Chamber-Dependent Expression of Brain Natriuretic Peptide and Its mRNA in Normal and Diabetic Pig Heart

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Abstract—Brain natriuretic peptide (BNP) is produced in cardiac myocytes, and increased secretion is closely associated with cardiac dysfunction. However, several fundamental aspects of BNP expression in the myocardium have not yet been resolved. In the present study, we report the presence of a precursor BNP mRNA transcript and a mature BNP mRNA transcript in normal porcine hearts. In normal pigs, the amount of precursor BNP mRNA was similar in atrial and ventricular myocardium, whereas the mature BNP transcript was 10- to 50-fold more abundant in atrial than in ventricular myocardium. Quantitation of proBNP in normal porcine hearts by radioimmunoassay disclosed abundant proBNP in the atria, whereas proBNP was undetectable in the ventricles. Laser confocal microscopy revealed proBNP in secretory granules of atrial but not in the ventricular myocardium of normal pigs. Mild streptozotocin-induced diabetes doubled the expression of BNP mRNA in porcine atrial myocardium ($P=0.03$), but was without effect on BNP mRNA in the ventricular myocardium. The data suggest that BNP mRNA processing and proBNP storage differ between the atrial and ventricular myocardium. The results also imply that diabetes increases cardiac BNP expression in a chamber-dependent manner. (Hypertension. 2002;40:54-60.)

Key Words: brain natriuretic peptide • diabetes • gene expression • heart

Brain natriuretic peptide (BNP) was discovered in 1988 by Sudoh et al1 in porcine brain tissue. Subsequent studies established that BNP is expressed and released mainly by the heart.2–4 In clinical cardiology, increased plasma BNP concentrations are now used as a marker of decreased left ventricular function. Nevertheless, several fundamental aspects of cardiac BNP expression have remained unknown.5–7

It is well established that mechanical stretching of myocytes increases BNP gene expression.8 This may involve angiotensin II and p38 mitogen-activated protein kinase–dependent mechanisms.9,10 Recent results imply that metabolic disturbances also affect BNP expression. Hence, streptozotocin-induced diabetes in rats increases cardiac BNP mRNA levels11 but only modestly affect cardiac function.12 In addition to transcriptional regulation, BNP mRNA concentrations are affected by mRNA stability.13 The relative importance of transcriptional regulation and posttranscriptional degradation is, however, unknown. Moreover, the porcine myocardium contains 2 BNP mRNA transcripts,14,15 but the relative expression15 of the 2 transcripts has not been determined, and it is not clear to what extent changes in mRNA levels correlate with the tissue content or release of BNP.

BNP is referred to as a ventricular hormone. This reflects observations of increased ventricular BNP expression in animal models with overt congestive heart failure.16–19 Severe cardiac failure in man doubles the ventricular BNP content but is without effect on atrial BNP.20 Nevertheless, early ventricular dysfunction has been associated with increased atrial BNP expression without any change in the ventricular BNP expression.21 Moreover, data are in conflict about the mRNA and protein expression in atrial versus ventricular myocardium in the normal heart.18,20–23 Thus, the primary site of cardiac BNP production at various stages of cardiac dysfunction remains to be settled.

Atrial natriuretic peptide (ANP) is localized in secretory granules of atrial myocytes.24 Because of analogies in structure and secretion pattern of ANP and BNP, it has been suggested that BNP might also be stored in secretory granules.24,25 Unlike atrial myocytes, normal ventricular myocytes, however, do not contain secretory granules. This may imply that the handling of BNP in atrial and ventricular myocytes differs.

In the present study, we measured the relative abundance of 2 BNP mRNA transcripts in porcine myocardium, variation between different regions of the porcine heart in BNP mRNA and proBNP levels, and the subcellular localization of proBNP in normal atrial and ventricular myocardium. In addition, we assessed to what extent diabetes affects cardiac BNP gene expression. The results suggest that the processing of BNP mRNA and proBNP differs in atrial and ventricular myocardium of normal pigs, and that diabetes affects BNP gene expression in a cardiac chamber-dependent fashion.
Methods

Animals
Hearts from domestic pigs (age, 5 to 6 months; body weight, 90±10 kg) were collected in a slaughterhouse (Steff-Houlberg, Ringsted, Denmark). Biopsies from left and right atrium, left and right ventricle, and interventricular septum were collected. In 4 pigs, ventricles were further divided into apex, mid ventricular, and basal sections. The mid ventricular and basal sections were each subdivided into anterior, lateral, and inferior wall segments of the left ventricle, interventricular septum, and right ventricle.

