (PDE-5) activity was inhibited with zaprinast (Sigma) at 100 μmol/L, directly dissolved into the medium. This concentration of zaprinast has been shown to significantly amplify the effects of atrial natriuretic factor and to increase intracellular cGMP levels in rabbit ventricular myocytes.12,13 In muscle strip preparations, zaprinast significantly reduced isometric tension development (data not shown). CGMP-dependent protein kinase (PKG-I) was inhibited with KT-5823 (Sigma) dissolved in ethanol (final concentration, 0.01%) and added to the medium at 1 μmol/L, a concentration previously shown to suppress PKG-I–mediated effects of PDE-5 inhibition and exogenous nitric oxide in rabbit ventricular myocytes.13,14 All agents were present from the onset of the 60-minute equilibration period and throughout the entire incubation period. Appropriate vehicle control groups were included for every investigated combination of agent and loading condition. Neither ethanol nor acetic acid in the concentrations used exerted any detectable effects on contractile function or gene expression.

**Quantitative mRNA Measurement in Rabbit Myocardium**

DNA-free total RNA was extracted from myocardial samples by a standard protocol with the RNAesy kit and RNase-free DNase Set (Qiagen, Hilden, Germany). First-strand cDNA synthesis was carried out with SuperScript II reverse transcriptase and random primer (Invitrogen) according to manufacturer’s instructions. Real-time polymerase chain reactions (PCRs) were performed on a LightCycler (Roche, Mannheim, Germany) in a volume of 20 μL in glass capillaries. The reaction mixture consisted of 2 μL cDNA, 0.5 μmol/L of each primer, 1 U of Taq DNA polymerase (Invitrogen), 2 μL of 10× PCR buffer (Invitrogen), 0.2 mmol/L of each deoxynucleoside triphosphate (BioLine, Luckenwalde, Germany) 2.5 mmol/L MgCl2, 500 mg/L bovine serum albumin (New England BioLabs, Frankfurt, Germany), 50 mL dimethyl sulfoxide (Sigma), and 1 μL of 1/1000 SYBR Green stock (Roche). PCR conditions and primer sequences used are provided in the online Data Supplement. cDNAs with known concentrations were used to generate quantification standard curves.

**Quantitative mRNA Measurement in Human Myocardium**

Left ventricular midmyocardial samples were obtained from 15 end-stage HF patients undergoing cardiac transplantation as a result of dilated cardiomyopathy (12 men, 3 women; age, 41.7±5.2 years; cohort 1) and from 23 end-stage HF patients (17 dilated cardiomyopathy, 6 ischemic cardiomyopathy; 21 men, 2 women; age, 55.9±2.3 years; cohort 2) for whom donor hearts were not immediately available and who therefore were treated with mechanical circulatory support by LVAD for a mean duration of 280 days (range, 30 to 741 days) to bridge the time until transplantation. Detailed patient characteristics for both cohorts are provided in Tables I and II respectively, in the online Data Supplement. The study was approved by the institutional ethics committee, and all patients provided written informed consent with the use of tissue samples. In cohort 2, 2 sequential samples were obtained at the times of device implantation and cardiac transplantation. The first sample consisted of left ventricular apical myocardium removed on cannulation of the chamber. The second sample was obtained on transplantation from a region adjacent to the cannulation site but not visibly affected by scarring. For SERCA2a and BNP mRNA quantification in human samples, the protocol described above for rabbit was modified. Reverse-transcribed cDNA was analyzed by real-time PCR with a commercial SYBR Green kit (FastStart DNA Master SYBR Green I, Roche, Basel, Switzerland). PCR conditions and primer sequences used are provided in the online Data Supplement. SERCA2a and BNP mRNA of each sample was analyzed in duplicate or triplicate, respectively.

