Vol. 177, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1991 Pages 40-47

CONCENTRATION AND MOLECULAR FORMS OF BRAIN NATRIURETIC PEPTIDE IN RAT PLASMA AND SPINAL CORD

Masahito ABURAYA[1,2], Etsu SUZUKI[1], Naoto MINAMINO[3], Kenji KANGAWA[1], Kenjiro TANAKA[2] and Hisayuki MATSUO[3*]

[1]Department of Biochemistry and [2]Department of Internal Medicine, Miyazaki Medical College, Kihara, Kiyotake, Miyazaki 889-16, Japan

[3]National Cardiovascular Center Research Institute, Fujishirodai, Suita, Osaka 565 , Japan

Received April 15, 1991

SUMMARY: To characterize the biological functions of rat brain (B-type) natriuretic peptide (BNP), which has been shown to be present mainly in the heart and only faintly in the spinal cord, the concentration and molecular forms of BNP in plasma and spinal cord were determined. The concentration of immunoreactive (ir-) BNP was 2.00 fmol/ml in normal rat and 13.29 fmol/ml in morphine-treated rat, being respectively about 1/20 and 1/80 those of ir-atrial (A-type) natriuretic peptide (ANP). In morphine-treated rats, ir-BNP was shown to circulate mainly as BNP-45 , which is identical to a major storage form found in cardiac atrium. In the spinal cord, BNP was also shown to be present as BNP-45, but its concentration was only 0.057 pmol/g, being about 1/60 that of spinal cord ANP. These results confirm that BNP mainly functions as a circulating hormone in the molecular form of BNP-45. Morphine stimulates secretion of ANP and BNP but by different ratios, suggesting different regulation systems for storage and secretion of ANP and BNP.

Our recent cloning and sequence analysis of cDNAs encoding rat and human BNP precursors have shown that BNP is also widely distributed in mammals, although amino acid sequences of mammalian BNPs are not as homologous as those of mammalian ANPs (1-4). Using a radioimmunoassay (RIA) established for rat BNP, we measured tissue concentration of ir-BNP in rat central nervous system, heart and other peripheral tissues (5). However, ir-BNP was not detected in brain and only at an extremely low concentration in spinal cord, while a high concentration of ir-BNP was found in heart, especially in atrium. This fact suggests that BNP mainly functions as a circulating hormone secreted from heart rather than as a neuropeptide in the central nervous system. In this study, we measured the concentration and molecular forms of ir-BNP in rat plasma and spinal cord, and compared them with those of

*To whom correspondence should be addressed.

Abbreviations: BNP, brain (B-type) natriuretic peptide; ANP, atrial (A-type) natriuretic peptide; ir, immunoreacitve; RIA, radioimmunoassay; TFA, trifluoroacetic acid; HPLC, high performance liquid chromatography; i.p., intraperitoneal(ly); IgG, immunoglobulin G; MW, molecular weight. γ -BNP, pro-BNP; BNP-45, pro-BNP[51-95]; BNP-32, pro-BNP[64-95] in the rat BNP system.

0006-291X/91 $1.50

ir-ANP to help elucidate physiological functions of BNP. Since the plasma concentration of ir-BNP was assumed to be extremely low, we treated the rats with morphine, which is known to increase plasma ir-ANP concentration, to obtain plasma of higher ir-BNP concentration (6-8).

MATERIALS AND METHODS

Peptides: Rat BNP-26, N-Tyr-BNP-26 and BNP-32 were synthesized with a peptide synthesizer (Applied Biosystems, 430A) in our laboratory. Rat BNP-45 was kindly donated by Dr. K. Inouye of Shionogi Research Laboratories.

