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NOVEL NATRIURETIC PEPTIDE, CNP, POTENTLY STIMULATES CYCLIC GMP PRODUCTION IN RAT CULTURED VASCULAR SMOOTH MUSCLE CELLS

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SUMMARY : The newly identified peptide C-type natriuretic peptide (CNP) caused only a slight elevation of cGMP in rat renal glomeruli. In contrast, CNP potently increased cGMP levels in cultured rat vascular smooth muscle cells (VSMC) and stimulated guanylate cyclase activity in the particulate fraction of the cells. The extent of maximum activation of the enzyme induced by CNP was 4-fold higher than that by human atrial natriuretic peptide (α-hANP) while CNP was 4- and 16-fold weaker than α-hANP in binding affinity for the putative receptors on VSMC and vasorelaxant activity for rat aorta, respectively. These results indicate that CNP is a potent stimulator of cGMP formation in VSMC but not in glomeruli and pharmacological feature of CNP is distinct from that of ANP.

Atrial natriuretic peptide (ANP) is known to play an important role in the regulation of body fluid and blood pressure and other physiological functions (1, 2). Identification of brain natriuretic peptide (BNP) in mammalian brain and heart (3-5), which has been reported to elicit pharmacological effects very similar to those of human ANP (α-hANP)(3, 6), discloses the possibility that water-salt and blood pressure homeostasis are regulated by both ANP and BNP. Very recently, a third type natriuretic peptide designated as CNP (C-type natriuretic peptide) was identified in porcine brain (7). Porcine CNP has amino acid sequences homologous to both ANP and BNP in a ring flanked by a disulfide linkage; however, it has a quite unique feature in N-terminal five amino acids, and ends at the second

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Abbreviations: ANP; atrial natriuretic peptide (A-type natriuretic peptide), BNP; brain natriuretic peptide (B-type natriuretic peptide), CNP; C-type natriuretic peptide, α-hANP; human pro-ANP[99-126], SDS; sodium dodecyl sulfate, PAGE; polyacrylamide gel electrophoresis.

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cysteine residue with no C-terminal tail. The amino acid sequences of porcine CNP along with porcine ANP and BNP are listed below.

CNP , G-L-S-K-G-C-F-G-L-K-L-D-R-I-G-S-M-S-G-L-G-C
ANP , S-L-R-R-S-S-C-F-G-G-R-M-D-R-I-G-A-Q-S-G-L-G-C-N-S-F-R-Y
BNP , S-P-K-T-M-R-D-S-G-C-F-G-R-R-L-D-R-I-G-S-L-S-G-L-G-C-N-V-L-R-R-Y

We found that porcine CNP elicited much less potent diuretic/natriuretic and hypotensive actions in rats than ANP and BNP, and that chick rectum relaxant activity of porcine CNP was 3 to 4 times more potent than that of ANP (7), suggesting that CNP plays unique functions which might be distinct from those of ANP and/or BNP. In the present report, we have focused on the potency for cGMP elevation and the binding activity of synthetic porcine CNP in rat renal glomeruli and cultured vascular smooth muscle cells, and compared with those of α-hANP which is structurally identical to porcine ANP (8).

