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RADIOIMMUNOASSAY FOR BRAIN NATRIURETIC PEPTIDE (BNP)

DETECTION OF BNP IN CANINE BRAIN

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SUMMARY: We established a highly sensitive and specific radioimmunoassay (RIA) for BNP. Our RIA detected BNP-like immunoreactivity (-LI) in the porcine and canine brains but did not detect BNP-LI in the human, monkey or rat brain. The widespread distribution of BNP-LI was demonstrated both in the porcine and canine brains, with the highest concentration in the medulla oblongata. In contrast, the highest concentration of ANP-LI determined simultaneously was in the midbrain and the olfactory bulb. High performance-gel permeation chromatography coupled with RIA revealed that the major component of BNP-LI was eluted at the position of synthetic BNP with a small molecular weight (3K). These results indicate that the RIA for BNP serves as a useful tool to investigate physiological roles of BNP.

Matsuo and his colleagues have recently isolated a novel natriuretic peptide from the porcine brain, which has 26 amino acid residues with an intramolecular disulfide linkage and shows a remarkable sequence homology to atrial natriuretic polypeptide (ANP) but is distinct from it (1). The authors designated the peptide 'brain natriuretic peptide (BNP)'. They also isolated a novel peptide

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Abbreviations used in this paper: BNP, brain natriuretic peptide; ANP, atrial natriuretic polypeptide; RIA, radioimmunoassay; -LI, -like immunoreactivity; HP-GPC, high performance-gel permeation chromatography.

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with 32 amino acids, carrying a BNP structure at the C-terminus. They designated this N-terminally extended form of BNP with 6 amino acids as BNP-32 and demonstrated that BNP and BNP-32 are the major forms of BNP family in the porcine brain (2).

Using a radioimmunoassay (RIA) specific for ANP and immunohistochemical methods, we demonstrated that ANP is a hormone not only secreted from the heart but also found in the brain (3). The major components of ANP in the rat brain were further shown to be N-terminally deleted forms of α-rat ANP (α-rANP), α-rANP[4-28] and α-rANP[5-28] (4). We and others have demonstrated that the intracerebroventricular (i.c.v.) administration of ANP suppresses water intake (5-7), salt appetite (8), pressor response (9) and secretion of vasopressin and ACTH (10,11). We also reported the inhibitory effect of the i.c.v. injection of ANP on central angiotensin II (AII)-induced ANP secretion from the heart (12,13). These results suggest an antagonistic relationship between ANP and renin-angiotensin systems in the central nervous system, as well as in the periphery, affecting the modulation of body fluid and blood pressure homeostasis (14,15).

More recently, we demonstrated that the i.c.v. administration of BNP exhibited the equipotent inhibitory actions on central AII-induced water intake (16), pressor response (17) and vasopressin secretion (18), when compared to ANP. These findings raise the possibility that central actions of ANP are shared by BNP.

In the present study, we have established a highly sensitive and specific RIA for BNP and examined BNP in the brain in several species.

MATERIALS AND METHODS

Peptides

BNP was synthesized by the solid phase method (1,7). BNP-32 (2) and BNP[17-26] were delivered by Shionogi Laboratories, Osaka, Japan. α-human ANP (α-hANP) and α-rANP were purchased from Protein Research Foundation, Osaka, Japan and Peninsula Laboratories Inc., Belmont, CA, USA, respectively. The homogeneity of these peptides was confirmed by reverse phase-high performance liquid chromatography and amino acid analysis.

Conjugation and immunization of BNP

BNP (50 mg) was conjugated to bovine thyroglobulin (18.3 mg, Sigma Chemical Co., St. Louis, MO, USA) using the carbodiimide coupling procedure as previously described (19). Conjugated BNP (15 μg) was emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI, USA) and injected, initially, intraperitoneally and subcutaneously, and then boosted by subcutaneous injections to ten BALB/c mice at the interval of 2-4 weeks.