**Western Immunoblot Analysis**

Rabbit muscle strips were thawed on ice and chilled in 50 μL homogenization buffer (containing in mmol/L: Na-HEPES 20, pH 7.4, EDTA 2, EGTA 2, DTT 1, phenylmethylsulfonyl fluoride 1, leupeptin 0.05, and iodoacetamide 1). After mechanical homogenization and sonication at 4°C, protein concentrations were determined in triplicate.15 Samples of 20 μg were denatured in electrophoresis buffer (containing in mmol/L: TRIS/HCl 100, pH 6.8, DTT 10, 2% SDS, 2% glycerol, and 0.5% bromophenol blue [ω/vol]) at 95°C and subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Proteins were electroblotted to nitrocellulose membranes, and membranes were blocked overnight at 4°C in 5%...
We assessed the effects of complete unloading and acutely elevated preload on SERCA2a expression in rabbit right ventricular muscle strips. For better comparability, all mRNA expression values were normalized for GAPDH mRNA expression because GAPDH mRNA levels were unaltered by mechanical loading conditions. Preparations were subjected to zero load or elevated preload over 6 hours by slack incubation or by stretching them to Lmax and allowing them to shorten isotonically from this level of passive stretch. Comparative mRNA expression levels in unloaded and stretched preparations (Figure 2A through 2C). Although stretch significantly reduced PLB mRNA expression (~31%; slack, n=8; stretch, n=8; P<0.01), no corresponding change in PLB protein was observed (slack, n=4; stretch, n=5; P=0.79) within 6 hours. These data show that the functional impact of SERCA2a upregulation was not corroborated by proportional upregulation of PLB.

Mathematical Methods
Force values were transferred to tension by normalizing to the cross-sectional area of a preparation, which was calculated assuming an elliptical cross section using this formula: Cross-sectional area = D1/2 × D2/2 × π, with D1 and D2 representing width and thickness. Gene expression was analyzed through the use of unpaired Student t test (paired in the case of human LVAD patients) and functional data (force-frequency relationship) using 2-way ANOVA, with values of P<0.05 considered statistically significant. The hyperbolic relationship between SERCA2a and BNP expression levels in human patients was obtained as follows. Reciprocal values of BNP expression were calculated and plotted versus SERCA2a expression, followed by linear regression analysis, yielding the correlation coefficient and significance level provided in the Results. Subsequently, the reciprocal BNP mRNA expression levels were retransformed, and the fitting parameters were used to create the depicted hyperbola.

The authors had full access to the data and take responsibility for their integrity. All authors have read and agree to the manuscript as written.

Results
Effect of Preload on SERCA2a Expression
We assessed the effects of complete unloading and acutely elevated preload on SERCA2a expression in rabbit right ventricular muscle strips. For better comparability, all mRNA expression values were normalized for GAPDH mRNA expression because GAPDH mRNA levels were unaltered by mechanical loading conditions. Preparations were subjected to zero load or elevated preload over 6 hours by slack incubation or by stretching them to Lmax and allowing them to shorten isotonically from this level of passive stretch. Compared with freshly isolated control myocardium (n=8), complete unloading over 6 hours (n=8) caused a 25% reduction in SERCA mRNA expression, whereas acute elevation of preload (n=8) potentiated SERCA mRNA expression by 102% (168% compared with unloaded preparations; Figure 1A; unloaded versus control, P<0.01; stretch versus both control and unloaded, P<0.001). The preload-induced upregulation of SERCA2a was confirmed at the protein level, which was significantly enhanced by 47% after 6 hours of stretch compared with unloaded preparations (Figure 1B and 1C; n=4 in each group; P<0.05). Because SERCA2a activity depends on the stoichiometry of its association with the endogenous inhibitor PLB, we also assessed PLB expression in unloaded and stretched preparations (Figure 2A through 2C). Although stretch significantly reduced PLB mRNA expression (~31%; slack, n=8; stretch, n=8; P<0.01), no corresponding change in PLB protein was observed (slack, n=4; stretch, n=5; P=0.79) within 6 hours. These data show that the functional impact of SERCA2a upregulation was not corroborated by proportional upregulation of PLB.

Analysis of Contractile Function
Assessment of muscle strip contractile function revealed that the frequency-dependent increase in developed tension was significantly steeper after 6 hours of stretch (n=5) compared with unloaded preparations (n=4) (Figure 3; P<0.005). The steepness of the force-frequency relationship correlates with myocardial SERCA2a activity. Therefore, although we did not directly assess SERCA2a pump activity, eg, in sarcoplasmic reticulum vesicles, this increase in steepness indicates that the observed expression changes are of functional relevance.