Male Göttingen minipigs (age, 12 to 17 months; body weight, 33±1 kg; Ellegaard Göttingen minipigs Aps, Dalmose, Denmark) were housed at Novo Nordisk A/S and fed twice daily 140 g SDS minipig diet (SDS) and 240 g commercial swine diet (Swinefoder 22, Slangerup, Denmark). The Animal Experiments Inspectorate, Ministry of Justice, Denmark, approved the study of minipigs.

Diabetes
In each minipig, 2 central venous catheters (Certo 455, B.Braun Melsungen AG) were introduced in the cranial caval vein under general anesthesia. After a 2-week recovery period, nicotinamide (45 mg/kg; Sigma-Aldrich) and streptozotocin (STZ) (125 mg/kg; Sigma-Aldrich) were injected intravenously. An oral glucose tolerance test was performed 1 to 2 weeks after injection of streptozotocin. After an 18-hour fast, the pigs were offered a mixed meal glucose tolerance test of 25 g SDS minipig diet (SDS) and 2 g/kg glucose (500 g/L, SAD, Copenhagen, Denmark). Plasma insulin and glucose were measured with a 2-site immunometric assay (catching antibody HUI-018 raised against the A-chain of human insulin) and glucose method (EBIO autoanalyser, Eppendorf), respectively. Five to 8 weeks after STZ treatment, the minipigs were killed by an intravenous injection of pentobarbital, and heart biopsies were collected.

mRNA Purification and cDNA Amplification
Cardiac biopsies were stored at −141°C (for mRNA analysis) or −80°C (for proBNP analysis). Total RNA was isolated with TRIzol (Life Technologies). The RNA integrity was assured by 1% agarose gel electrophoresis. First-strand cDNA was synthesized from 1 μg total RNA with M-MULV reverse transcriptase (40 U, Roche A/S). Parallel analyses of dilutions of heart cDNA pooled from 5 normal domestic pigs were used to determine the relation between the time point of the log-linear increase of the fluorescence signal and the concentration of an mRNA transcript. The mRNA copy numbers of BNP670 and BNP1479 in porcine heart were estimated by parallel real-time PCR analysis of purified BNP cDNA. BNP and β-actin mRNA contents of a cardiac biopsy were quantitated in duplicate; interassay coefficients of variation were 10% and 12%, respectively.

Porcine proBNP Antiserum
Peptides corresponding to the N-terminal decapeptide of porcine proBNP extended C-terminally with cysteine (for immunization) or tyrosyl (for tracer/standards) were synthesized (Cambridge Biochemicals). Their identities were verified by mass spectrometry and amino acid analysis. For immunization of white rabbits (Ssc: CPH-CRH strain; State Serum Institute, Copenhagen, Denmark), proBNP 1 to 10 Cys was coupled to bovine serum albumin, mixed with 0.9% NaCl, emulsified with complete Freund’s adjuvant (State Serum Institute), and injected subcutaneously. Booster injections were performed with proBNP 1 to 10 Cys emulsified in incomplete Freund’s adjuvant (State Serum Institute). Analysis of porcine heart extracts after chromatography as well as synthetic N-terminal truncated forms of proBNP peptide confirmed the specificity of porcine proBNP antiserum.

ProBNP Radioimmunoassay
Cardiac biopsies were minced on dry ice, immersed into boiling water for 30 minutes (10 mL/g tissue), homogenized with a polytron, and centrifuged at 10 000g for 30 minutes at 4°C. The pellets were resuspended in an equal volume of ice-cold acetic acid, left for 20 minutes at 20°C to 22°C, and centrifuged as above. For radioimmunoassay (RIA), proBNP1 to 10 Tyr was labeled with iodine 125 and purified by reversed-phase high performance liquid chromatography (Aquamure C-8 column, 222×4.6 mm).33 Heart extracts were incubated with the tracer and antiserum (1:50 000) for 3 days at 4°C. The antibody-bound and free fractions were separated by centrifugation after a 30-minute incubation with charcoal and human albumin. Radioactivity was determined with a Wizzard γ-counter (Wallac).