Preparation of plasma samples and spinal cord extracts: Plasma samples were collected from male Wistar rats (10 weeks old; body weight, 350-380 g). Morphine sulfate (10 mg/ml in 0.9% saline) was injected intraperitoneally (i.p.) into some of the rats (30 mg/kg), and 0.9% saline was injected into other rats that served as the control animals. For time-course experiments, 5 morphine-treated rats and 6 groups of control rats (3 rats in each group) were decapitated at 0, 15, 30, 60 and 120 min. For measurements of plasma concentration of ir-BNP and ir-ANP, 8 morphine-treated rats and 6 control rat groups were decapitated at 30 min. Whole blood was collected in centrifuge tubes containing 1/10 volume of 1.5% EDTA and 5,000 KIU/ml aprotinin. Clear plasma was obtained by centrifuging the whole blood twice at 3,000 rpm for 10 min at 4 °C . Each plasma was loaded onto a Sep-pak C-18 cartridge (2.5 ml, Waters), and the cartridge was thoroughly washed with 0.1% trifluoroacetic acid (TFA). The adsorbed materials on the cartridge were then eluted with 60% CH_3CN in 0.1% TFA. A portion of each eluate was submitted to RIA for ANP. The remaining portion was subjected to anti-BNP IgG immunoaffinity chromatography (column volume: 100 μl). The adsorbed materials on the affinity column were treated with Sep-pak C-18 cartridges and then submitted to RIA for BNP. Rat spinal cord (cervical to sacral portion) was collected from 40 male Wistar rats. Spinal cord tissue was diced and boiled for 10 min in 10 volumes of water, and then homogenized with a Polytron mixer after addition of glacial acetic acid (final concentration = 1.0 M). Aliquots of the supernatants, obtained by centrifugation at 16,000 x *g* for 30 min, were treated with Sep-pak C-18 cartridges, and then submitted to RIA for BNP and ANP.

Immunoaffinity chromatography: An anti-BNP IgG immunoaffinity column was prepared as reported (9). In brief, the IgG fraction of antiserum #179-3 was coupled with AFFIGEL-10 (Bio-Rad) according to the product manual. Samples dissolved in 0.1M sodium phosphate buffer (pH 7.4) containing 0.001% Triton X-100 were loaded onto the column. After washing the column with the same buffer, the adsorbed materials were eluted with 1M acetic acid containing 10% CH_3CN and 0.002% Triton X-100.

Characterization of ir-BNP and ir-ANP in rat plasma and spinal cord: From 93.5 ml of morphine-treated rat plasma, the peptide fraction was extracted with Sep-pak C-18 cartridges. The peptide fraction was purified by anti-BNP IgG immunoaffinity chromatography (column volume, 500 μl), and the adsorbed peptides were then subjected to Sephadex G-50 gel filtration (fine, 1.2 x 104 cm). The peptide fraction of the spinal cord extracts was also concentrated with reverse phase C-18 column (Chemco LC-SORB SPW-C-ODS, 15 ml) and then separated by Sepahdex G-50 column (fine, 1.8 x 134 cm). Aliqouts of fractions from each chromatography were submitted to RIA for BNP and ANP. The fractions exhibiting ir-BNP were further analyzed by reverse phase high performance liquid chromatography (HPLC) and CM ion exchange HPLC. The reverse phase HPLC was performed on a Hi-Pore RP-318 column (4.6 x 250 mm, Bio-Rad) with a linear gradient elution of CH_3CN from 10% to 60% in 0.1% TFA at a flow rate of 1.0 ml/min, while the ion exchange HPLC was carried out on a TSK gel CM-2SW column (4.6 x 250 mm, Tosoh) with a linear gradient elution of $HCOONH_4$ (pH 6.6) from 10mM to 0.6M in the presence of 10% CH_3CN. Aliquots of all fractions were submitted to RIA for BNP.

RIAs for BNP and ANP: RIAs for rat BNP and ANP were performed as reported previously (5,10). Antiserum #179-3 against rat BNP-26 showed 0.002% crossreactivity with rat α-ANP when used at a final dilution of 1:480,000, and peptides were measurable in the range of 2~150 fmol/tube. In the RIA for rat ANP, BNP-26 showed less than 0.0003% crossreactivity.