Effect of BNP on SERCA2a Expression
To elucidate whether BNP may be directly involved in the regulation of preload-dependent SERCA2a expression, muscle strips treated with exogenous rhBNP were unloaded or stretched over 6 hours, and SERCA2a expression was assessed (Figure 4). rhBNP treatment resulted in a significant concentration-dependent reduction in SERCA2a mRNA in both unloaded and stretched preparations. At 100 pmol/L, rhBNP completely suppressed the preload-dependent SERCA upregulation in stretched preparations (n=8; P<0.0001 versus stretch control). We found that 100 pmol/L BNP is equivalent (350 pg/mL) to plasma levels that are not uncommonly found in patients with severe HF and have been correlated with reduced exercise capacity.
demonstrate that BNP suppresses myocardial SERCA2a expression, especially preload-induced stimulation of SERCA expression. There also is a direct negative inotropic effect of BNP, resulting in an 18\% reduction in contractile force at 100 pmol/L (n=5; P<0.05).

BNP is assumed to be load-dependently regulated. We therefore assessed the effect of mechanical load on endogenous BNP mRNA expression (Figure 5A). Mean BNP mRNA expression in muscle strips stretched over 6 hours (n=8) was higher than in slack preparations (n=8), but this effect was not significant (P=0.24). When the period of stretch was extended to 24 hours, however, BNP mRNA expression was several-fold potentiated (n=6; P<0.01 versus both unloaded and stretched over 6 hours). SERCA2a mRNA expression, which was upregulated after 6 hours, had returned to baseline levels after 24 hours (Figure 5B), indicating that upregulation of SERCA2a by enhanced preload is transient. These findings indicate that BNP antagonizes myocardial SERCA2a expression not only after it is added exogenously but potentially also when it is endogenously expressed at high levels.

Assessment of cGMP-Dependent Signaling
Natriuretic peptides exert biological effects via the guanylyl cyclase-A receptor, subsequent formation of cGMP and activation of PKG-I.5 To assess whether this pathway is involved in mediating BNP effects on load-dependent SERCA2a expression, we tested whether downstream interventions in this pathway modulate the SERCA2a expression response to load (Figure 6). Treatment with the PDE-5 inhibitor zaprinast (100 \mu mol/L), which is expected to increase cGMP levels, suppressed the upregulation of SERCA2a in preparations stretched for 6 hours to 49\% (n=9; P<0.005 versus slack; P<0.01 versus stretch control), thus mimicking the effect of rhBNP (Figure 6A). In slack preparations, SERCA2a mRNA was not affected by PDE-5 inhibition. Coadministration of the PKG-I inhibitor KT-5823 (1 \mu mol/L) with 100 pmol/L rhBNP, on the other hand, restored the ability of the preparations to upregulate SERCA2a during 6 hours of stretch, with SERCA2a mRNA values being 71\% higher than in the absence of KT-5823 (Figure 6B; n=8 in each group; P<0.001 versus rhBNP alone). This indicates that PKG-I plays a critical role in mediating BNP effects on load-dependent SERCA2a expression. Because the protein phosphatase calcineurin and its downstream mediator NFAT have been implicated in mediating the effects of mechanical load on myocardial gene expression, we studied the effect of the calcineurin inhibitor CsA. Pretreatment with CsA (1 \mu mol/L) suppressed the SERCA2a upregulation afforded by 6 hours of stretch (n=9; P<0.0001 versus stretch control; Figure 6C), suggesting that acutely elevated mechanical load upregulates SERCA2a expression via the calcineurin/NFAT signaling pathway.

Relationship Between BNP and SERCA2a Expression in Human Myocardium
We assessed myocardial BNP and SERCA2a mRNA expression levels in 15 consecutive patients undergoing cardiac transplantation for end-stage HF caused by dilated cardiomyopathy (cohort 1; patient data in Table I in the online Data Supplement). SERCA2a and BNP mRNA levels showed an inverse correlation best described by a hyperbolic function (correlation coefficient of SERCA2a versus 1/BNP=0.64; P<0.05). Those patients expressing the highest myocardial BNP levels exhibited the lowest SERCA2a expression levels and vice versa (Figure 7). A separate group of 23 consecutive end-stage HF patients (cohort 2; patient data in Table II of the
online Data Supplement), for whom donor organs were not immediately available, were mechanically supported by LVADs to bridge the time period until transplantation. LVAD treatment, which is targeted at mechanically unloading the failing heart, has been reported to induce reverse remodeling of the heart and thus may cause downregulation of myocardial BNP expression and upregulation of SERCA2a. We therefore analyzed BNP and SERCA2a mRNA expression levels in sequential myocardial biopsies obtained at the times of device implantation and transplantation and for both genes assessed relative changes in expression between implantation and transplantation (Figure 8). The response of individual patient BNP expression levels to LVAD treatment was variable (Figure 8A). When the subgroup of patients who downregulated BNP (represented by solid symbols in Figure 8A) was analyzed separately, SERCA2a mRNA expression significantly increased during the period of LVAD support (Figure 8B), indicating that downregulation of BNP indeed was accompanied by SERCA2a recovery in patients supported by LVADs.

