Light and Electron Microscopic Localization of Brain Natriuretic Peptide in Relation to Atrial Natriuretic Peptide in Porcine Atrium

Immunohistocytochemical Study Using Specific Monoclonal Antibodies

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Background. Although first isolated from the porcine brain, brain natriuretic peptide (BNP) is also found in the heart, particularly in the atria.

Methods and Results. We examined the immunocytochemical localization of BNP in relation to atrial natriuretic peptide (ANP) in five porcine atria using highly specific monoclonal antibodies. With the use of an indirect immunoperoxidase method, serial sections examined by light microscopy showed foci of multihormonal myocytes containing both ANP and BNP localized in the subendocardium. Monohormonal myocytes containing ANP only were observed in the subepicardium and part of the subendocardium. The staining intensity of ANP and BNP showed a transmural gradient from the subendocardium to the subepicardium. At the electron microscopic level, double immunocytochemistry using a two-face immunogold-staining method revealed two types of granules: Type 1 is a monohormonal granule containing ANP alone, and type 2 is a multihormonal granule containing both ANP and BNP. Although most atrial myocytes containing both, type 1 granules were frequently observed in the epicardial side, and type 2 granules were frequently observed in the endocardial side. We observed a few type 2 granules even in the light microscopically monohormonal myocytes.

Conclusions. These data suggest that the atrial myocyte population comprises single, multipotential cells able to synthesize both natriuretic peptides in varying proportions. The atrial transmural gradient of BNP and ANP may be related to responses to wall stress. (Circulation 1991;84:1203–1209)

In 1988, a new 26-amino-acid peptide showing a remarkable sequence homology to atrial natriuretic peptide (ANP) was isolated from the porcine brain.1 Designated “brain natriuretic peptide” (BNP), its pharmacological activity is similar to that of ANP, including diuretic-natriuretic, hypotensive, and vasorelaxant effects. This strongly suggests that in mammals, BNP and ANP act as a dual-control mechanism in body fluid and electrolyte homeostasis. ANP is a cardiac hormone secreted from the heart; its tissue distribution has been examined in both physiological and pathological states.2–6 Using a specific radioimmunoassay (RIA), BNP has also been found in the heart, particularly in the atria, although at concentrations of only 2% of those of ANP.7 However, we still have no light and electron microscopic data on the distribution of BNP.

We used highly specific monoclonal antibodies to immunocytochemically localize BNP in relation to ANP in the atria of the pig. At the light microscopic level, we used the indirect immunoperoxidase method and compared distribution in serial sections. At the electron microscopic level, we double-stained ANP and BNP with a two-face immunogold-staining method to determine whether BNP and ANP coexist in the same secretory granule.
Methods

Animals

The hearts of five adult pigs (100–120 kg) obtained from a slaughterhouse were dissected. For the light microscopic study, the left and right atrial walls were immediately cut transmurally. For the electron microscopic study, the atrial walls were immediately cut into small pieces.

Preparation and Characterization of Monoclonal Antibodies

α-Human ANP and porcine BNP were synthesized by the solid-phase method. The homogeneity of these peptides was confirmed by reverse-phase high-performance liquid chromatography and amino-acid analysis.

Porcine α-ANP is identical to human α-ANP.8 A monoclonal antibody to α-human ANP was prepared as previously described.9 The recognized epitope of the monoclonal antibody was located in the N-terminal half of the ring structure of α-human ANP including the Met12 residue. This RIA can recognize not only human α-ANP but also β-ANP with a cross-reactivity of 80% on a molar basis and γ-ANP on an equimolar basis, but cross reactivities with porcine BNP, BNP-32, and γ-BNP were less than 0.002%.

A monoclonal antibody to BNP has been produced by fusion of a nonproducing mouse myeloma cell line, X63-Ag8.653, with spleen cells from BALB/c mice (Shizuoka Animal Center, Shizuoka, Japan).10 These mice had been immunized with synthetic BNP conjugated to bovine thyroglobulin (Sigma Chemical, St. Louis, Mo.) using the carbodiimide (Nakarai Chemicals, Kyoto, Japan) coupling procedure. Hybridomas were screened for antibody production by RIA using culture media and [125I]BNP. They were cloned by the limiting dilution technique, expanded in culture, and injected intraperitoneally into BALB/c mice. Antibody obtained belonged to the immunoglobulin G1 subclass. Analysis by a Scatchard plot revealed high affinity for BNP, with an association constant of 2.0×1010 M⁻¹. Specific RIA with this antibody showed cross reactivity with porcine BNP-32 and γ-BNP of 33% on a molar basis, but cross reactivities with human α-ANP, β-ANP, and γ-ANP were less than 0.001%.10

