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DISTRIBUTION OF C-TYPE NATRIURETIC PEPTIDE AND ITS MESSENGER RNA IN RAT CENTRAL NERVOUS SYSTEM AND PERIPHERAL TISSUE

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SUMMARY: In rat, the highest concentration of immunoreactive (ir–) C–type natriuretic peptide (CNP) was found in the central nervous system, as is the case in pig and human. Although its concentration in peripheral tissue was much lower than that in brain, CNP was present mainly as CNP–53 in ileum–jejunum, colon–cecum, stomach, kidney, lung, testis and submaxillary gland, but not in heart. By Northern blot analysis, CNP mRNA was detected in ileum–jejunum, testis, thymus, adrenal gland and submaxillary gland as well as in brain and spinal cord. CNP mRNA was further verified by reverse transcription–polymerase chain reaction to be present in most peripheral tissue, including aorta and bone marrow. These results indicate that CNP is synthesized in peripheral tissue and possibly functions as a local regulator in addition to acting as a neuropeptide in the central nervous system. © 1993 Academic Press, Inc.

C–type natriuretic peptide (CNP) is a third member of the natriuretic peptide family, being comprised of A–type (atrial) natriuretic peptide (ANP), B–type (brain) natriuretic peptide (BNP) and CNP (1). CNP has the most highly conserved amino acid sequence among the three natriuretic peptides, but exerts a pharmacological spectrum distinct from that of ANP and BNP (1–3). Regional distribution studies of CNP in porcine and human tissue have demonstrated that CNP is localized in brain but not in heart, and that its concentration in brain is highest among the three peptides (4,5). These results suggest that CNP functions as a neuropeptide in the central nervous system (CNS) in regulating homeostatic balance of body fluid volume and blood pressure. On the other hand, a CNP–specific receptor, guanylate cyclase B (GC–B), is widely distributed not only in the CNS but also in the peripheral system (6–8). This fact strongly suggests that CNP is also synthesized and functions in peripheral tissue, although

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Abbreviations: ANP, A–type (atrial) natriuretic peptide; BNP, B–type (brain) natriuretic peptide; CNP, C–type natriuretic peptide; ir–, immunoreactive; RIA, radioimmunoassay; HPLC, high performance liquid chromatography; RP, reverse phase; IEX, ion exchange; RT–PCR, reverse transcription–polymerase chain reaction; TFA, trifluoroacetic acid; MW, molecular weight; GC, guanylate cyclase.

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CNP-producing tissues or cells have not yet been identified. While measuring CNP concentration in rat tissue, we noted significant levels of ir-CNP in gastrointestinal tract and kidney (9). We systematically searched for CNP and its mRNA in rat peripheral tissue by using radioimmunoassay (RIA) for CNP, Northern blot analysis and reverse transcription-polymerase chain reaction (RT-PCR) to identify CNP-producing tissue.

MATERIALS AND METHODS

Materials: CNP-22 and [Tyr^0]-CNP-22 were synthesized in our laboratory with a peptide synthesizer (Applied Biosystems, 430A). Rat CNP-53 was kindly donated by Dr. Y. Minamitake of Suntory Institute for Biomedical Research.

RIA for CNP: Since rat CNP-22 is identical to porcine CNP-22, RIA established for porcine CNP was used in this study (4,10). In the RIA for CNP, antiserum #171-4 was used at a final dilution of 1:60,000, and peptides were measureable in a range of 1~50 fmol/tube. Rat α-ANP showed 0.001% and BNP-45 less than 0.001% crossreactivity in this RIA.

RIAs for rat ANP and BNP: RIAs for rat ANP and BNP were performed as reported (11,12). CNP-22 showed less than 0.001% crossreactivity in these two RIAs.