Immunohistochemistry
Paraformaldehyde-fixed sections (4 μm) were incubated anti-proBNP antiserum (1:100) or preimmune rabbit serum. Bound antibodies were visualized with antirabbit antiserum directly conjugated with fluorescein isothiocyanate (Dako). Sections were examined with confocal laser scanning microscopy (LSM510, Zeiss).

Statistics
Student 2-tailed t tests were used for 2-group comparisons. Values are mean±SEM.

Results
Two BNP Transcripts in Porcine Heart
A previous study described the presence of a 1479-bp-long BNP mRNA (BNP1479) in pig hearts,14 whereas another study described a 670-bp BNP mRNA (BNP670).15 BNP1479 is similar to BNP670 but contains 2 sequence insertions of 246 bp and 554 bp, respectively. The sequence insertions correspond to the sequences of the 2 introns of the BNP gene. The 2 BNP transcripts were detected and quantified with 2 sets of primers. One set of primers (BNP670A, 5′-GTGCTCTGCTCCTGTGTTCT-3′; BNP670B, 5′-TCCAGCGCTTGTTGAGG-3′) spans the 246-bp sequence insertion and amplifies a 197-bp fragment of BNP670, and a 443-bp fragment of BNP1479. The other set of primers (BNP670A, 5′-TGGACATCAGGAAGGACCTC-3′; BNP670B, 5′-ACCTGGACCACTACCTGTG-3′) amplifies a 207-bp fragment of the 246-bp sequence insertion in BNP1479. To exclude a contamination of the RNA preparations database contains 2 porcine BNP transcripts of 670 bp and 1479 bp (BNP 670 and BNP 1479 transcript differs from the BNP 670 transcript by 2 sequence insertions; reference Nos. M23596 and M25547, respectively). 14,15 The

LightCycler and DNAmaster SYBR Green kit (Roche A/S). Each PCR reaction contained cDNA synthesized from 20 or 200 ng total RNA. Parallel analyses of dilutions of heart cDNA pooled from 5 normal domestic pigs were used to determine the relation between the time point of the log-linear increase of the fluorescence signal and the concentration of an mRNA transcript. The mRNA copy numbers of BNP670 and BNP1479 in porcine heart were estimated by parallel real-time PCR analysis of purified BNP cDNA. BNP and β-actin mRNA contents of a cardiac biopsy were quantitated in duplicate; interassay coefficients of variation were 10% and 12%, respectively.
BNP, The other assay measured the copy number of BNP plus BNP670. BNP670 was 100 times more abundant than BNP in atrial myocardium, but only 10 to 20 times more abundant in ventricular myocardium (Figure 1C).

ProBNP Is Present Only in Atrial Myocardium

A second aim of the study was to assess possible variations among the regions of the normal porcine heart in BNP mRNA and proBNP concentrations. BNP670 mRNA levels were 10-fold higher in atrial tissue than in ventricular tissue of normal pigs (Figure 2A). ProBNP concentrations were similar in left and right atrium but undetectable in any of the ventricular biopsies (Figure 2B). The detection limit of the proBNP RIA was 0.5 pmol/g wet weight, i.e., 20-fold less than the average proBNP level in atrial biopsies. BNP expression is generally believed to be regulated by chronic pressure or volume overload. As the wall tension differs among different regions of the normal left ventricle, we subdivided the left ventricle of 4 porcine hearts into 9 segments. However, ProBNP was undetectable by RIA even in the segments of the left ventricle with highest wall stress.