Iodination

BNP was radioiodinated by the chloramine T method as previously described (19). Labeled BNP was purified by applying the reaction mixture to a Sep-Pak C_{18} cartridge (Waters Associates Inc., Milford, MA, USA) and eluting the labeled peptide with a solution of 50 % acetonitrile in 5 mM trifluoroacetic acid (TFA). The specific activity of [^{125}I]BNP ranged from 400 to 700 μCi/μg.

RIA for BNP

RIA for BNP was performed following the method of RIA for ANP as previously described (19). The RIA incubation mixture consisted of 100 μl of standard or sample, 100 μl of a final 1:20,000 dilution of antiserum (F-8), 100 μl of [^{125}I]BNP (approximately 10,000 cpm) and 200 μl of the standard buffer. The antiserum and standard or sample were incubated for 24 hours at 4°C, after which [^{125}I]BNP was added and incubated for additional 24 hours at 4°C. Bound and free ligands were separated by adding 1.0 ml of a suspension of dextran-coated charcoal consisting of 250 mg of Norit SX Plus (Norit, Holland) and 25 mg of Dextran T-70 (Pharmacia Fine Chemicals, Uppsala, Sweden) in 0.05 M phosphate buffer (pH 7.4).

RIA for ANP

ANP levels were measured by a RIA that recognizes the C-terminal sequence of α-ANP, as described previously in detail (19). The cross-reactivity of BNP in the RIA was less than 0.02 %.

Brain tissues and extraction procedure

Brains of pigs (n=4), dogs (n=4), rats (n=3), monkeys (n=2) and humans (n=2) were studied. Canine brains were obtained from neonatal Beagles. Brains were dissected on ice at 4°C into 11 regions referring to the rat brain dissection method of Glowinski and Iversen (3). The dissected tissue samples were weighed, frozen in liquid nitrogen and stored at -70°C until extraction. Brain tissues were extracted in 10 volumes of 0.1 M acetic acid, as described previously in detail (3,4).

High performance-gel permeation chromatography (HP-GPC)

HP-GPC was performed on a TSK-GEL G2000 SW (Toyo Soda, Tokyo, Japan) column (7.5 x 600 mm) and eluted with 10 mM TFA containing 0.3 M sodium chloride and 30 % acetonitrile, as a solvent (3,4,19). The flow rate was 0.3 ml/minute and the fraction volume was 0.36 ml. The column was calibrated with a polypeptide molecular weight calibration kit (Pharmacia Fine Chemicals, Uppsala, Sweden) and synthetic BNP.

RESULTS AND DISCUSSION

RIA for BNP

A typical standard curve of BNP in the RIA using a mouse antiserum against BNP, F-8, is shown in Figure 1. The minimal detectable quantity in the RIA was as little as 0.5 pg(0.17 fmol)/tube of synthetic BNP and the 50 % binding intercept was 15 pg(5 fmol)/tube.

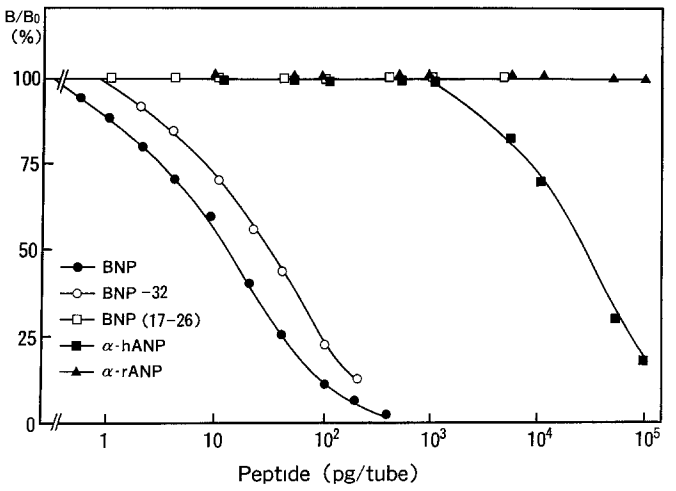

Figure 1
A representative standard curve of BNP (closed circles) and dilution curves of BNP-related peptides, α-hANP and α-rANP.

