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Research Article

C-type natriuretic peptide (CNP), the third member of the natriuretic peptide family, is thus far known to be distributed mainly in the central nervous system and is considered to act as a neuropeptide, in contrast to atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), which act as cardiac hormones. Recently, we and others have demonstrated that the ANP-B receptor, which is selectively activated by CNP, is localized not only in the central nervous system but in peripheral tissues, including blood vessels. This finding has made us speculate regarding the peripheral production of CNP. In the present study, cultured endothelial cells were examined for CNP production by RIA and Northern blot analysis. CNP-like immunoreactivity was detected in the conditioned media of endothelial cells. Northern blot analysis detected CNPmRNA with a size of 1.2 kb. In addition, transforming growth factor (TGF)-beta, one of the key growth factors for vascular remodeling, markedly stimulated the expression of CNPmRNA and induced a tremendous increase in CNP secretion. We could also detect CNP transcript in the bovine thoracic aorta using the reverse transcription-polymerase chain reaction method. The present study demonstrates the endothelial production of CNP and suggests that a member of the natriuretic peptide family may act as a local regulator in vascular walls. Since evidence for the pathophysiological importance of the vascular renin-angiotensin system […]

Endothelial Production of C-Type Natriuretic Peptide and Its Marked Augmentation by Transforming Growth Factor-β

Possible Existence of "Vascular Natriuretic Peptide System"

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Abstract

C-type natriuretic peptide (CNP), the third member of the natriuretic peptide family, is thus far known to be distributed mainly in the central nervous system and is considered to act as a neuropeptide, in contrast to atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), which act as cardiac hormones. Recently, we and others have demonstrated that the ANP-B receptor, which is selectively activated by CNP, is localized not only in the central nervous system but in peripheral tissues, including blood vessels. This finding has made us speculate regarding the peripheral production of CNP. In the present study, cultured endothelial cells were examined for CNP production by RIA and Northern blot analysis. CNP-like immunoreactivity was detected in the conditioned media of endothelial cells. Northern blot analysis detected CNPmRNA with a size of 1.2 kb. In addition, transforming growth factor (TGF)-β, one of the key growth factors for vascular remodeling, markedly stimulated the expression of CNPmRNA and induced a tremendous increase in CNP secretion. We could also detect CNP transcript in the bovine thoracic aorta using the reverse transcription-polymerase chain reaction method.

The present study demonstrates the endothelial production of CNP and suggests that a member of the natriuretic peptide family may act as a local regulator in vascular walls. Since evidence for the pathophysiological importance of the vascular renin-angiotensin system has been accumulating and the natriuretic peptide system is known to be antagonistic to the renin-angiotensin system, the possible existence of "vascular natriuretic peptide system" may prove to be of physiological and clinical relevance. (*J. Clin. Invest.* 1992. 90:1145–1149.) Key words: natriuretic peptides • endothelial cells • natriuretic peptide receptors • growth factors • vascular remodeling

Introduction

Since the discovery of atrial natriuretic peptide (ANP)[1] and brain natriuretic peptide (BNP) in the mammalian heart and brain, ANP and BNP have been considered to form a natriuretic peptide family responsible for body fluid homeostasis and blood pressure control, both as cardiac hormones and as neuropeptides (1–7). The third member of the natriuretic peptide family, C-type natriuretic peptide (CNP), which has recently been isolated from the porcine brain, has 22 amino acid residues (8). CNP has a ring structure formed by an intramolecular disulfide bond, which is conserved in ANP and BNP. An NH_2-terminally elongated form of CNP with 53 amino acid residues (CNP-53) has been subsequently isolated from the porcine brain (9). While the major production site for ANP and BNP is the heart (2–4, 6, 7), it has been reported thus far that CNP is distributed not in the heart but mainly in the central nervous system (10, 11), and thus it has been claimed that CNP acts principally as a neuropeptide.