Light Microscopic Immunohistochemistry

Samples were immediately immersed in chilled Zamboni’s fixative (2% paraformaldehyde and 0.2% picric acid in 0.1 mol/l phosphate buffer adjusted to pH 7.4) for 3 hours. After overnight washing at 4°C, the fixed specimens were dehydrated through graded alcohols and xylene and then embedded in paraffin. Serial 4-μm sections were made from each block. After dewaxing, every third section was stained with hematoxylin and eosin to assess general morphology. The other sections were alternately immunostained with antibodies to ANP and BNP. Immunohistochemical reactions according to an indirect immunoperoxidase method were performed as previously described.3,11 Briefly, the first step involved inhibition of intrinsic peroxidase activity by the addition of 0.3% H2O2 in 0.01 mol/l phosphate-buffered saline (PBS). Nonspecific binding was blocked with normal goat serum. As the primary antibody, the monoclonal antibody described above (dilution of the ascite, 1:1,000) was added to the sections for 48 hours at 4°C. In the second step, peroxidase-conjugated F(ab')2 fragment of the secondary antibody (goat antiserum IgG[H+L], Jackson Immunoresearch Laboratories) was added for 45 minutes at room temperature. Sections were then stained with 45 mg 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical) and 0.05% H2O2 in 100 ml of 0.05 mol/l Tris buffer solution (adjusted to pH 7.6) for 3 minutes at room temperature. Sections were washed four times (10 minutes each) with PBS between each step. Finally, counterstaining with hematoxylin was performed.

Double Immunocytochemistry at Electron Microscopic Level

After fixation in chilled Zamboni’s fixative for 3 hours, specimens were washed three times (15 minutes each) in PBS containing 10% sucrose at 4°C, dehydrated through graded ethanol, vacuum infiltrated with LR white resin, and polymerized at 50°C. The general morphology was assessed in semithin sections after toluidine blue staining to distinguish the endocardial side from the epicardial side. Thin sections were cut using a diamond knife on an ultramicrotome and mounted on bare 300-mesh nickel grids. For double staining, sequential immunogold staining of the two faces of the ultrathin section was performed according to Bendayan.12 In the first staining sequence, only one side of the section was exposed to the solutions. The grids were floated for 30 minutes, tissue sections facing down, on drops of PBS containing 3% normal goat serum before incubation with anti-BNP antibody (diluted 1:500) for 20 hours at 4°C. After washing in PBS, the sections were placed on drops of 10 nm gold-labeled goat antimouse IgG (diluted 1:20) (Janssen Life Product) for 1 hour and then washed in PBS, rinsed in distilled water, and dried. The grids were then turned over, and the other sides of the sections were labeled according to the same procedure using anti-ANP antibody (diluted 1:250) for 20 hours at 4°C and 5 nm gold-labeled anti-mouse IgG (diluted 1:20) (Janssen) for 1 hour at room temperature. Care was taken to not wet both sides of the grid with the same reagents. After completion of the incubation procedures, the grids were rinsed in PBS and distilled water and counterstained with uranyl acetate and lead citrate before examination with a Hitachi HU-12A electron microscope.

Controls

Although both monoclonal antibodies showed high degrees of specificity on RIA,3,10 control experiments were done to verify the specificity of the reaction in light and electron microscopic immunocytochemistry.
These included preabsorption of the primary antibody with the corresponding peptide antigen (1 nmol/peptide/ml diluted antiserum) and substitution of the primary antibody with nonimmune mouse serum. In addition, to exclude the possibility of cross reaction between ANP and BNP, the primary anti-
body was preabsorbed with heterologous antigen (1 nmol/ml).

The following controls were performed in double immunocytochemistry at the electron microscopic level to exclude the possibility of cross reaction between the first and second staining sequence: substitution of the first primary antibody with nonimmune serum or primary antibody absorbed with homologous antigen (1 nmol/ml) and substitution of the primary antibody with nonimmune serum or primary antibody absorbed with homologous antigen (1 nmol/ml) in the second run of the double-staining procedure.

**Results**

Control sections incubated with nonimmune mouse serum as a first antibody or with primary antibody preabsorbed with homologous antigen showed no specific immunoreaction at either the light or electron microscopic level. Furthermore, staining with antibody to BNP was not reduced by preabsorption with ANP, nor was the reverse true. Control study in double immunocytochemistry at the electron microscopic level also showed no cross reaction between the first and second staining sequences.

**Light Microscopic Immunohistochemistry**

ANP or BNP immunoreaction products were observed in the atrial myocytes but not in the endocardium, epicardium, or any of the connective tissues. Almost all atrial myocytes showed immunoreactivity for ANP, although staining intensity was slightly stronger in the endocardial (Figure 1A) than the epicardial side (Figure 1C). On the other hand, BNP was localized in the subendocardium of both atria (Figure 1B) and was rare in the epicardial side (Figure 1D). Positivity faded from the endocardium toward the epicardium (Figure 1B). The distribution of BNP in the subendocardium was not diffuse but formed foci of positive cells. Some myocytes of the subendocardium were positive for BNP (Figure 1B), and others were negative (Figure 1F). In the positive cell, BNP immunoreactive products (Figure 1H) resembling those of ANP (Figure 1G) were fine, granular, and dark brown, and strong concentrations were
located in the paranuclear region as well as the peripheral cytoplasm. On both ANP and BNP, the immunoreactivity was stronger in auricles than in posterior walls, but there was no significant difference between the left and right sides.