Statistical analyses: One way analysis of variance (ANOVA) followed by the Bonferroni method was used for time-course comparison of the plasma concentrations of ir-BNP and

ir–ANP. Linear regression was evaluated by the least–squares method. Data are represented as the mean ± standard error of the mean (s.e.m.), and a level of $p<0.01$ was considered to be significant.

RESULTS AND DISCUSSION

Since the plasma concentration of ir–BNP was assumed to be very low, we pre–treated the rats with morphine, which is known to increase the plasma ir–ANP concentration. Although the mechanism for the increase of plasma ir–ANP concentration by i.p. injection of morphine has not been well–elucidated, this reaction is thought to be mediated by opioid receptors and not by direct action on the heart (11,12). Figure 1**a** shows the time–course of the plasma concentration of ir–BNP and ir–ANP after i.p. injection of morphine (30 mg/kg). Plasma concentrations of both ir–BNP and ir–ANP had already increased significantly at 15 min, reaching a maximum at 30 min, in a similar profile; and the ir–BNP concentration at 30 min was 6~7 times higher than that at 0 min. Thus, morphine treatment was shown to be useful for preparing rat plasma containing a higher concentration of ir–BNP.

The peptide fraction obtained from 93.5 ml of morphine–treated rat plasma was first purified by immunoaffinity chromatography and then subjected to Sephadex G–50 gel filtration. As shown in Fig. 2, most ir–BNP (more than 75%) emerged at an elution position of BNP–45, corresponding to a MW of 5K, along with a minor peak at the MW of 3K. Fractions **a** (fractions #35–39) and **b** (fractions #40–44) were each pooled and subjected to reverse phase HPLC on a C–18 column. The ir–BNP of fraction **a** emerged at an elution time identical to that of BNP–45, while the ir–BNP in fraction **b** was detected at the elution time of BNP–32 (Figs. 3**a** and 3**b**). In CM ion exchange HPLC, the ir–BNP in fraction **a** was observed completely as a single peak at the elution time of BNP–45 (Fig. 3**c**). On the other hand, the ir–BNP in fraction

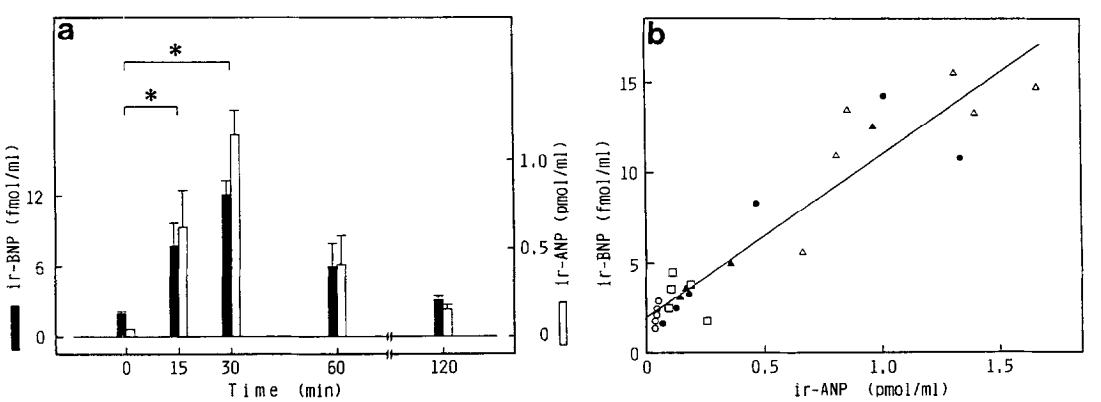

Figure 1. (**a**) Time course of plasma ir–BNP and ir–ANP concentrations after intraperitoneal injection of morphine into rats. *) $p<0.01$.
(**b**) Relationship between ir–BNP and ir–ANP concentrations in morphine–treated rat plasma. ○— 0 min, ●— 15 min, △— 30 min, ▲— 60 min, □— 120 min.