BNP-32 showed 66.2 % of cross-reactivity on a molar basis. The cross-reactivity of the C-terminal fragment of BNP, BNP[17-26], was less than 0.004 %. α-hANP exhibited only 0.12 % of cross-reactivity and the cross-reactivity of α-rANP was less than 0.002 %. Intra- and interassay coefficients of variation were 5.1 % (n=6) and 11.5 % (n=10), respectively. Since the C-terminal fragment of BNP, BNP[17-26], did not show significant cross-reactivity, and the only difference in the amino acid sequence between α-hANP and α-rANP is Met at the position of 12 in the ring structure of α-ANP, our antiserum F-8 in the RIA was presumed to recognize the N-terminal portion of BNP.

Detection of BNP-LI in the porcine and canine brains and its characterization

The serial dilution curves of the porcine and canine brain extracts were parallel to the standard curve of BNP as depicted in Figure 2. The brain extracts of rats, monkeys or humans, however, did not exhibit cross-reactivity in the RIA. Figures 3A and 3B show the gel filtration profiles of BNP-LI in the porcine and canine brain extracts, respectively. In both cases, BNP-LI was eluted as one major

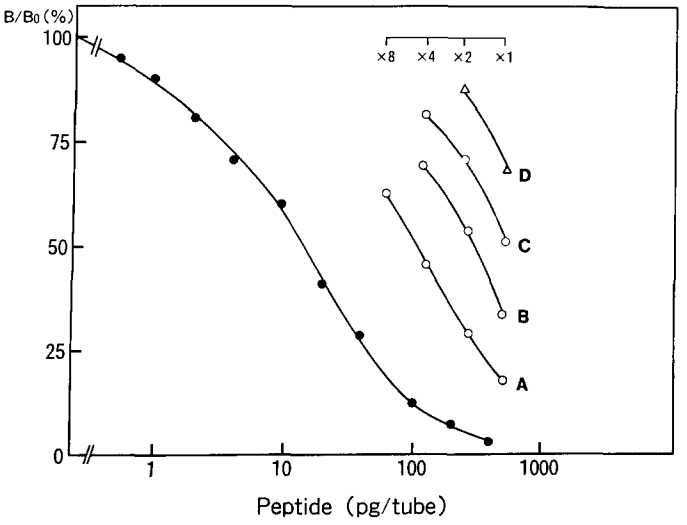

Figure 2
A standard curve of BNP (closed circles) and dilution curves of the extracts from porcine and canine brain regions. (A) caudate nucleus, pig, (B) medulla oblongata, pig, (C) lenticular nucleus, pig, (D) medulla oblongata, dog.

peak with an approximate molecular weight of 3,000 at the elution position of synthetic BNP or BNP-32, and in the porcine brain, we also detected a minor peak with a higher molecular weight (between 8.2K and