Discussion

In this study, we describe a previously unidentified and unexpected effect of BNP as a negative regulator of SERCA expression in the myocardium. This is based on the following observations. First, SERCA expression both at the mRNA and protein level is upregulated by preload in rabbit multicellular muscle strips. This increase in SERCA2a expression is associated with improved myocardial performance, underlining the functional relevance of the preload-induced expression changes. Second, exogenously applied BNP concentration-dependently reduces SERCA expression in unloaded myocardium and abolishes SERCA upregulation in mechanically loaded myocardium, which establishes a causal relationship. Third, during a prolonged period of mechanical load in vitro (24 hours), BNP expression is highly induced and SERCA upregulation is suppressed. Fourth, signal transduction analysis indicates that BNP-dependent regulation of SERCA expression occurs through PKG-I activation with subsequent inhibition of calcineurin. Finally, the potential relevance for the pathophysiology of human heart disease is demonstrated because downregulation of myocardial BNP in end-stage HF patients supported by LVADs is accompanied by recovery of SERCA expression.

We performed this investigation to characterize the signaling mechanisms mediating downregulation of SERCA as a
consequence of mechanical overload and were surprised by the discovery that in the short term, an elevation of preload alters SERCA expression in the opposite direction. Furthermore, we found that under physiological conditions, preload appears to be essential for maintaining normal SERCA expression levels because we observed SERCA to be downregulated in myocardium completely unloaded for 6 hours. This is in line with SERCA downregulation in heterotopically transplanted (and thus hemodynamically unloaded) hearts in rats. Functionally, this regulation of SERCA expression by mechanical load represents an adaptive response enabling the myocardium to compensate for the enhanced demand. BNP upregulation and SERCA downregulation occur on remodeling during the development of heart failure. Consequently, an inverse relationship between SERCA and BNP in failing myocardium has been reported previously by us and others. Likewise, BNP expression has been shown to decrease and SERCA expression to recover during mechanical support in LVAD-treated patients. However, to the best of our knowledge, we are the first to investigate and demonstrate a direct causal relationship between BNP levels and SERCA expression in the myocardium. This mechanistic connection also may resolve why SERCA upregulation on LVAD support has not always been observed because we found that SERCA mRNA expression recovery was confined to the subgroup of patients that downregulated BNP with the

Figure 6. Signaling pathways relevant to preload-induced SERCA2a upregulation. A, Effect of PDE-5 inhibition. *P<0.005 vs slack control; #P<0.05 vs stretch control; †P<0.05 vs slack/zaprinast. B, Effect of BNP in the presence of PKG-I inhibition. #P<0.05 vs slack; †P<0.01 vs stretch +100 pmol/L BNP. C, Effect of calcineurin inhibition. *P<0.0001.

Figure 7. Relationship between SERCA2a and BNP expression levels in human failing myocardium. SERCA/GAPDH is plotted vs BNP/GAPDH mRNA expression ratios in 15 end-stage HF patients and fitted with a hyperbolic function. Correlation coefficient for SERCA2a vs 1/BNP = 0.64; P<0.05.

Figure 8. Relationship between SERCA2a and BNP expression levels during LVAD treatment in human failing myocardium. A, Percent change in SERCA vs BNP mRNA expression in 23 HF patients between the times of LVAD implantation and cardiac transplantation. B, Change in SERCA/GAPDH mRNA expression ratio in patients downregulating BNP mRNA during LVAD support (subset of data presented in A). ● Indicates patients downregulating BNP mRNA; ○, patients upregulating BNP mRNA. *P<0.01.
limitation that, because of the availability of tissue from the time of LVAD implantation, we were not able to confirm this recovery also at the protein level. We acknowledge that our clinical data do not prove causality. However, that BNP indeed suppresses SERCA expression has been established by our 6-hour in vitro experiments, and we interpret our clinical findings as supportive evidence suggesting that this mechanism also may be relevant in the clinical situation.