At least three receptors for the natriuretic peptide family, namely the ANP-A receptor, ANP-B receptor, and clearance receptor, have been identified (12). We and others have demonstrated that the ANP-B receptor is selectively activated by CNP (13, 14). Interestingly, the ANP-B receptor is distributed not only in the central nervous system but also in peripheral tissues, including blood vessels (13, 15, 16). These findings have led us to speculate that CNP is locally synthesized in peripheral organs besides the heart, and that this locally synthesized hormone may serve as a regulator of the "extracardiac peripheral natriuretic peptide system," like the extrarenal local renin-angiotensin system (RAS) (17–19). We focused on the blood vessels, since accumulating evidence suggests the significant role of the vascular RAS for the vascular tone and remodeling (19, 20), and it has been recently demonstrated that natriuretic peptides can modulate not only vascular tone, but also vascular growth in an antagonistic way to the vascular RAS (21).

The present study demonstrates the production of CNP in cultured endothelial cells (EC), its marked augmentation by transforming growth factor (TGF)-β, and CNP gene expression in intact aorta by reverse transcription-polymerase chain reaction (RT-PCR) method.

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1. *Abbreviations used in this paper:* ANP, atrial natriuretic peptide; AVP, arginine-vasopressin; bFGF, basic fibroblast growth factor; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; EC, endothelial cells; ET, endothelin; HP-GPC, high performance gel permeation chromatography; LI, like immunoreactivity; PDGF, platelet-derived growth factor; RAS, renin-angiotensin system; RT-PCR, reverse transcription-polymerase chain reaction; SMC, smooth muscle cells; TGF-β, transforming growth factor-β; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

Methods

Cell culture. EC were isolated from adult bovine carotid artery by scraping the intimal surface with a scalpel and cultured in DME (Flow Laboratories, Irvine, Ayrshire, Scotland) supplemented with 10% FCS (Hazleton Biologics, Inc., Lenexa, KS) at 37°C in a humidified atmosphere containing 5% CO_2 (22). Cells at passages 20–25 were used in the present study. EC were identified by the uptake of fluoresceinated acetylated low density lipoprotein. Vascular smooth muscle cells (SMC) were obtained from thoracic aortae of male Wistar rats, as previously reported (13, 16).

Preparation of conditioned media and cell extracts. EC and vascular SMC were grown to confluence in 6-cm culture dishes. The cells were washed twice with serum-free DME and maintained in 2 ml of DME containing 0.5% FCS for 24 h before experiments. During the experiments, the dying cells were < 1% of total cells, as confirmed by trypan blue uptake. To study the release of natriuretic peptides, media from cells were replaced with 2 ml of fresh DME containing 0.5% FCS and the cells were then incubated at 37°C for indicated time with or without following agents: TGF-β (R & D Systems, Inc., Minneapolis, MN), basic fibroblast growth factor (bFGF) (R & D Systems, Inc.), platelet-derived growth factor (PDGF)-AA (BACHEM Feinchemikalien AG, Bubendorf, Switzerland), PDGF-BB (BACHEM Feinchemikalien AG), human thrombin (Sankyo Pharmaceutical Co., Tokyo, Japan), arginine-vasopressin (AVP) (Peptide Institute, Inc., Minoh, Japan), 8-bromo cyclic GMP (cGMP) (Sigma Chemical Co., St. Louis, MO), 8-bromo cyclic AMP (cAMP) (Sigma Chemical Co.), and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (Sigma Chemical Co.). After the incubation, conditioned media were collected and centrifuged at 600 *g* for 10 min. The supernatants were stored at −20°C. Cells were washed twice with ice-cold Dulbecco's PBS, mechanically scraped, boiled for 7 min in 0.1 M acetic acid containing 0.1% Triton X-100, and disrupted by sonication (Handy Sonic; Tomy Seiko Co., Ltd., Tokyo, Japan). The cell homogenates were centrifuged at 10,000 *g* for 30 min at 4°C and the supernatants were stored at −20°C.

RIA for CNP. The RIA for CNP was performed as previously reported (11). The cross-reactivities with α-ANP, porcine BNP, rat BNP, and CNP-53 were 0.2%, 14%, < 0.01%, and 100% on a molar basis, respectively.

RIA for ANP. ANP concentrations were measured using the specific RIA for ANP (23). The cross-reactivities with porcine BNP, rat BNP, and CNP were < 0.1% on a molar basis.