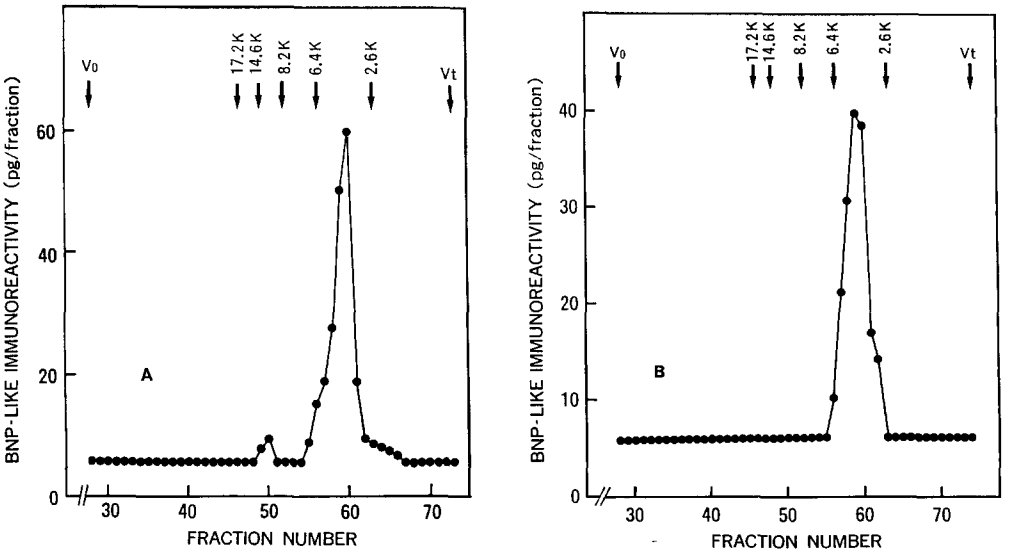

Figure 3
Gel filtration profiles on a TSK-GEL G2000 SW column of BNP-LI in extracts from the medulla oblongata in pigs (A) and from the whole brain in dogs (B). Arrows denote the elution positions of a series of myoglobins of a polypeptide molecular weight calibration kit (Pharmacia Fine Chemicals, Uppsala, Sweden), void volume (Vo) and total volume (Vt).

14.6K). The elution pattern of BNP-LI in the porcine brain is compatible with that in the previous report (1,2). The BNP-immunoreactive peak with a higher molecular weight is possibly considered to be BNP of 12 K daltons, the precursor form of BNP, recently identified in the porcine heart (20). The finding that BNP-LI was also detected in the dog suggests that the presence of BNP is not restricted to the pig and that BNP is a common neuropeptide among species. Further characterization of BNP-LI in the canine brain is now in progress in our laboratory.

Tissue levels of BNP-LI and ANP-LI in the porcine and canine brains

Table 1 and Table 2 give the regional distributions of BNP-LI and ANP-LI determined simultaneously in the same tissue samples, in the porcine and canine brains, respectively.

In the porcine brain, the rank order of BNP-LI concentrations was; caudate nucleus > medulla oblongata > lenticular nucleus > olfactory bulb > cerebral cortex > hypothalamus > midbrain > thalamus > pons > hippocampus. BNP-LI was not detectable in the cerebellum and the pituitary gland. This finding is agreed with the previous report (21) and supports the validity of our RIA for BNP.

Table 1

Regional distribution of BNP-like immunoreactivity (-LI) and ANP-LI in porcine brain

Brain region	BNP-LI	ANP-LI
Olfactory bulb	2.48 ± 1.51	0.78 ± 0.10
Cerebral cortex	1.47 ± 0.75	< 0.1
Hippocampus	0.19 ± 0.11	< 0.1
Lenticular nucleus	3.75 ± 0.45	< 0.1
Caudate nucleus	5.77 ± 0.72	< 0.1
Thalamus	0.37 ± 0.10	< 0.1
Hypothalamus	0.91 ± 0.38	0.13 ± 0.05
Midbrain	0.61 ± 0.13	1.50 ± 1.37
Pons	0.32 ± 0.14	0.11 ± 0.05
Medulla oblongata	4.08 ± 2.11	< 0.1
Cerebellum	< 0.1	< 0.1
Pituitary gland (pooled)	< 0.1	< 0.1

Values are the mean ± S.E.M. (n=4; ng/g wet tissue)

Table 2

Regional distribution of BNP-like immunoreactivity (-LI) and ANP-LI in canine brain