Peptide extraction: Rat tissue was collected from 6 male Sprague-Dawley rats (12~14 weeks old), except that pituitaries were collected from 18 rats and divided into 3 groups. Brains were dissected into 9 regions referring to the method of Glowinski and Iversen (13). Cardiac atrium, ventricle and other tissue were carefully collected to avoid inter-tissue contamination. Tissue was diced and boiled for 10 min in 10 volumes of water. Glacial acetic acid was added (final concentration = 1 M) after cooling, and boiled tissue was homogenized with a Polytron mixer for 4 min. The supernatants, obtained by centrifugation at 16,000 x g for 20 min, were loaded onto Sep-pak C_{18} cartridges (Waters). After washing with 0.5M acetic acid and 0.1% trifluoroacetic acid (TFA), adsorbed materials were eluted with 60% CH_3CN containing 0.1% TFA. The eluted materials were lyophilized and submitted to RIAs for ANP, BNP and CNP.

Characterization of ir-CNP : C_{18} column-treated tissue extracts (1~10 g wet wt equivalents) were subjected to gel filtration on a Sephadex G-50 column (1.9 x 134 cm). An aliquot of each fraction was submitted to RIA for CNP. Fractions exhibiting CNP-immunoreactivity were further analyzed by reverse phase high performance liquid chromatography (RP-HPLC) on a Chemcosorb $_5C_{18}$-300 column (4.6 x 250 mm, Chemco) using a linear gradient elution of CH_3CN from 10% to 60% in 0.1% TFA and partially by CM ion exchange (IEX) HPLC on a TSK gel CM-2SW (4.6 x 250 mm, Tosoh) using a gradient elution of $HCOONH_4$ (pH 6.5) containing 10% of CH_3CN (2). Aliquots of all fractions were submitted to RIA for CNP.

RNA extraction and Northern blot analysis: Rat tissue was collected from 3~20 male Sprague-Dawley rats (10~12 weeks old) soon after decapitation. Collected tissue was immediately frozen in liquid nitrogen and stored at $-85°C$ before extraction. Total RNA was extracted by the acid-guanidium thiocyanate-phenol-chloroform method (14). Poly(A^+)RNA was isolated by using Oligotex-dT30 (Nippon Roche). Poly(A^+)RNA (10~20 μg) was denatured with 1M glyoxal and 50% dimethylsulfoxide, electrophoresed on a 1.4% agarose in 10mM sodium phosphate buffer (pH 7.0), and then transferred to a Zeta Probe membrane (Bio-Rad) and fixed by ultraviolet irradiation. The membrane was prehybridized and hybridized at 65 °C in 1.5x SSPE containinig 0.5% Blott, 1.0% SDS and 0.5 mg/ml of salmon sperm DNA. A full-length rat CNP cDNA of 1020 bp was labeled by the random-primed method and used for hybridization (10). The blot was washed at 37°C successively with 2x SSC/0.1% SDS, 0.5x SSC/0.1% SDS and 0.1x SSC/0.1% SDS. Autoradiography was carried out at $-80°C$ for 4 days.

RT-PCR: Two primers (5'-primer, TTCGGGACCTGCGTGTGGACA; 3'-primer, CCCTCCC CAAATAATAATAAA) for PCR were designed using a primer analysis software (Oligo, National Biosciences) and synthesized with a DNA synthesizer (Applied Biosystems, 391). First stand cDNA was synthesized with poly(A^+)RNA (0.25 μg), the 3'-primer and M-MLV reverse transcriptase, and the resulting cDNA was amplified 20, 30 and 40 cycles by the PCR

method with AmpliTaq polymerase (GeneAmp RNA PCR kit, Takara). The PCR products were electrophoresed on a 2.5% low melting agarose gel (SeaPlaque, FMS) in 1x TBE buffer. Southern blot analysis of the PCR products was carried out in a manner similar to that of Northern blot analysis.