MATERIALS AND METHODS

Peptides: Alpha-hANP and porcine CNP were synthesized by solid phase method as described previously (7, 9). We also synthesized a CNP derivative which was elongated with a tyrosine residue at the N-terminal, called Y^0-CNP for radioiodination. Equivalent potency of Y^0-CNP to CNP was confirmed in cGMP accumulation and binding activity in VSMC, and vasorelaxant activity in rat aorta. Iodination of α-hANP and Y^0-CNP were carried out by the lactoperoxidase method, and the specific activities of both labeled peptides were equally 22.2MBq/μg.
Preparation of rat renal glomeruli: Kidneys were dissected from male Sprague-Dawley rats of 8-9 weeks old. Renal glomeruli were isolated by sieving method (10). Purity and the number of glomeruli were evaluated by light microscopy.
Culture of rat vascular smooth muscle cells (VSMC): VSMC were derived from explants of Sprague-Dawley rats aorta (11), and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco). Cells were used for experiments between 3th to 7th passage.
cGMP accumulation in glomeruli and VSMC: A. Glomeruli; Isolated renal glomeruli were incubated with peptides in 125μl Krebs-Henseleit solution containing 1mM 1-methyl 3-isobutylxanthine (MIX) at 30°C for 2min. Concentration of cGMP in the glomeruli was quantified by using a cGMP radioimmunoassay kit (Yamasa Shoyu) (12). B. VSMC; Cells were incubated with peptides in 0.25ml DMEM containing 0.1% BSA, 20mM HEPES and 0.5mM MIX, pH 7.4 at 37°C for 10min. Cellular cGMP concentration was determined by radioimmunoassay (13).
Guanylate cyclase activity in VSMC membrane: Confluent VSMC were scrapped and homogenized in 8 volumes of 50mM Tris-HCl, pH 7.4 containing 0.25M sucrose, 1mM EDTA, 1mM dithiothreitol, 0.2mM phenylmethylsulfonyl fluoride (PMSF) and 20μg/ml aprotinin. The homogenates were centrifuged at 100,000xg for 60min, and particulate fraction was washed and resuspended in the same buffer. The activity of guanylate cyclase in particulate fraction was determined at 37°C in 50mM Tris-HCl, pH 7.6, containing 1mM GTP, 5mM $MgCl_2$, 7.5mM phosphocreatine, 13units/ml creatine kinase and 0.5mM MIX with or without 0.5mM ATP. Concentration of cGMP generated during 3min incubation was determined by radioimmunoassay.
Receptor binding assay: A. Glomeruli; Isolated glomeruli were incubated with 5x 10^{-10}M [^{125}I]α-hANP (ca. 3x10^5cpm) in the presence of unlabeled peptide in 0.25ml of 10mM Tris-HCl, pH 7.4 containing 10mM $MgCl_2$, 1mM EDTA, 0.25M sucrose, 0.2% bovine serum albumin (BSA) and 1mM PMSF at 0°C for 30 min. At the end of the incubation, 1ml of the ice-cold buffer was added and aliquots were centrifuged

at 10,000xg for 5min. Radioactivity within the pellet was counted by a γ-spectrophotometer (12). B. VSMC; Cells were incubated with indicated concentrations of [^{125}I]α-hANP or [^{125}I]Y^{0}-CNP in the presence or absence of the unlabeled peptide in 0.25ml DMEM containing 0.1% BSA and 20mM HEPES, pH 7.4 for 45min at 25°C. Following wash with the same medium, the cells were solubilized with 0.5M NaOH and radioactivity was measured (13).

Affinity-crosslinking of labeled peptide with VSMC: VSMC were incubated with $5x10^{-10}$M [^{125}I]α-hANP or [^{125}I]Y^{0}-CNP in the presence or absence of $5x10^{-7}$M unlabeled peptide in the same medium described above at 25°C for 45min. After washing for 3 times with 1ml of phosphate-buffered saline (PBS), cells were then added with 0.25ml of 0.1mM disuccinimidyl suberate (DSS) and incubated on ice for 30min. The DSS solution was aspirated and cells were scraped from each well in 0.5ml of PBS, followed by centrifugation. Cell pellets were resuspended in 0.1ml SDS sample buffer (60mM Tris-HCl, pH 6.8, 27% glycerol, 0.3% SDS) with 5% 2-mercaptoethanol. After boiling for 5min, samples were run on 7.5% polyacrylamide gels (14). Dried gels were exposed to Kodak X-Omat AR film at -80°C for 7 days.

Vasorelaxant assay: Aortic strips were prepared from thoracic aorta of Sprague-Dawley rats and mounted in a 7ml chamber containing Krebs-Henseleit solution at 37°C. The strips were contracted with $2x10^{-7}$M norepinephrine and once the contractile response stabilized, they were exposed to peptide cumulatively.