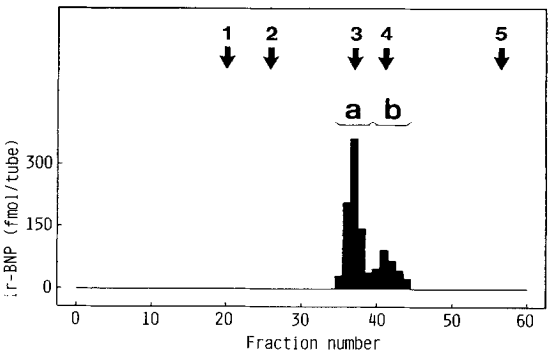

Figure 2. Sephadex G–50 gel filtration of morphine–treated rat plasma.
Sample: Morphine–treated rat plasma (93.5 ml equivalents). Details of the sample preparation method are described in Methods. Solvent system: 1M acetic acid. Column: 1.2 x 104 cm, Sephadex G–50, fine. Fraction size: 2 ml/tube. Flow rate: 4 ml/hr. Arrows indicate elution positions of 1) Vo, 2) γ–BNP, 3) BNP–45, 4) BNP–32 and 5) Vt, respectively.

b was separated into at least 2 peaks and only a portion of the ir–BNP emerged at the time of BNP–32 (Fig. 3**d**). Based on these results, ir–BNP was mainly present as BNP–45 in morphine–treated rat plasma, and the remaining portion was deduced to be a mixture of secondary products including BNP–32.