RESULTS

Distribution of ir–CNP: Regional distribution of ir–CNP in rat is summarized in Table 1, along with that of ir–ANP and ir–BNP. In brain and spinal cord, ir–CNP was distributed in a range of 1.2~4.3 pmol/g, with the highest concentration of ir–CNP being observed in olfactory bulb. Cerebellum, midbrain–thalamus, medulla–pons and hypothalamus contained ir–CNP at the second highest level (about 3.0 pmol/g), with a high ir–CNP concentration in cerebellum as is the case in porcine brain (4). Throughout the brain, ir–ANP was present in the highest concentration among the three natriuretic peptides, about 3 times higher than that of ir–CNP, while ir–BNP was not detected in a significant concentration. In each brain region, ir–CNP was

Table 1. Regional distribution of immunoreactive ANP, BNP and CNP in rat

region	ir–ANP (pmol/g wet tissue)	ir–BNP (pmol/g wet tissue)	ir–CNP (pmol/g wet tissue)
brain			
olfactory bulb	4.58	< 0.04	4.33
hippocampus	3.31	< 0.04	2.30
striatum	2.13	< 0.04	1.50
cerebral cortex	3.73	< 0.04	1.95
cerebellum	1.05	< 0.04	3.70
midbrain–thalamus	10.31	< 0.04	2.86
medulla–pons	3.21	< 0.04	3.65
hypothalamus	14.17	< 0.04	3.05
septum	8.08	< 0.04	1.21
spinal cord	3.96	0.06	1.74
pituitary			
anterior lobe	1.70	< 0.3	2.56
posterior lobe	2.41	< 0.3	2.37
heart			
atrium	87360	625.66	6.26
ventricle	30.10	3.07	0.61
submaxillary gland	1.61	< 0.06	0.11
stomach	0.54	< 0.06	0.14
ileum–jejunum	0.27	< 0.06	0.17
colon–cecum	0.63	< 0.06	0.33
lung	1.35	< 0.06	0.66
liver	0.35	< 0.06	0.23
pancreas	0.33	< 0.06	0.25
kidney	4.15	< 0.06	0.75
spleen	0.45	< 0.06	0.77
thymus	0.60	0.10	0.26
testis	0.28	< 0.06	0.16
adrenal gland	0.51	0.14	0.31
bone marrow	0.81	< 0.06	2.12
thoracic aorta	0.15	< 0.06	0.07
skeletal muscle	0.11	< 0.06	0.17

distributed in relatively equal concentrations, while there were large regional differences in ir–ANP concentration. In pituitary, the two peptides were found in similar amounts with about 2.5 pmol/g of ir–CNP being detected.

In peripheral tissue, ir–CNP was detected in every examined tissue. Among them, cardiac atrium contained the highest concentration. However, this immunoreactivity was verified by RP–HPLC to be derived from crossreactivity of ANP, which was present in atrium at a concentration 15,000 times higher than that of ir–CNP (data not shown). In bone marrow and spleen, ir–CNP was also detected at a relatively high level, but this immunoreactivity was found to be non–specific. In submaxillary gland, ileum–jejunum, colon–cecum, lung and kidney as well as in stomach, adrenal gland and testis, at least a portion of detected ir–CNP was shown to be comprised of CNP by chromatographic characterization, as described below. Thus, the ir–CNP concentration in peripheral tissue listed in Table 1 should be recognized as apparent values containing various ratios of non–specific immunoreactivity. Based on the characterization data of ir–CNP, kidney and gastrointestinal tract were shown to contain the highest levels of ir–CNP among all peripheral organs (about 0.1~0.5 pmol/g).

BNP is present mainly in heart but is not found in significant concentrations in other peripheral tissue except for adrenal gland (14). ANP has also been shown to be produced and stored in the peripheral tissue, such as kidney and lung (10,11), but ir–ANP concentrations listed in Table 1 also contain some non–specific immunoreactivity.