RESULTS

The effect of CNP on accumulation of cGMP was investigated using rat isolated renal glomeruli and cultured rat vascular smooth muscle cells (VSMC). In renal glomeruli, 1.6-fold increase of cGMP concentration was induced by an application of 10^{-5}M of CNP. Whereas, α-hANP increased cGMP contents up to 5.4-fold with an EC50 of $3.6x10^{-7}$M (Fig. 1A). In contrast to renal glomeruli, a marked accumulation of cGMP was evoked by CNP in rat VSMC (Fig. 1B). CNP increased cGMP concentration up to 160-fold of basal level at 10^{-6}M with an EC_{50} of $7.7x10^{-8}$M. Alpha-hANP was less effective than CNP, and the EC_{50} was $3.7x10^{-7}$M and maximum

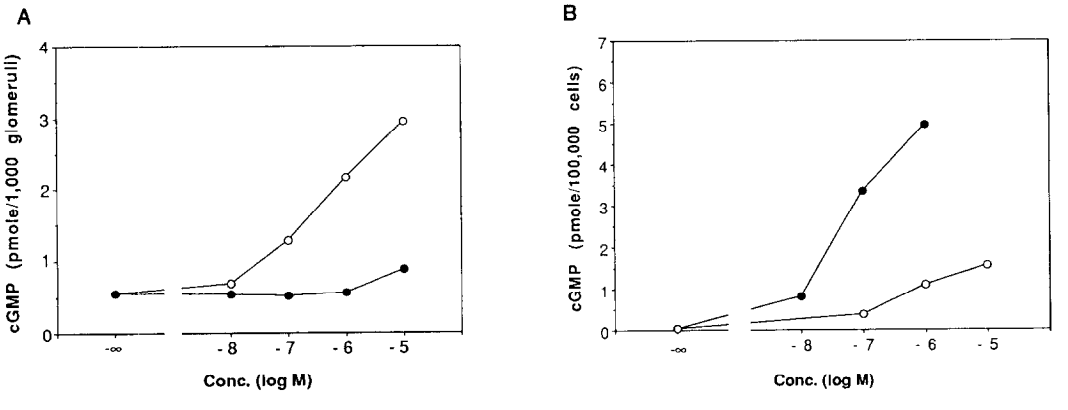

Figure 1. Augmentation of cGMP concentrations induced by CNP (closed circles) or ANP (open circles) in rat renal glomeruli (A) and rat vascular smooth muscle cells (VSMC) (B). Renal glomeruli or VSMC were incubated with the peptide in the presence of MIX as described in "Materials and Methods". Data represent mean values of triplicate determinations from two or three separate experiments in glomeruli or VSMC, respectively.

elevation was 50-fold at 10^{-5}M. When the cells were exposed to 10^{-6}M of CNP with 10^{-5}M of α-hANP, partial reduction of the cGMP response to CNP was observed (none, 0.04±0.02; α-hANP, 1.84±0.10; CNP, 6.98±0.55; CNP plus α-hANP, 4.51±0.01 pmole/10^5cells, mean±S.E. of 3 determinations).

Membrane fraction of VSMC was prepared, and subjected to the assay of particulate guanylate cyclase activity. CNP activated the enzyme more potently than α-hANP (Fig. 2). Maximum activation induced by CNP was about 4-fold greater than that of α-hANP, and the EC_{50} for CNP and α-hANP was $1.1x10^{-7}$M and $8.0x10^{-7}$ M, respectively. The stimulation induced by CNP and α-hANP was both potentiated by an addition of 0.5mM ATP without a shift in the EC_{50}.

In both renal glomeruli and VSMC, CNP inhibited the binding of [^{125}I]α-hANP with a less potent affinity than α-hANP (Fig. 3A, B). The IC_{50} values for CNP and α-hANP were $8.3x10^{-9}$M and $1.0x10^{-9}$M in glomeruli, and $3.5x10^{-9}$M and $1.1x10^{-9}$M in VSMC, respectively.