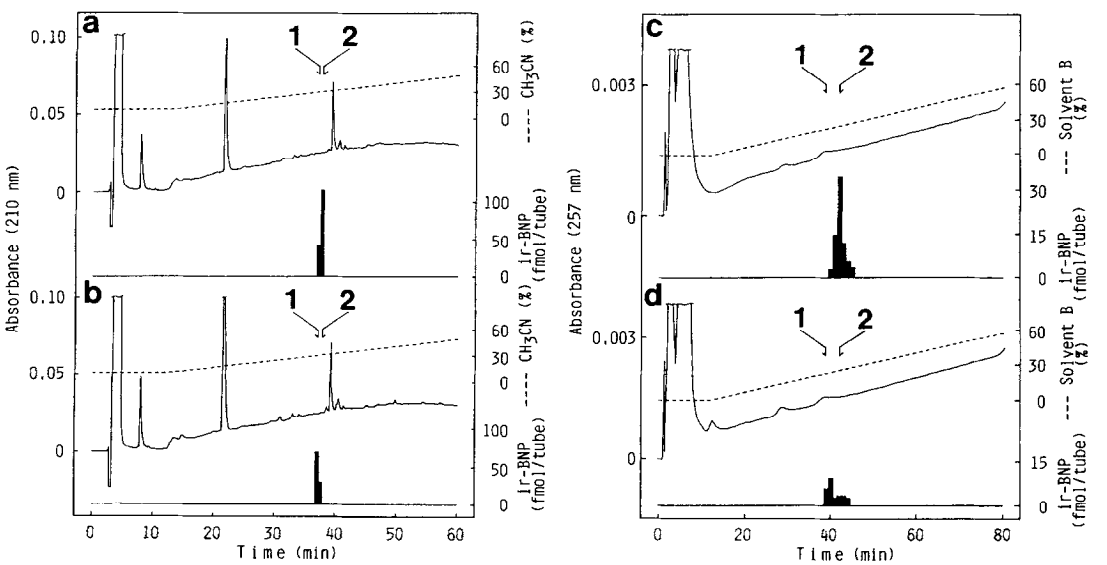

Figure 3. Reverse phase HPLC (**a,b**) and CM ion exchange HPLC (**c,d**) of morphine–treated rat plasma.
Sample: **a,c**) Fraction **a** (fractions #35–39, 0.8 ml/tube) and **b,d**) fraction **b** (fractions #40–44, 0.8 ml/tube) in Fig. 1. Column: **a,b**) Hi–Pore RP–318 (4.6 x 250 mm, Bio–Rad). **c,d**) TSK gel CM–2SW (4.6 x 250 mm, Tosoh). Flow rate: 1.0 ml/min. Solvent system: **a,b**) Linear gradient elution of CH_3CN in 0.1% TFA from 10% to 60% over 60 min. **c,d**) Linear gradient elution of $HCOONH_4$ (pH 6.6) from 10mM to 0.6M in the presence of 10% CH_3CN over 72 min.
Arrows indicate elution positions of 1) BNP–32 and 2) BNP–45.

Molecular forms of ir–ANP in control and morphine–treated rat plasma were characterized by the same method. In reverse phase HPLC and CM ion exchange HPLC, most of the ir–ANP emerged at the elution time of α–ANP, and no apparent difference was observed between ir–ANP in the control and morphine–treated groups (data not shown), suggesting that the molecular form of ir–BNP in control rat plasma was identical to that of morphine–treated rat plasma.

Plasma concentrations of ir–BNP was then measured after condensation and desalting rat plasma with reverse phase C–18 cartridges and immunoaffinity columns. Recovery yields of this condensation method were estimated to be about 65% by adding synthetic BNP–45 (5.0 fmol/ml) to fresh plasma. Table 1 summarizes the plasma concentration of ir–BNP and ir–ANP in the control and morphine–treated rats. In the control rats, the ir–BNP concentration was 2.00 $\pm$ 0.19 fmol/ml and that of ir–ANP was 45.10 $\pm$ 2.33 fmol/ml. Injection of morphine into conscious rats increased the plasma concentration of ir–ANP about 20 times and that of ir–BNP about 7 times at 30 min after injection. In Fig. 1**b**, the plasma concentrations of ir–BNP and ir–ANP in the control and morphine–treated rats at all sampling times are compared. An apparent correlation (r=0.94) was observed between plasma concentrations of ir–BNP and ir–ANP. These results demonstrated that morphine simultaneously stimulates BNP and ANP secretion from the heart at definite ratios.

In cardiac atrium, ir–BNP and ir–ANP exist at a ratio of 1:140 (5). In plasma, the ratio increased to 1:20 in the control rats and 1:80 in the morphine–treated rats. Although we have to consider the turnover rates of BNP and ANP as well as the contribution of cardiac ventricle, the other major source of plasma ir–BNP, these ratios suggest that BNP is not stored in tissue in a high concentration, but is secreted rather continually into the blood stream, while ANP is stored

Table 1. Concentration of immunoreactive BNP and ANP in rat plasma

	control		morphine–treated	
group No.	ir–BNP (fmol/ml)	ir–ANP (fmol/ml)	ir–BNP (fmol/ml)	ir–ANP (fmol/ml)
1	2.32	46.53	12.98	1353
2	1.29	38.29	14.75	1302
3	1.66	39.05	10.80	1062
4	1.99	45.30	15.00	1057
5	2.80	55.58	12.72	992
6	1.96	45.87	13.58	1124
7			13.73	1155
8			12.83	693
mean $\pm$ s.e.m.	2.00 $\pm$ 0.19	45.10 $\pm$ 2.33	13.29 $\pm$ 0.43	1092 $\pm$ 67

Plasma concentrations of ir–BNP and ir–ANP in morphine–treated rat were measured at 30 min after i.p. injection of morphine (30 mg/kg body weight).

in the atrium at a relatively high ratio and secreted in response to acute stimuli such as morphine injection. Although both BNP and ANP are secreted into plasma by morphine injection, these two natriuretic peptides may have distinct mechanisms of production, storage and secretion, which suggests separate physiological roles for ANP and BNP.