Characterization of ir–CNP: Figure 1 shows the results of Sephadex G–50 gel filtration of rat tissue extracts. In brain and spinal cord, ir–CNP was separated into four peaks of molecular weight (MW) 12K, 6K, 3K and void volume (Figs. 1a and 1b). Among them, ir–CNP of MW 6K and 3K were identified to be CNP–53 and CNP–22 by RP–HPLC (Figs. 2a and 2b) and CM IEX–HPLC (data not shown). 12K CNP was deduced to be pro–CNP based on the MW and elution time in RP–HPLC, while ir–CNP at the void volume was due to non–specific immunoreactivity. Based on these data, CNP–53 was found to be a major endogenous form in the CNS, and more than 70% of ir–CNP in brain and spinal cord was derived from CNP. In ileum–jejunum and colon–cecum, ir–CNP was mainly composed of CNP–53 and CNP–22, which were present at a ratio of 3:1 (Figs. 1d, 1e, 2c and 2d) and constituted 60% of the total ir–CNP. In kidney, 12K and 6K CNP were observed in gel filtration, but 3K CNP was not detected. 6K CNP in kidney was identified to be CNP–53 and its oxide form by RP–HPLC (Fig. 2c). In lung and testis, only a portion of ir–CNP emerged at MW 6K region, which was shown to be CNP–53 by RP–HPLC (Figs. 1f, 1g and 2e). We further characterized ir–CNP in other peripheral tissues and have summarized the results in Table 2.

Distribution of CNP mRNA: Distribution of CNP mRNA was first examined by Northern blot analysis using large amounts of poly(A^+)RNA. As shown in Fig. 3, CNP gene was highly

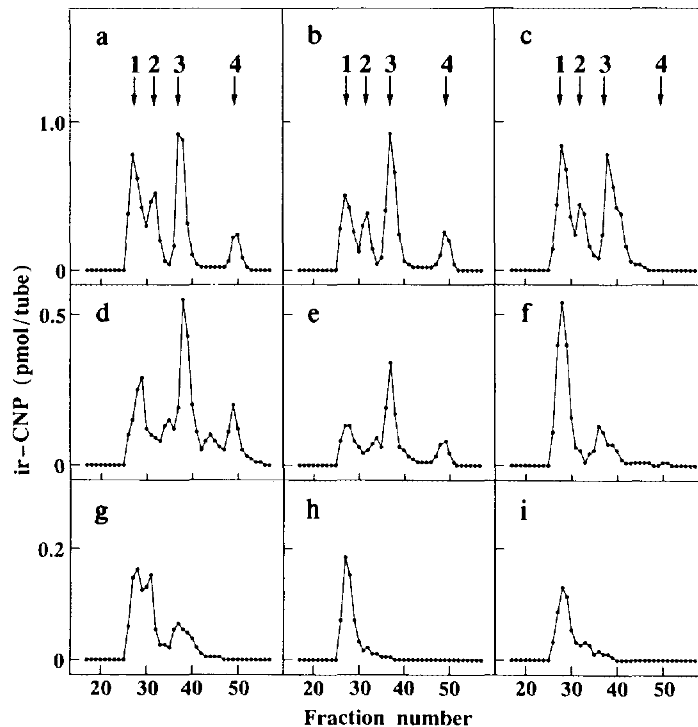

Figure 1. Sephadex G-50 gel filtration of rat tissue extracts.
Sample: Reverse phase C_{18} column treated extracts of (a) brain (5 g), (b) spinal cord (10 g), (c) kidney (10 g), (d) ileum-jejunum (10 g), (e) colon-cecum (10 g), (f) lung (5 g), (g) testis (10 g), (h) bone marrow (1 g), and (i) skeletal muscle (10 g).
Column: Sephadex G-50 (fine, 1.9 x 134 cm, Pharmacia). Flow rate: 12 ml/hr.
Fraction size: 6 ml/tube. Solvent: 1M CH_3COOH. Arrows indicate elution positions of (1) Vo, (2) 12K CNP, (3) CNP-53 and (4) CNP-22, respectively.

expressed in brain, cerebellum and spinal cord. In peripheral tissue, CNP mRNA was detected in ileum-jejunum, testis, thymus, adrenal gland, cardiac atrium and ventricle, stomach, submaxillary gland and skeletal muscle, although their gene expression levels were much lower than those in the CNS.