Localization of proBNP in the Myocardium

Laser confocal microscopy was used to study the subcellular localization of proBNP within the normal porcine myocardium. Immunohistochemistry of atrial sections stained with a proBNP
specific antiserum revealed proBNP in the myocyte cytoplasm (Figure 3, LA). On high-power magnification, proBNP staining in atrial myocytes predominated in granules of 100 to 300 nm in diameter (Figure 3, AM). Stained granules were longitudinally orientated along the myofibrils (Figure 3, AM). In concordance with the RIA results, there was no proBNP staining in ventricular myocardium (Figure 3, LV).

Effects of Diabetes on BNP Expression in Porcine Heart
To examine how diabetes might affect cardiac BNP expression in porcine myocardium, we treated minipigs with a combination of nicotinamide and the pancreatic β-cell toxin STZ. Although STZ did not increase the fasting glucose levels significantly, 6 out of 12 minipigs developed a grossly abnormal plasma insulin and plasma glucose response after an oral glucose tolerance test (Figure 4A and 4B). Atrial BNP mRNA levels were ~2 times higher in STZ-treated minipigs with decreased insulin secretion capacity compared with STZ-treated minipigs with nearly normal insulin secretion capacity (P=0.03; Figure 4C). A similar trend was observed for atrial proBNP concentrations, although the difference between the 2 groups of minipigs was not statistically significant (Figure 4D). In the ventricle, BNP mRNA levels were similar in the 2 groups of minipigs, whereas proBNP was undetectable. In contrast to the finding in normal pigs, the BNP mRNA level was similar to or slightly lower in left atrial compared with left ventricular biopsies of STZ-treated minipigs.

Discussion
The present study revealed several novel aspects of BNP biology in normal and diabetic hearts. Thus, although the immature BNP 1479 transcripts constituted <1% of the mature BNP 670 transcripts in the atrium, they constituted up to 20% in the ventricle. Of note, the abundance of BNP 1479 mRNA was similar in atrial and ventricular myocytes. This might reflect that the transcription of the BNP gene occurs at similar rate in the 2 tissue types, but the turnover of the mature BNP 670 in the cytosol occurs more rapidly in normal ventricular myocytes. That possibility agrees with in vitro studies, suggesting that mRNA stabilization via an AUUUA motif in the 5′-untranslated region of the BNP mRNA affects BNP mRNA levels in cultured rat myocytes. It is also possible that the difference in BNP 1479 mRNA between atrial and ventricular myocardium reflects different nuclear export rates of BNP mRNA in the 2 tissues. Regulation of mRNA export from the cell nucleus can be an important determinant of gene expression, eg, in the case of the Rev protein and HIV-1 genes. The present results imply that further studies of BNP mRNA
trafficking and processing would be worthwhile to improve our understanding of how BNP mRNA levels are regulated.

The concentrations of proBNP differed markedly between atrial and ventricular myocardium as assessed both with RIA measurements and immunohistochemistry. Notably, this difference was observed both in normal domestic pigs and in STZ-treated minipigs. In contrast, the relative levels of BNP mRNA were markedly higher in the atrium than in the ventricle of normal pigs, whereas BNP mRNA levels were almost similar in atrium and ventricle of STZ-treated minipigs. The divergent results on the relative BNP mRNA levels in atrium and ventricle may reflect STZ-treatment; differences in age, strain, or diets; stress before removal of the heart; or a combination of ≥2 factors. Nevertheless, the observation that mRNA levels in atrium and ventricle were similar in STZ-treated minipigs while proBNP concentrations differed markedly indicates that cellular processing of proBNP differs in atrial and ventricular myocardium. This idea was further supported by results of laser confocal microscopy of proBNP stained sections of normal porcine hearts. We could detect proBNP in atrial myocytes in a granular pattern resembling what previously has been described for ANP. Because such granules are not found in normal ventricular myocytes and because proBNP was undetectable in ventricular myocardium, the results are compatible with the idea that ventricular proBNP normally is constitutively secreted (or perhaps rapidly degraded). Interestingly, BNP positive granules have been observed in ventricular myocytes of patients with cardiac failure, suggesting that the ventricular myocytes may acquire atrial myocyte-like features during the development of cardiac failure.