Brain region	BNP-LI	ANP-LI
Olfactory bulb	0.55 ± 0.22	0.30 ± 0.05
Cerebral cortex	< 0.06	< 0.06
Hippocampus	< 0.06	0.07 ± 0.01
Lenticular nucleus	< 0.06	0.06 ± 0.03
Caudate nucleus	< 0.06	0.24 ± 0.11
Thalamus	0.27 ± 0.13	0.10 ± 0.01
Hypothalamus	0.34 ± 0.10	0.18 ± 0.01
Midbrain	0.71 ± 0.12	0.06 ± 0.02
Pons	0.81 ± 0.14	0.13 ± 0.02
Medulla oblongata	0.92 ± 0.01	0.10 ± 0.01
Cerebellum	< 0.06	< 0.06

Values are the mean ± S.E.M. (n=4; ng/g wet tissue)

The concentration of ANP-LI was lower than that of BNP-LI in the porcine brain. Furthermore, the distribution pattern of ANP-LI in the porcine brain differs from that of BNP-LI. The highest concentration of ANP-LI was observed in the midbrain, followed by the olfactory bulb and the hypothalamus. The rank order of ANP-LI in the porcine brain was consistent with that in the human and monkey brains as we previously reported (22). We also reported that in the rat brain, ANP concentration is the highest in the hypothalamus and septum, followed by the midbrain (3). The different rank order of ANP in the brain may be due to inter-species differences.

In the canine brain, the highest concentrations of BNP-LI and ANP-LI were observed in the medulla oblongata and the olfactory bulb, respectively (Table 2). In contrast to the pig, BNP-LI was not detectable in the corpus striatum (the caudate nucleus or the lenticular nucleus) of the canine brain. This result may be explained by the fact that we studied the canine brain at the neonatal stage. Further studies are in progress in our laboratory.

The finding that the concentration of BNP-LI is higher than that of ANP-LI both in the porcine and canine brains in the present study further indicates the functional significance of BNP as a neuropeptide

comparable to ANP, and suggests that possible functions of brain ANP reported so far are shared by BNP. Moreover, the different distribution of BNP and ANP observed in the present study also raises the possibility that BNP possesses different unknown functions from those suggested in ANP. Since the large amount of BNP-LI was detected in the medulla oblongata, one of the crucial regions for central cardiovascular control, both from pigs and dogs, BNP may play a critical role in blood pressure homeostasis, including the integration of baroreflex. Taken together, the present findings indicate that our RIA for BNP serves as a useful tool to further elucidate physiological roles of BNP. Moreover, using our antiserum against BNP, immunohistochemical studies on the precise localization of BNP and the possible co-localization with other neurotransmitters in neurons are ongoing in our laboratory.

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REFERENCES

1. Sudoh, T., Kangawa, K., Minamino, N., and Matsuo, H. (1988) Nature 332, 78-80.
2. Sudoh, T., Minamino, N., Kangawa, K., and Matsuo, H. (1988) Biochem. Biophys. Res. Commun. in press.
3. Morii, N., Nakao, K., Sugawara, A., Sakamoto, M., Suda, M., Shimokura, M., Kiso, Y., Kihara, M., Yamori, Y., and Imura, H. (1985) Biochem. Biophys. Res. Commun. 127, 413-419.
4. Shiono, S., Nakao, K., Morii, N., Yamada, T., Itoh, H., Sakamoto, M., Sugawara, A., Saito, Y., Katsuura, G., and Imura, H. (1986) Biochem. Biophys. Res. Commun. 135, 515-520.
5. Nakamura, M., Katsuura, G., Nakao, K., and Imura, H. (1985) Neurosci. Lett. 58, 1-6.
6. Katsuura, G., Nakamura, N., Inouye, K., Kono, M., Nakao, K., and Imura, H. (1986) Eur. J. Pharmacol. 121, 285-287.
7. Itoh, H., Nakao, K., Katsuura, G., Morii, N., Shiono, S., Yamada, T., Sugawara, A., Saito, Y., Watanabe, K., Igano, K., Inouye, K., and Imura, H. (1987) Neurosci. Lett. 74, 102-106.