To further measure low levels of CNP gene expression semiquantitatively, we employed the RT-PCR method and selected primer set and amplification conditions with high sensitivity and low background. Three PCR cycles were used for quantitative estimation. As shown in Fig. 4, weak bands were observed with 20 cycle amplification in brain, cerebellum, spinal cord and 10 pg of CNP cDNA. With 30 cycle amplification, pituitary, testis, adrenal gland, aorta, cardiac atrium, ventricle, stomach, and 10 fg~1 pg of the control template were positive. With 40 cycle amplification, 1 fg of CNP cDNA was detected, and weak bands which are not clearly visible in Fig. 4 were observed in lung, kidney, ileum-jejunum, colon-cecum, thymus and submaxillary gland. However, no band was detected in liver, skeletal muscle,

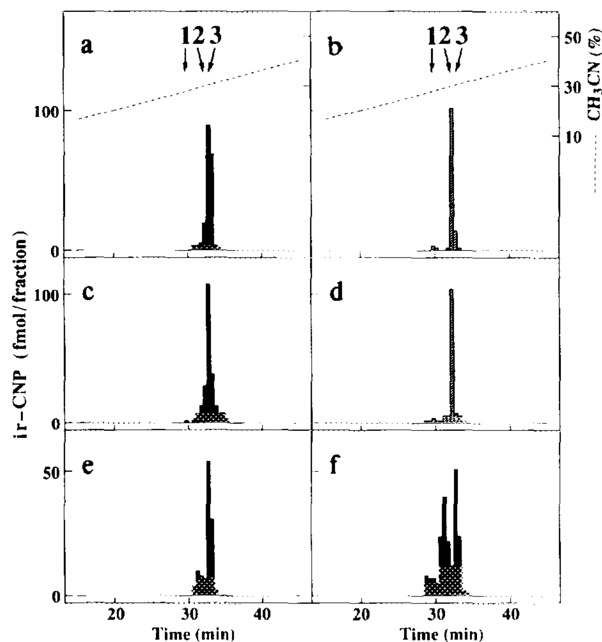

Figure 2. Reverse phase HPLC of immunoreactive CNP in rat tissue.
Sample: (a) 6K CNP in brain (1/20 of fractions #36–39 in Fig. 1a), (b) 3K CNP in brain (1/5 of fractions #48–51 in Fig. 1a), (c) 6K CNP in ileum–jejumun (1/10 of fractions #37–40 in Fig. 1d), (d) 3K CNP in ileum–jejunum (1/3 of fractions #48–51 in Fig. 1d), (e) 6K CNP in lung (1/2 of fractions #37–38 in Fig. 1f) and (f) 6K CNP in kidney (1/10 of fractions #37–42 in Fig. 1c).
Column: Chemcosorb $_5C_{18}$–300 (4.6 x 250 mm, Chemco). Flow rate: 1.0 ml/min.
Solvent system: Linear gradient elution from (A) to (B) over 60 min.
H_2O : CH_3CN : 10% TFA = (A) 90 : 10 : 1, (B) 40 : 60 : 1 (vol./vol.)
Arrows indicate elution times of (1) methionine sulfoxide form of CNP–22, (2) CNP–22 and (3) CNP–53, respectively. Black and hatched columns indicate 6K and 3K immunoreactivity, respectively.

Table 2. Distribution of CNP and its mRNA in rat tissue

tissue	peptide		mRNA	
	apparent	characterized	Northern	RT-PCR
brain	+++	+++	+++	+++
cerebellum	+++	+++	+++	+++
spinal cord	+++	+++	+++	+++
pituitary	+++	nd	nd	+++
heart atrium	+++	–	+	++
ventricle	+	nd	+	++
submaxillary gland	+	+	+	+
stomach	+	+	+	++
ileum/jejunum	+	++	+	+
colon/cecum	+	++	–	+
lung	+	+	–	+
liver	+	–	–	–
kidney	+	+	+	+
spleen	+	–	–	–
thymus	+	–	+	+
testis	+	+	++	++
adrenal gland	+	+	+	++
bone marrow	++	–	–	+
thoracic aorta	+	nd	nd	++
skeletal muscle	+	–	+	–

nd: not determined.