Fig. 4A demonstrates a specific and saturable binding of [^{125}I]Y^0-CNP in VSMC. Analysis of the saturation binding data according to Scatchard resulted in a linear plot, suggesting the presence of a single class receptor with an apparent dissociation constant (Kd) of $3.8x10^{-9}$M and a maximum binding (Bmax) of 42 fmole/10^5cells (Fig. 4B). [^{125}I]α-hANP bound to the cells with a higher affinity of a Kd of $0.9x10^{-9}$M and an almost comparable Bmax of 33 fmole/10^5cells (data not shown).

Affinity-crosslinking of either [^{125}I]α-hANP or [^{125}I]Y^0-CNP with VSMC resulted in a detection of only one radioactive band of approximately 70kDa under a reduced condition. Labeling of the protein was inhibited almost completely by both CNP and α-hANP at $5x10^{-7}$M (Fig. 5).

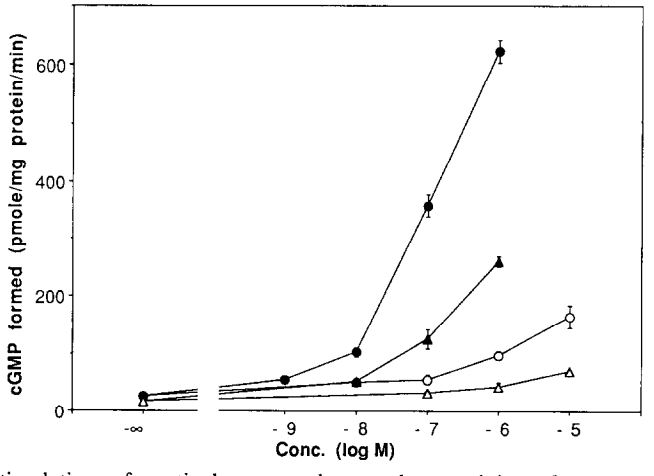

Figure 2. Stimulation of particulate guanylate cyclase activity of rat VSMC by CNP (closed symbols) in comparison with ANP (open symbols). Peptides were added to the reaction mixture for the enzyme assay at the indicated concentrations and incubated for 3 min at 37°C in the absence (triangles) or presence (circles) of 0.5mM ATP. Each value represents mean±S.E. of triplicate determinations.

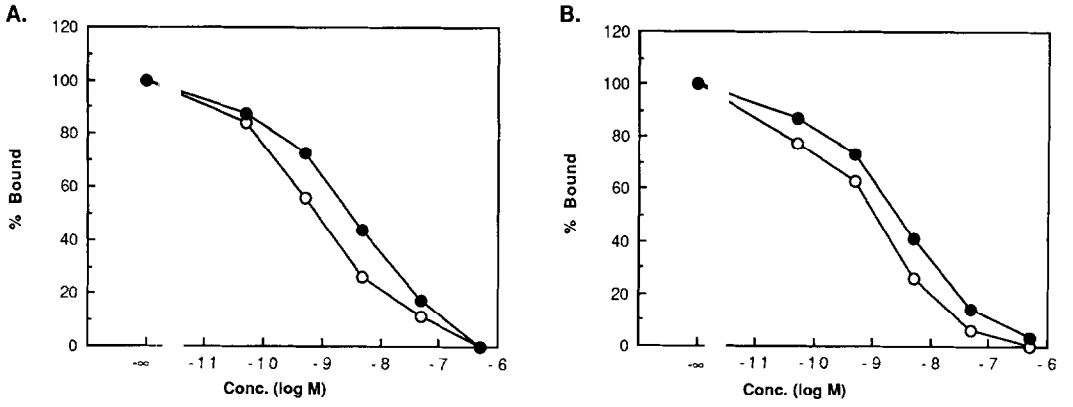

Figure 3. Displacement of [^{125}I]α-hANP from binding sites by unlabeled CNP (open circles) or ANP (closed circles) in renal glomeruli (A) and VSMC (B). Renal glomeruli or VSMC were incubated with 5x10^{-10}M [^{125}I]α-hANP in the presence of unlabeled peptides. Data represent means of triplicate determinations from two or four separate experiments in glomeruli or VSMC, respectively.