The present results suggest that mild diabetes (ie, decreased insulin secretion capacity on an oral glucose tolerance test) is associated with an increase in atrial but not ventricular BNP mRNA expression. This further supports the concept of a differential regulation of BNP gene expression in atrium and ventricle. A selective effect of STZ-induced diabetes on atrial BNP expression in minipigs is in agreement with a finding in dogs that early cardiac failure increased atrial BNP expression and that overt cardiac failure mainly affected ventricular function. Nevertheless, the lack of effect of STZ-treatment on ventricular BNP gene expression in minipigs was in apparent contrast to recent findings in STZ-treated rats. Whole-heart BNP mRNA levels of STZ-treated rats with blood glucose values of ≈35 mmol/L were increased 3- to 4-fold. In rats, BNP mRNA expression is higher in the ventricle than in the atrium, and the mass of the ventricles is much higher than the mass of the atria. Thus, these previous results reflect an effect of diabetes on ventricular BNP expression. In a separate study, we found no

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**Figure 4.** Effect of STZ-treatment on plasma glucose and insulin concentrations and heart BNP expression in minipigs. A and B, Plasma insulin (A) and glucose (B) after oral glucose (2 g/kg). STZ-treated minipigs were stratified into nonresponders, with nearly normal insulin and glucose profiles (n=6), and responders (n=6), with abnormal insulin and glucose profiles. Insulin and glucose profiles of normal minipigs that had not been treated with STZ are shown for comparison. C and D, BNP mRNA (C) and proBNP (D) content in left atrium (LA) and left ventricle (LV) in responding (filled bars) and nonresponding (open bars) minipigs. BNP mRNA values have been normalized with β-actin mRNA values. ND indicates nondetectable (ie, <0.5 pmol/g wet weight).
difference in ventricular BNP mRNA levels between STZ-treated mice with a median blood glucose of ≈ 16 mmol/L compared with nondiabetic control mice. However, the ventricular BNP mRNA level was significantly higher in STZ-treated mice with blood glucose > 16 mmol/L compared with mice with blood glucose < 16 mmol/L (C. Christoffersen and L.B. Nielsen, unpublished observations, 2002). Thus, although the data should be interpreted with caution because of possible species differences in cardiac response to STZ-induced diabetes, the available evidence may reflect that ventricular BNP gene expression is affected mainly at extremely high blood glucose concentrations. In contrast, atrial BNP gene expression may be affected even at very mild stages of diabetes.

With the prospect that plasma BNP measurements will gain wide use as a biochemical marker of cardiac dysfunction, it is pertinent to improve understanding on how BNP regulation and secretion from myocytes occur. This study suggests that BNP mRNA turnover and proBNP processing are different in atrial and ventricular myocardium. Moreover, the data suggest that diabetes can affect BNP gene expression in a cardiac chamber-dependent fashion.

Perspectives

Although this study illustrates that atrial myocytes store proBNP in secretory granules, the secretion of proBNP from ventricular myocytes is most likely constitutive. This implies that a disease state that confers increased BNP expression in the ventricle probably also confers increased plasma proBNP concentrations. This idea has been strongly supported by a recent observation in patients undergoing heart surgery, ie, that the plasma proBNP concentration is closely correlated with the ventricular BNP mRNA expression (J.P. Goetze and L.B. Nielsen, unpublished observations, 2002). It is more difficult to predict how an increase in atrial BNP expression may affect plasma proBNP and BNP concentrations. The present results imply that mild diabetes mainly affects atrial proBNP expression. Unfortunately, our porcine proBNP RIA was unsuited for plasma proBNP measurements. Thus, further studies are needed to resolve to what extent diabetes per se causes an increase in the plasma proBNP concentration or whether increased plasma proBNP concentration in a diabetic individual primarily reflects development of diabetic cardiomyopathy. Recent studies identified a novel cardiac serine protease, corin, that converts proANP and proBNP into ANP and BNP, respectively. Interestingly, corin might be expressed at a higher level in atrial than in ventricular myocytes. Thus, it is possible that the molecular forms of atrial- and ventricular-derived proBNP in plasma differ. Such differences could prove of diagnostic value, eg, in diabetes and mild cardiac failure, and extend the applications of plasma BNP measurements.

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