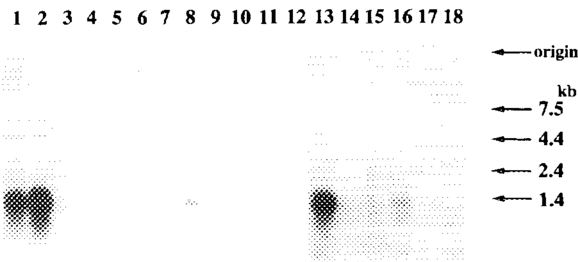

Figure 3. RNA blot analysis of rat CNP transcripts in rat tissue.
Sample: 20 μg of poly(A^+)RNA were denatured and electrophoresed in each lane, except for cardiac atrium (10 μg) and ventricle (10 μg). (1) Brain, (2) cerebellum, (3) lung, (4) liver, (5) kidney, (6) colon–cecum, (7) ileum–jejunum, (8) testis, (9) thymus, (10) adrenal gland, (11) cardiac atrium, (12) ventricle, (13) spinal cord, (14) submaxillary gland, (15) stomach, (16) skeletal muscle, (17) spleen, (18) bone marrow. Numbers on the right side are molecular sizes shown in kilobases. Autoradiography was at -80°C for 4 days. Brain and spinal cord were deduced to have comparable intensity based on internal standards, although CNP cDNA probes of different specific radioactivity were used in lanes (1)~(12) and (13)~(18).

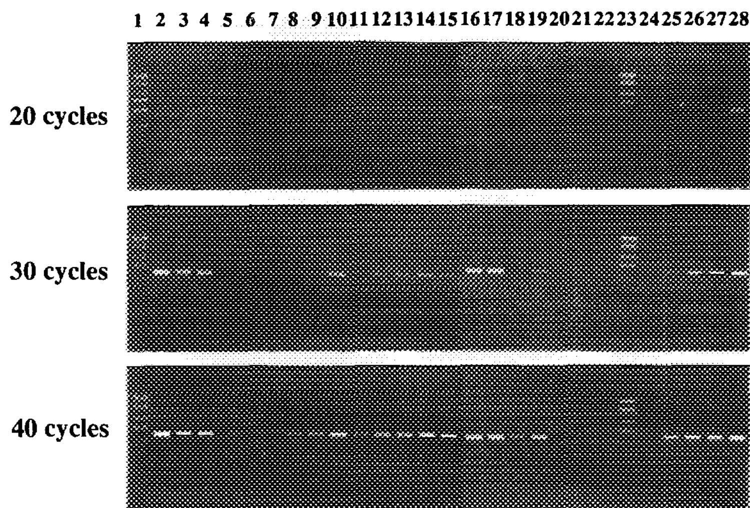

Figure 4. Electrophoretic analysis of RT–PCR products of CNP transcripts in rat tissue.
0.25 μg of poly(A^+)RNA was reverse transcribed and then amplified by the PCR method. (1,23) Size marker (1000, 700, 500, 400, 300bp from up to down), (2,16) brain, (3) cerebellum, (4) pituitary, (5) lung, (6) liver, (7) kidney, (8) colon–cecum, (9) ileum–jejunum, (10) testis, (11) thymus, (12) adrenal gland, (13) aorta, (14) cardiac atrium, (15) ventricle, (17) spinal cord, (18) submaxillary gland, (19) stomach, (20) skeletal muscle, (21) spleen, (22) bone marrow. In lanes (24)~(28), different amounts of rat CNP cDNA were amplified by the PCR method to estimate CNP mRNA levels. (24) 1 fg, (25) 10 fg, (26) 100 fg, (27) 1 pg, (28) 10 pg of rat CNP cDNA. Lanes (1)~ (15) and (16~28) were electrophoresed in separate experiments.

spleen and bone marrow under any of these conditions. The PCR products had a molecular size of 350bp corresponding to the expected product of 346bp and were shown by Southern blot analysis to hybridize to rat CNP cDNA.