**Double Immunocytochemistry at Electron Microscopic Level**

Gold particles were concentrated on the myocytes’ secretory granules but not on other organelles. Most secretory granules were classified into two types according to their staining pattern. Type 1 is a monohormonal granule labeled only with small gold particles, indicating ANP (Figure 2A). Type 2 is a multihormonal granule containing both small and large gold particles, indicating ANP and BNP, respectively (Figure 2B). There were no differences between these granules in size or density. The labeling intensities of ANP in type 1 and 2 granules were relatively uniform, but that of BNP in type 2 granules varied (Figure 2C). Monohormonal granules containing BNP alone or nonimmunoreactive granules were observed in a very small population (approximately 1% of all granules). Although type 1 and 2 granules were both found in most atrial myocytes (Figure 2C), their ratios in different atrial tissue areas varied greatly. Most granules were type 1 on the epicardial side, whereas on the endocardial side, most were type 2, with type 1 predominating in some areas. Those type 2 granules occasionally seen on the epicardial side were less intensely labeled than those of the endocardial side, indicating a lower BNP content.

**Discussion**

We believe this is the first report on the immunohistochemical distribution of BNP in the atria. BNP has such a close homology to ANP in amino acid sequence that it is difficult to immunohistochemically differentiate them. However, we verified the high specificity of the monoclonal antibodies by control studies. Furthermore, the distribution of BNP was localized and heterogeneous, indicating that its antibody does not cross react with ANP.

**Subcellular Storage Patterns**

Double immunocytochemistry using two-face immunogold staining revealed two types of granules: monohormonal granules containing ANP alone (type 1) and multihormonal granules containing both ANP and BNP (type 2). Previously, specific heart granules were categorized as A, B, and D types on the basis of the usual ultrastructural appearance. However, Skepper and Navaratnam showed that these types did not represent different populations but rather different section planes through a uniform set of granules. The present study also found no ultrastructural differences between type 1 and 2 granules and thus supports the hypothesis of Skepper and Navaratnam.

The presence of multihormonal granules containing both ANP and BNP indicates that BNP is secreted with ANP. These data suggest that stimulators of ANP secretion, such as mechanical stretch of atrial myocytes, also stimulate BNP secretion.

Monohormonal granules containing BNP alone or nonimmunostained granules were observed in a very small population. However, this staining procedure has some disadvantages. First, the antigenic determinants may have been inaccessible, slightly denatured, or partially extracted during the fixation-and-embedding process. Second, for a secretory granule to be double stained, antigenic sites must be available at both sides of the section. If antigenic sites are exposed on only one side, a false impression of single staining will be obtained. Therefore, there will be fewer monohormonal or nonimmunostained granules than actually immunocytochemically observed. Thus, BNP monohormonal or nonimmunoreactive granules may be only methodological artifacts, although we cannot rule out their presence.

**Histological Immunostaining Patterns**

Using an indirect immunoperoxidase method, serial sections examined by light microscopy showed foci of multihormonal myocytes containing both ANP and BNP distributed in the subendocardium. Monohormonal myocytes containing ANP alone were found in the subepicardium and part of the subendocardium. Their staining intensity showed a transmural gradient from the subendocardium to the subepicardium. The immunoelectron microscopic findings of type 1 granules in myocytes of the epicardial side and type 2 granules in myocytes of the endocardial side are consistent with those of light microscopy. However, we observed type 2 granules even in light microscopically monohormonal myocytes of the epicardial side, although their numbers were small. This discrepancy may be due to higher resolutions in the gold technique than in light microscopic immunohistochemistry. These data support the idea that the atrial myocyte population comprises single, multipotential cells that can synthesize ANP and BNP at varying ratios. Wall stress is stronger in the endocardial than the epicardial side. Thus, their transmural staining gradient may be explained by responses to wall stress during development. However, this does not explain the variability of BNP distribution in the subendocardium.

Recently, we reported that the plasma levels of ANP and BNP increase together in patients with congestive heart failure but that the increase was more prominent in BNP than in ANP. In such pathological conditions, ANP increases not only in the atria but also in the ventricles, where its secretion markedly decreases after birth. BNP amino acid sequence varies greatly among animal species. Thus, further studies are needed on changes in the ratio and distribution of type 1 and 2 granules and monohormonal and multihormonal myocytes in failing human and animal hearts.

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References

23. Mirsky I: Left ventricular stresses in the intact human heart. Biophys J 1969:918

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