CNP relaxed aortic strips precontracted by norepinephrine with an EC_{50} of 71.7±1.8 x10^{-9}M (mean±S.E. of 5 preparations), being approximately 16-fold higher than that of α-hANP (EC_{50}=4.6±0.1 x10^{-9}M, mean±S.E. of 5 preparations). When aortic tissues were subjected to the assay after rubbing out the endothelium, the vasorelaxant responses to both CNP and α-hANP were not affected (data not shown).

DISCUSSION

In renal glomeruli, CNP competed for the binding of [^{125}I]α-hANP, whereas, the increment of the cGMP concentration was very limited. This low activity in cGMP

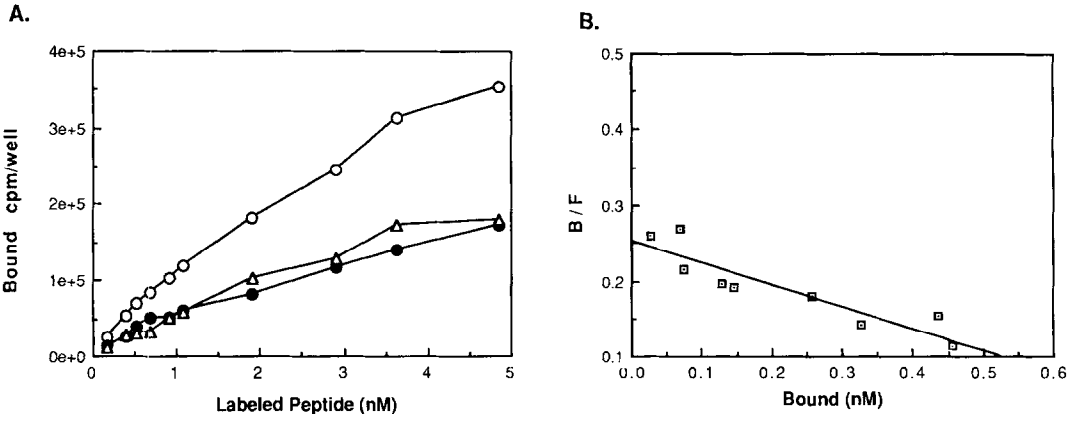

Figure 4. (A) Saturation curve of [^{125}I]Y^{0}-CNP binding to rat VSMC. VSMC were incubated at 25°C for 45min with increasing concentrations of [^{125}I]Y^{0}-CNP, and total (open circles), nonspecific (closed circles) and specific binding (open triangles) were measured. Nonspecific binding was determined by incubating the cells with [^{125}I]Y^{0}-CNP in the presence of 10^{-6}M unlabeled CNP. (B) Scatchard plot of the specific binding. Each point represents mean of quadruplicate determinations.

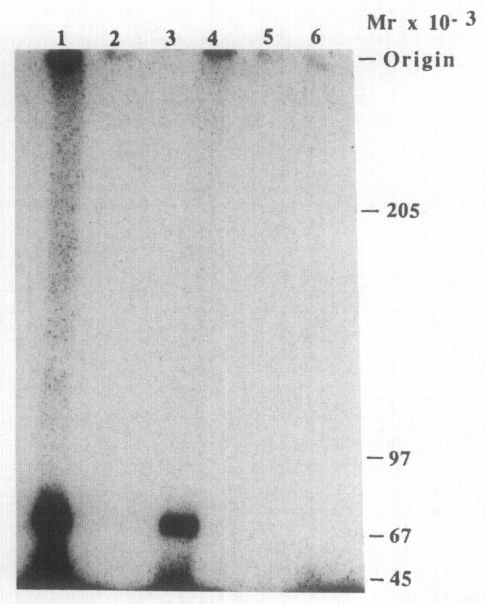

Figure 5. Autoradiography of SDS-PAGE analysis under reduced condition of [^{125}I]α-hANP and [^{125}I]Y^{0}-CNP covalently coupled to the surface of intact cultured rat VSMC. Confluent cells were incubated with 5x10^{-10}M [^{125}I]α-hANP or [^{125}I]Y^{0}-CNP in the absence (lane 1 and 4), or presence of ANP (lane 2 and 5) or CNP (lane 3 and 6) at each 5x10^{-7}M, respectively. The migration of known molecular weight markers are shown to the right.