Our observation that in a subgroup of LVAD-treated HF patients, SERCA upregulation was induced by unloading appears to contradict our in vitro findings of SERCA upregulation caused by elevated load. However, LVAD treatment does not result in preload elimination, and it predominantly reduces afterload. Thus, LVAD treatment should not eliminate the load-dependent stimulation to express SERCA, and the net effect on SERCA expression may depend on the changes in BNP levels during LVAD treatment. Our in vitro findings that BNP is strongly upregulated while SERCA expression is suppressed after 24 hours of preloaded isotonic contractions would be consistent with this notion.

Load-induced myocardial hypertrophy and failure are associated with expression changes of various Ca\(^{2+}\)-regulatory proteins, among which decreased expression and Ca\(^{2+}\) uptake function of SERCA play a prominent role, resulting in increased diastolic tone, decreased systolic myofilament activation, and impaired frequency-dependent potentiation of contractile force.\(^4\)\(^5\)\(^6\)\(^7\) Although these are well-established findings, the molecular mechanisms responsible for SERCA downregulation are still unclear. Enhanced biomechanical load causes the heart to react with a compensatory hypertrophic response. The Ca\(^{2+}\)-dependent phosphatase calcineurin, acting via dephosphorylation and nuclear translocation of the transcription factor NFAT, has been implicated as a key mediator of hypertrophy.\(^8\)\(^9\)\(^10\) The SERCA2a promoter region possesses a consensus site for members of the GATA family possessing a consensus site for members of the GATA family and NFAT is able to associate with NFAT activation.\(^11\)\(^12\)\(^13\)\(^14\)\(^15\)\(^16\)\(^17\)\(^18\)\(^19\)\(^20\)\(^21\)\(^22\)\(^23\)\(^24\)\(^25\)\(^26\)\(^27\)\(^28\)\(^29\)\(^30\)\(^31\)\(^32\)\(^33\)\(^34\)\(^35\) The SERCA2a promoter region possesses a consensus site for members of the GATA family and NFAT is able to associate with GATA-4 to activate gene transcription, thereby enhancing SERCA2a expression. Because of the lack of available NFAT antibodies that cross-react with rabbit NFAT, we were not able to directly demonstrate an involvement of the calcineurin/NFAT-signaling pathway. However, from the indirect evidence that upregulation of SERCA by acute load was abolished on treatment of muscle strips with the calcineurin inhibitor CsA, we conclude that this effect is at least partially mediated by calcineurin. Acute preload-induced upregulation of SERCA mediated by calcineurin/NFAT can be viewed as part of the compensatory hypertrophic response. Transgenic mice overexpressing a constitutively active mutant of calcineurin indeed exhibited enhanced SERCA2a expression.\(^22\)

By which mechanisms does BNP influence SERCA expression? We demonstrate here that PDE-5 inhibition with zaprinast, which causes accumulation of cGMP, mimicked the effects of BNP on SERCA expression. Furthermore, we show that PKG-I inhibition with KT-5823 restored the ability of the myocardium to upregulate SERCA in response to preload in the presence of high concentrations of BNP. These findings suggest that BNP acts via increasing intracellular cGMP levels, which, in turn, will activate PKG-I. Natriuretic peptides are able to stimulate the generation of cGMP by binding to the sarcolemmal guanylyl cyclase-A receptor.\(^5\)\(^33\) This pathway has been reported to mediate antihypertrophic cardiac actions of natriuretic peptides and could explain why BNP and zaprinast in our study exerted similar effects on SERCA expression.

How could PKG-I affect SERCA expression? If steady-state myocardial SERCA expression depends on calcineurin activity, any mechanism reducing the latter will decrease SERCA expression and therefore represents a potential candidate to mediate SERCA downregulation during the transition to HF. Indeed, cGMP generated by application of nitric oxide reduces myocardial calcineurin activity and NFAT signaling via PKG-I.\(^6\) Activation of PKG-I has been shown to inhibit L-type Ca\(^{2+}\) current.\(^34\)\(^35\) Overexpression of PKG-I reduced cardiac myocyte Ca\(^{2+}\) transient amplitude and resulted in impaired Ca\(^{2+}\)/calmodulin-dependent calcineurin/NFAT activation.\(^36\) This reduction in Ca\(^{2+}\) transient amplitude also could explain the mild negative inotropic effect exerted by 100 pmol/L BNP in this study.