RIA for BNP. Since amino acid sequences of BNP are divergent among species, in contrast to those of ANP and CNP (6, 7, 13), concentrations of bovine BNP and rat BNP were determined by specific RIA for each peptide. Bovine BNP concentrations were measured by the RIA for porcine BNP (24) because the COOH-terminal 26-amino acid sequence of bovine BNP is identical to porcine BNP (25). The cross-reactivities with α-ANP and CNP were less than 0.2% on a molar basis. Measurement of rat BNP concentrations was performed as we reported (7). The cross-reactivities with α-ANP and CNP were less than 0.1% on a molar basis.

RIA for endothelin (ET). Measurement of ET-1 concentrations was performed as described (26). The cross-reactivities with ET-2, ET-3, and human big ET-1 in the RIA were 80%, 20%, and 80% on a molar basis, respectively.

High performance-gel permeation chromatography (HP-GPC). HP-GPC was performed on a TSK-GEL G2,000 SW column (7.5 × 600 mm; Toyo Soda, Tokyo, Japan) eluted with 10 mM trifluoroacetic acid containing 0.3 M sodium chloride and 30% acetonitrile as a solvent, as we reported (6, 23). The flow rate was 0.3 ml/min and the fraction volume was 0.36 ml.

RNA extraction and Northern blot analysis. RNA was extracted from confluent cultured bovine EC and the rat brain by guanidinium thiocyanate CsCl method (6, 13), and was subjected to poly $(A)^+$ RNA enrichment. Northern blot analysis was performed as reported elsewhere (6, 13), using the rat CNPcDNA probe (27) and human β-actin genome probe (Wako Pure Chemical, Osaka, Japan).

PCR and Southern blot analysis of RT-PCR products. Fresh bovine thoracic aorta was obtained from a local slaughterhouse and periadventitial tissues were carefully removed. Based on the nucleotide sequence of the human CNP gene and rat CNPcDNA (10, 27), two oligonucleotide primers for PCR were prepared (sense, 5'-ACCATGCACCTCTCCCAGCT-3'; antisense, 5'-CTAACATCCCAGGCCGCTCA-3'), which correspond to the 5'- and 3'- ends of the CNP coding sequence, respectively. After reverse transcription of 10 μg of total RNA from the bovine aorta by oligo (dT) priming and Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories Inc., Gaithersburg, MD), the resulting single-stranded cDNA was subjected to PCR as reported (13, 27). To further confirm the specificity of PCR products, Southern blot analysis was performed using ^{32}P-labeled synthetic oligomer (5'-CAACAAGAAGGGCTTGTCCAA-3') as a probe (28). The nucleotide sequence of this probe is conserved in human and rat preproCNP (10, 27).

Peptides. α-Human ANP, porcine BNP, rat BNP, CNP and ET-1 were purchased from Peptide Institute, Inc. CNP-53 was donated by Shionogi Research Laboratories (Shionogi & Co., Ltd., Osaka, Japan).

Statistics. Values are expressed as the means±SEM. Statistical analysis of data was performed using Student's *t* test or one way analysis of variance.

Results

Detection of CNP-like immunoreactivity (LI) in EC and EC-conditioned media. The serial dilution curve of the conditioned media of EC was parallel to the standard curve of CNP, as depicted in Fig. 1 *A*. CNP-LI in the EC-conditioned media increased time dependently (Fig. 1 *B*), and the concentration of CNP-LI in the conditioned media was 17.8±1.6 fmol/10^6