In the rat central nervous system, a significant concentration of ir-BNP was detected only in the spinal cord (5,13); thus, we characterized the ir-BNP of the spinal cord. In Sephadex G-50 gel filtration, ir-BNP in the spinal cord extracts was observed as a single peak in fractions #40-43, corresponding to a MW of 5K , while ir-ANP emerged as two peaks with MWs of 13K and 3K (Fig. 4a). Fractions #40-43 from the gel filtration column were then analyzed by reverse phase HPLC and CM ion exchange HPLC. BNP immunoreactivity was eluted as a single peak at the elution time of BNP-45 in the two different HPLC systems, and no other immunoreactivity was detected by either HPLC (Fig. 4b). On the other hand, high-MW ir-ANP was identified as γ-ANP, and low-MW ir-ANP was found to be a mixture of α-ANP[4-28], α-ANP[5-28] and α-ANP. In the present study, the tissue concentration of

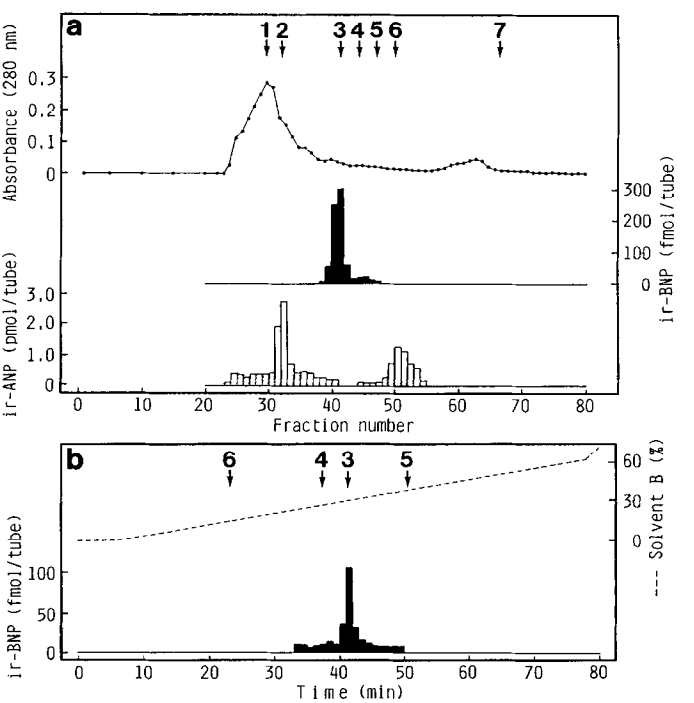

Figure 4. (a) Sephadex G-50 gel filtration of rat spinal cord extracts.
Sample: Rat spinal cord extracts (10 g equivalents). Fraction size: 5 ml/tube.
Column: 1.8 x 134 cm, Sephadex G-50, fine. Solvent: 1M acetic acid. Flow rate: 6 ml/hr.
Arrows indicate elution positions of 1) Vo, 2) γ-ANP, 3) BNP-45, 4) BNP-32,
5) α-ANP, 6) α-ANP[4-28] and 7) Vt, respectively.
(b) CM ion exchange HPLC of ir-BNP of 5K-MW.
Sample: Fractions #40-43 (2.37 ml/tube) in (a). Chromatographic conditions were identical to those in Figs. 3c and 3d. Arrows are the same as in (a).