Recently, it was shown that in patients with end-stage HF, despite unaltered mRNA expression of the guanylyl cyclase-A receptor, cGMP production in response to atrial natriuretic peptide (ANP) was strongly reduced, indicating receptor desensitization, whereas LVAD support restored ANP sensitivity.\(^22\) This may represent an adaptive mechanism protecting the heart against the unfavorable effects of natriuretic peptides on gene expression. However, no information is available on local concentrations of BNP released in an autocrine/paracrine manner into the myocardial interstitium. Excessive BNP release could well overcome receptor desensitization or enhanced natriuretic peptide clearance. Interestingly, it also has been reported that although hemodynamic responses to ANP were blunted in a dog rapid-ventricular-pacing model of HF, those to BNP were preserved, raising the possibility that an additional BNP-selective receptor may exist in the cardiovascular system.\(^36\)

Taking together these reports and our own data, we propose that BNP depresses myocardial SERCA expression via guanylyl cyclase-A (or an alternative BNP-selective, natriuretic peptide receptor), cGMP, and PKG-I, followed by a reduced Ca\(^{2+}\)/calmodulin-dependent calcineurin/NFAT activation. Thus, upregulation of myocardial BNP expression with its autocrine/paracrine actions on cardiac myocytes may contribute to the transition from beneficial hypertrophy to HF. Interestingly, the expression of BNP also is stimulated by GATA transcription factors.\(^37\) Hence, upregulation of BNP after activation of the calcineurin/NFAT-signaling pathway appears to represent a negative feedback system capable of limiting the hypertrophic response provided by calcineurin activation.

A recent study reported that the HF phenotype in mice challenged with pressure overload as a result of transversal aortic constriction was favorably affected by chronic PDE-5 inhibition with sildenafil,\(^38\) which would not be in line with our concept. In that study, however, myocardial protection was associated with reduced instead of increased cGMP levels, and additional effects of sildenafil on the PI3-kinase/Akt/GSK-3β pathway were identified. These observations
make it unlikely that the PDE-5 inhibitory action of the drug was responsible for the favorable effects.

In summary, we suggest that BNP not merely represents a surrogate marker for HF but may play a critical role in the pathogenesis of HF. By blunting the adaptive response to mechanical load, it decreases myocardial SERCA expression, thereby contributing to disturbed myocyte Ca\textsuperscript{2+} homeostasis, which are key features of the transition into HF. With this in mind, long-term effects of the clinical application of BNP in experimental heart failure treatment need to be carefully assessed, especially because a recent meta-analysis of clinical trials evaluating the effects of recombinant BNP in decompensated HF identified a tendency for mortality to increase in BNP-treated subjects.\textsuperscript{39}

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

One of the pathophysiological hallmarks of congestive heart failure (HF) is an impaired calcium homeostasis of the cardiac myocytes, resulting in both contractile dysfunction and arrhythmogenesis. Reduced expression and function of the Ca\(^{2+}\)-ATPase of the sarcoplasmic reticulum (SERCA) is a major mechanism of defective calcium cycling. In myocardial hypertrophy and HF, brain natriuretic peptide (BNP) is expressed in the ventricular myocardium and released into the systemic circulation. Elevated serum levels of BNP, a diagnostically used criterion of HF, are correlated with poor prognosis. Nevertheless, myocardial production of BNP, as a result of its vasodilating, natriuretic, and antihypertrophic effects, has been considered beneficial until now. In the present study, we report that in isolated rabbit myocardium, acutely elevated myocardial preload triggers enhanced expression of SERCA, resulting in improved myocardial function. This compensatory response to mechanical load is completely suppressed by BNP at levels typically found in human HF patients. Furthermore, we report that in end-stage HF patients transiently treated with a left ventricular assist device as a bridge to transplantation, only those patients who downregulate BNP expression exhibit recovery of SERCA expression, which is a surrogate for improved myocardial calcium homeostasis. Our findings suggest that patients with HF, because of their endogenously expressed levels of BNP, may be lacking an important mechanism relevant for short-term compensation of elevated preload. Thus, rather than being purely beneficial, BNP also could contribute to the clinical progression of HF through this newly identified role.
Relevance of Brain Natriuretic Peptide in Preload-Dependent Regulation of Cardiac Sarcoplasmic Reticulum Ca\(^{2+}\) ATPase Expression

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