8. Itoh, H., Nakao, K., Katsuura, G., Morii, N., Shiono, S., Sakamoto, M., Sugawara, A., Yamada, T., Saito, Y., Matsushita, A., and Imura, H. (1986) Circ. Res. 59, 342-347.
9. Itoh, H., Nakao, K., Morii, N., Yamada, T., Shiono, S., Sakamoto, A., Sugawara, A., Saito, Y., Katsuura, G., Shiomi, T., Eigyo, M., Matsushita, A., and Imura, H. (1986) Brain Res. Bull. 16, 745-749.
10. Yamada, T., Nakao, K., Morii, N., Itoh, H., Shiono, S., Sakamoto, M., Sugawara, A., Saito, Y., Ohno, H., Kanai, A., Katsuura, G., Eigyo, M., Matsushita, A., and Imura, H. (1986) Eur. J. Pharmacol. 125, 453-456.
11. Itoh, H., Nakao, K., Katsuura, G., Morii, N., Yamada, T., Shiono, S., Sakamoto, M., Sugawara, A., Saito, Y., Eigyo, M., Matsushita, A., and Imura, H. (1986) Neurosci. Lett. 69, 254-258.
12. Itoh, H., Nakao, K., Yamada, T., Morii, N., Shiono, S., Sugawara, A., Saito, Y., Mukoyama, M., Arai, H., and Imura, H. (1988) Hypertension 11[Suppl I], I-57-I-61.
13. Itoh, H., Nakao, K., Yamada, T., Morii, N., Shiono, S., Sugawara, A., Saito, Y., Mukoyama, M., Arai, H., and Imura, H. (1988) Can. J. Physiol. Pharmacol. 66, 255-262.
14. Nakao, K., Morii, N., Itoh, H., Yamada, T., Shiono, S., Sugawara, A., Saito, Y., Mukoyama, M., Arai, H., Sakamoto, M., and Imura, H. (1986) J. Hypertension 4[Suppl 6], S492-S496.
15. Itoh, H., Nakao, K., Morii, N., Sugawara, A., Yamada, T., Shiono, S., Saito, Y., Mukoyama, M., Arai, H., Sakamoto, M., and Imura, H. (1987) Jpn. Circ. J. 51, 1208-1215.
16. Itoh, H., Nakao, K., Yamada, T., Shirakami, G., Kangawa, K., Minamino, N., Matsuo, H., and Imura, H. (1988) Eur. J. Pharmacol. 150, 193-196.
17. Shirakami, G., Nakao, K., Yamada, T., Itoh, H., Mori, K., Kangawa, K., Minamino, N., Matsuo, H., and Imura, H. (1988) Neurosci. Lett. 91, 77-83.
18. Yamada, T., Nakao, K., Itoh, H., Shirakami, G., Kangawa, K., Minamino, N., Matsuo, H., and Imura, H. (1988) Neurosci. Lett. in press.
19. Nakao, K., Sugawara, A., Morii, N., Sakamoto, M., Suda, M., Soneda, J., Ban, T., Kihara, M., Yamori, Y., Shimokura, M., Kiso, Y., and Imura, H. (1984) Biochem. Biophys. Res. Commun. 124, 815-821.
20. Minamino, N., Aburaya, M., Ueda, S., Kangawa, K., and Matsuo, H. (1988) Biochem. Biophys. Res. Commun. 155, 740-746.
21. Ueda, S., Minamino, N., Sudoh, T., Kangawa, K., and Matsuo, H. (1988) Biochem. Biophys. Res. Commun. 155, 733-739.
22. Itoh, H., Nakao, K., Mukoyama, M., Hosoda, K., Shiono, S., Morii, N., Yamada, T., Sugawara, A., Saito, Y., Arai, H., Shirakami, G., and Imura, H. (1988) J. Hypertension in press.