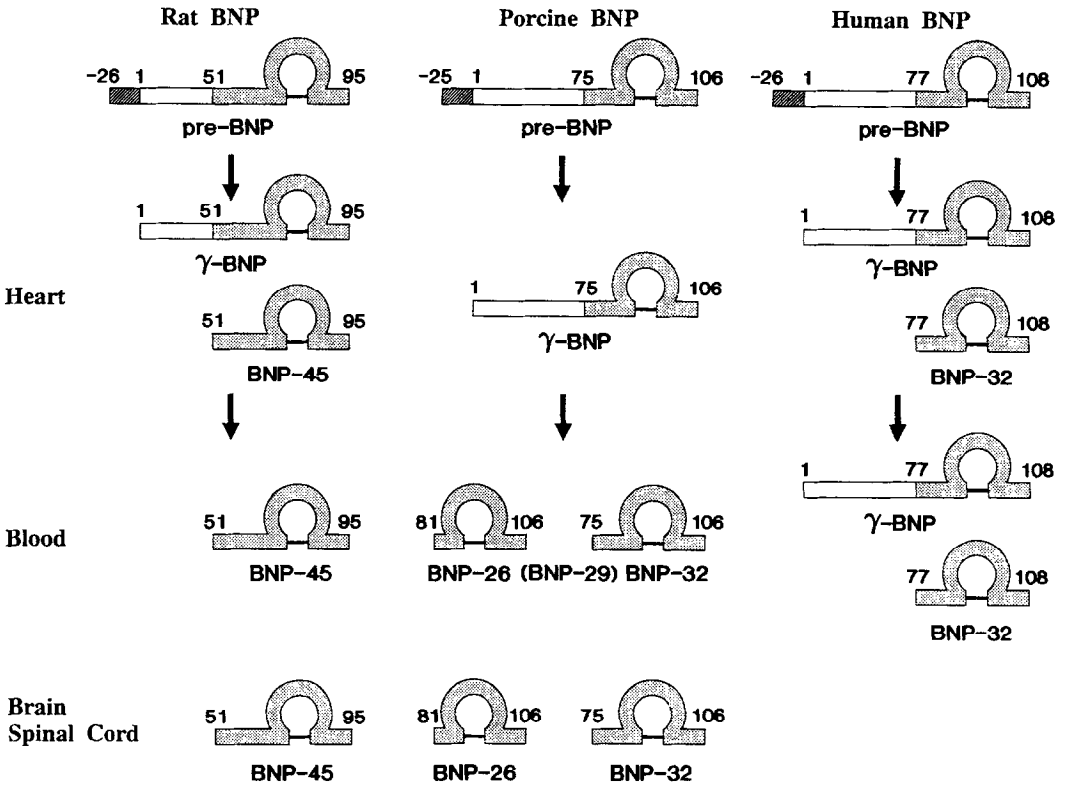

Figure 5. Processing and biosynthetic pathways of rat, porcine and human BNPs.

ir-BNP was re-measured and determined to be 57 fmol/g wet wt, which was only 1/50 that of ir-ANP in the spinal cord. Thus, the neuronal function of rat BNP should be very limited as compared to that of rat ANP.

As summarized in Fig. 5, rat BNP is mainly present as BNP-45 in the cardiac atrium, plasma and spinal cord. Only in the cardiac ventricle is γ-BNP a major form, where it is present at a concentration a little higher than that of BNP-45 (5,14). These data indicate that BNP functions as the molecular form of BNP-45 in rats. In the case of pigs, porcine BNP is stored mainly as γ-BNP in the heart and circulates in the blood stream as a mixture of BNP-32, BNP-29 and BNP-26 (15). In the central nervous system, BNP-32 and BNP-26 are the major molecular forms, and no other molecular form has been found (16). Human BNP is thought to function as BNP-32, since no other low-MW form of BNP has been detected so far (17,18). In addition to BNP-32, however, high MW γ-BNP is present in the heart and probably in plasma. Based on the data summarized in Fig. 5, it is concluded that the proteolytic processing system of mammalian BNP is different in each species and that BNP functions as BNP-45 in rats, but as BNP-32 and/or BNP-26 in pigs and humans. This is in sharp contrast to mammalian ANP that has an extremely uniform processing system; i.e., ANP functions as α-ANP in the circulation system and as α-ANP[4-28] and α-ANP[5-28] in the central nervous

system. So far, nothing is known about differences in biological activity between rat BNP–45 and rat BNP–32, although mammalian BNP has been found to show very different potencies in different bioassays (19). Whether interspecies differences in the molecular form of BNP are derived from specificity of the BNP receptor of each species or just from differences in the amino acid sequence of BNP precursors has yet to be elucidated.