DISCUSSION

Tissue distribution of ir–CNP in rat CNS is generally similar to that in porcine CNS (2), indicating a wide distribution of CNP in rat CNS without large concentration differences. Immunoreactive CNP concentration in rat CNS is much higher than that in peripheral tissue even when compared with apparent values shown in Fig. 1. These data also support the idea that CNP is a natriuretic peptide functioning as a neuropeptide in the CNS, although ANP is more abundant than CNP in the case of rat CNS.

CNP has not so far been recognized to be present in peripheral tissue. In fact, in our characterization of ir–CNP in porcine peripheral tissue, we could not detect a significant level of CNP–53 and CNP–22 except in adrenal medulla (4). Komatsu et al. reported the presence of ir–CNP in rat kidney, ileum and colon, but this immunoreactivity was not well characterized (15). In the present study, CNP was demonstrated to be present as a peptide in intestine, kidney, stomach, lung, testis, adrenal gland and submaxillary gland. In these tissues, CNP is mainly present as CNP–53 as in the case of the CNS. These results indicate wide distribution of CNP in rat peripheral tissue in a low but significant concentration.

By Northern blot analysis, high levels of CNP mRNA were observed in the CNS as expected from peptide concentrations. In peripheral tissue, CNP mRNA was detected in submaxillary gland, kidney, adrenal gland, ileum–jejunum, thymus and testis at levels much lower than those in the CNS. By the RT–PCR method, which could detect as low as 1 fg of cDNA, CNP gene was shown to be expressed in every examined tissue except for liver, spleen and skeletal muscle. It should be noted that aorta, cardiac atrium and ventricle have positive bands in this analysis. Although skeletal muscle showed a positive band in Northern blot analysis, it had no band in the RT–PCR (Figs. 3 and 4) and no ir–CNP in the RIA, indicating the absence of CNP and its mRNA in this tissue. These data demonstrate that CNP gene is widely expressed in most peripheral tissue, far more than we had expected. Some discordances were observed between the distribution of CNP and its mRNA. In cardiac atrium, thymus and bone marrow, CNP was not present as a peptide, while CNP mRNA was detected by Northern blot analysis and/or RT–PCR. This suggests that CNP mRNA may not be actively translated in these tissues, although CNP gene is expressed significantly.

Biologically active natriuretic peptide receptors, GC–A and GC–B, are distributed not only in the CNS but also in the peripheral system. Although GC–A receptor ligands, ANP and BNP, are known to be produced in heart and function as circulating hormones, CNP–producing

tissue and its target organs had not been identified prior to the present study. As for the function of CNP in peripheral tissue, several studies have recently reported that CNP inhibits (*i*) proliferation of cultured vascular smooth muscle cells, (*ii*) intimal thickening after vascular injury, (*iii*) water and electrolyte absorption from jejunum, and (*iv*) causes relaxation in canine veins (16–19). During this study, we identified CNP in the culture medium of human monocytic leukemia cell line (THP-1)(20). Nakao et al. has reported the production of CNP in bovine vascular endothelial cells (21). These data indicate the production of CNP in the peripheral system, such as vascular tissue and the monocyte–macrophage system. Garbers et al. also reported the presence of a high concentration of CNP in porcine seminal plasma (22). In the present study, we detected significant levels of CNP and its mRNA in rat aorta, gastrointestinal tract and testis. The coincidence of the reported functions with the distribution data of CNP in rat confirms that CNP is generally produced in mammalian peripheral tissue. In the present study, CNP was shown to be produced and to be present in most peripheral tissue at different levels. These results will provide invaluable clues in searching and elucidating a variety of physiological functions of CNP in the peripheral system.

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