accumulation in glomeruli is assumed to be corresponded to the low diuretic activity of the peptide in vivo (7).

On the other hand, CNP potently increased cGMP concentrations in rat VSMC. Since the simultaneous addition of α-hANP with CNP at a maximum effective dose resulted in a reduction of cGMP elevation induced by CNP, it can be suggested that both peptides stimulate cGMP production through, at least partly, a same mechanism. This would be supported by the findings that CNP as well as α-hANP stimulated particulate guanylate cyclase activity in VSMC, and the effects of the peptides were both accelerated in the presence of ATP. Furthermore, methylene blue, an inhibitor of soluble guanylate cyclase, did not affect the cGMP responses to these two peptides in VSMC (data not shown).

Although the potency of CNP for cGMP formation in VSMC was greater than that of α-hANP, kinetic binding study revealed that apparent binding affinity of CNP was 4-fold weaker than that of α-hANP. Crosslinking of the peptides to VSMC demonstrated that the detectable natriuretic peptide receptor on VSMC was mostly a 70kDa protein, which does not involve guanylate cyclase domain in the molecule (15). One interpretation for the discrepancy between the binding to the receptor and the cGMP elevation would be that CNP shares a common, 70kDa receptor with ANP in VSMC and it elicits guanylate cyclase activation through a more efficient signal transduction than ANP. Hirose et al. have demonstrated the presence of

dissociative complexes of ANP receptor and guanylate cyclase by radiation inactivation analysis, and suggested that 70kDa receptor would also be responsible for the activation of guanylate cyclase (16, 17). They have proposed that ANP may activate guanylate cyclase through a dissociation of the putative receptor-enzyme complex ; Rc•GC (inactive) + ANP → Rc•ANP + GC (active) (Rc: receptor, GC: guanylate cyclase). According to the model, CNP might be able to dissociate the receptor enzyme complex more effectively than ANP. Another interpretation is that the action of CNP might be mediated through a certain subtype of natriuretic peptide receptors, which is preferably occupied and activated by CNP rather than ANP and is only a minority of the total binding sites in VSMC. Recently, multiple natriuretic peptide receptors have been cloned and sequenced (15, 18-20). One of these has been found to require relatively high concentrations of both ANP and BNP to give a remarkable cGMP response, and it has been suggested that an unidentified natural ligand for this receptor may be present (20). CNP might be a candidate for the ligand.

To the potent effect of CNP on cGMP production in VSMC, vasorelaxant activity in isolated rat aorta was 16-fold less potent than that of α-hANP. We have found that some ANP analogs in which cysteine residues were substituted by penicilamine residues also stimulated cGMP production effectively in VSMC with weak vasorelaxant activities (21). It has also been reported that an ANP analog, Lys^{11} [7-23]ANP possessed similar property (22). These observations suggest possibilities that i) cGMP elevation may not be a sole factor sufficient to promote vasorelaxation of natriuretic peptides, or ii) natriuretic peptide receptors in the cultured cells may be different from those in intact aortic smooth muscles as reported previously (23, 24).

In conclusion, it must be noteworthy that CNP significantly increases cGMP contents in VSMC but not in glomeruli, and shows very weak vasorelaxant activity in aorta. Such properties of the peptide have never observed in ANP and BNP, suggesting unique physiological functions of CNP other than diuresis and hypotension. This peptide is originally found in porcine brain, and is assumed to be present in a higher concentration than ANP. The studies on the activities of CNP in various tissues including central nervous system are now going on.

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