Acknowledgments: The authors are grateful to Dr. H. Masuda of Hyogo College of Medicine for comments on the manuscript. This work was supported in part by research grants from the Ministry of Education, Science and Culture and the Ministry of Health and Welfare of Japan.

REFERENCES

1. Sudoh, T., Kangawa, K., Minamino, N. & Matsuo, H. (1988) Nature, 332, 78–81.
2. Maekawa, K., Sudoh, T., Furusawa, M., Minamino. N., Kangawa, K., Ohkubo, H., Nakanishi, S. & Matsuo, H. (1988) Biochem. Biophys. Res. Commun., 157, 410–416.
3. Sudoh, T., Maekawa, K., Kojima, M., Minamino, N., Kangawa, K. & Matsuo, H. (1988) Biochem. Biophys. Res. Commun., 159, 1427–1434.
4. Kojima, M., Minamino, N., Kangawa, K. & Matsuo, H. (1989) Biochem. Biophys. Res. Commun., 159, 1420–1426.
5. Aburaya, M., Minamino, N., Hino, J., Kangawa, K. & Matsuo, H. (1989) Biochem. Biophys. Res. Commun., 165, 880–887.
6. Gutkowska, J., Racz, K., Garcia, R., Thibault, G., Kuchel, O., Genest, J. & Cantin, M. (1986) Eur. J. Pharmacol., 131, 91–94.
7. Crum, R.L. & Brown, M.R. (1988) Life Sci., 43, 851–858.
8. Masuda, H. (1988) Acta Medicica Hyogonensia, 13, 11–20.
9. Sudoh, T., Minamino, N., Kangawa, K. & Matsuo, H. (1988) Biochem. Biophys. Res. Commun., 155, 726–732.
10. Miyata, A., Kangawa, K. & Matsuo, H. (1986) J. Hypertens, 4 (Suppl. 2) S9–S11.
11. Stasch, J., Grote, H., Kazdu, S. & Hirth, C. (1989) Eur. J. Pharmacol., 159, 101–102.
12. Vollmar, A.M., Ardendt, R.M. & Schulz, R. (1987) Eur. J. Pharmacol., 143, 315–321.
13. Ogawa, Y., Nakao, K., Mukoyama, M., Shirakami, G., Itoh, H., Hosoda, K., Saito, Y., Arai, H., Suga, S., Jougasaki. M., Yamada, T., Kambayashi, Y., Inouye, K. & Imura, H. (1990) Endocrinology, 126, 2225–2227.
14. Aburaya, M., Minamino, N., Hino, J., Kangawa, K. & Matsuo, H. (1989) Biochem. Biophys. Res. Commun., 163, 226–232.
15. Aburaya, M., Minamino, N., Kangawa, K., Tanaka, K. & Matsuo, H. (1989) Biochem. Biophys. Res. Commun., 165, 872–879.
16. Ueda, S., Minamino, N., Sudoh, T., Kangawa, K. & Matsuo, H. (1988) Biochem. Biophys. Res. Commun., 155, 733–739.
17. Tateyama, H., Hino, J., Minamino, N., Kangawa, K., Ogihara, T. & Matsuo, H. (1990) Biochem. Biophys. Res. Commun., 166, 1080–1087.
18. Hino, J., Tateyama, H., Minamino, N., Kangawa, K. & Matsuo, H. (1990) Biochem. Biophys. Res. Commun., 167, 693–700.
19. Kambayashi, Y., Nakao, K., Kimura, H., Kawabata, T., Nakamura, M., Inouye, K., Yoshida, N. & Imura, H. (1990) Biochem. Biophys. Res. Commun., 173, 599–605.