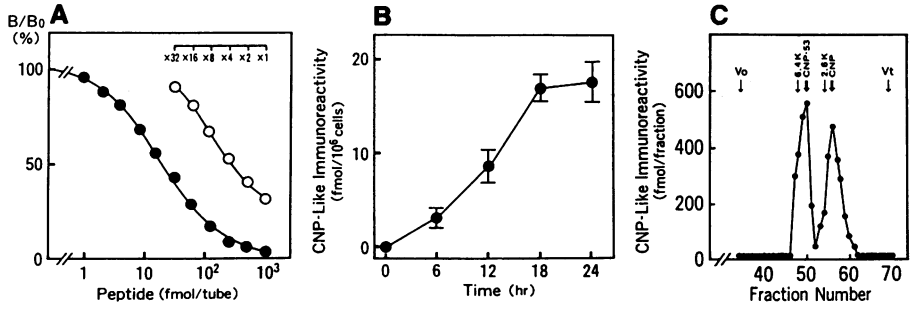

Figure 1. (*A*) A typical standard curve of CNP (*closed circle*) and a dilution curve of the conditioned media of cultured bovine EC (*open circle*). (*B*) The amount of CNP-LI accumulated in the media as function of incubation time. Each point represents mean±SEM of three separate experiments. (*C*) A typical HP-GPC profile of CNP-LI in the conditioned media of cultured bovine EC. Arrows denote elution positions of a series of myoglobins of a polypeptide molecular weight calibration kit (Pharmacia, Uppsala, Sweden), void volume (Vo), and total volume (Vt). Elution positions of synthetic CNP-53 and CNP are also indicated.

cells per 24 h. In contrast, both ANP-LI and BNP-LI concentrations in the conditioned media were undetectable (below 0.1 fmol/10^6 cells per 24 h). The cellular content of CNP-LI was 0.15±0.05 fmol/10^6 cells, but neither ANP-LI nor BNP-LI was detectable in cell extracts. The amount of CNP-LI in EC-conditioned media for 24 h incubation was ~ 100-fold larger than the cellular content of CNP-LI. The ratio of CNP-LI in the media to that in the cells was similar to the ratio of ET-1-LI (3.87±0.12 pmol/0.0637±0.0036 pmol/10^6 cells per 24 h). Fig. 1 *C* shows a representative HP-GPC profile of CNP-LI in EC-conditioned media. CNP-LI was composed of two components. One peak eluted at the position of 2–3 kD, corresponding to synthetic CNP, and the other peak eluted at the position with an apparent molecular weight of 5–6 kD, corresponding to synthetic CNP-53. The presence of two components of CNP-LI corresponding to CNP and CNP-53 is consistent with our previous observation on CNP-LI in the rat and human brain (11, 27).

In contrast to EC, we could not detect ANP-LI, BNP-LI, nor CNP-LI in vascular SMC-conditioned media.

Regulation of CNP secretion from EC. Table I shows the effects of various agents that affect vascular tone and/or growth on CNP-LI concentrations in EC-conditioned media. Among growth factors, TGF-β caused a tremendous increase in the CNP-LI concentration in the conditioned media. As shown in Fig. 2, TGF-β stimulated the accumulation of CNP-LI in EC-conditioned media in a concentration-dependent manner (10^{-12}–10^{-9} M). TGF-β (10^{-9} M) augmented the accumulation of CNP-LI more than two orders of magnitude. The stimulatory effect of TGF-β seems to be potent and specific for CNP, when compared to that of TGF-β on ET-1-LI secretion (1.5-fold increase at 10^{-9} M TGF-β), which is consistent with the result reported previously (29). Neither ANP-LI nor BNP-LI was detected in the conditioned media and cell extracts from EC exposed to TGF-β.

While PDGF-AA and PDGF-BB had insignificant effects on the CNP-LI concentration, bFGF also elicited a significant increase in the CNP-LI concentration. Thrombin and AVP caused an increase of the CNP-LI concentration in EC-conditioned media. TPA, 8-bromo cGMP and 8-bromo cAMP also significantly stimulated the accumulation of CNP-LI in EC-conditioned media.

Table I. Effects of Various Agents on CNP-LI and ET-LI Concentrations in Conditioned Media of Bovine EC

	CNP-LI	ET-LI
	fmol/10^6 cells	*pmol/10^6 cells*
Vehicle	17.2±1.2	4.17±0.11
TGF-β 10^{-9} M	2230±180*	6.32±0.21*
bFGF 10^{-9} M	43.7±4.0*	4.51±0.14*
PDGF-BB 10^{-9} M	21.8±4.5	4.24±0.25
PDGF-AA 5×10^{-9} M	20.1±1.5	4.30±0.21
8-bromo cGMP 10^{-3} M	31.2±4.2*	3.95±0.11
8-bromo cAMP 10^{-3} M	56.3±7.9*	4.31±0.23
TPA 10^{-6} M	50.6±2.5*	4.04±0.09
AVP 10^{-6} M	37.2±5.5*	4.96±0.17*
Thrombin 10 U/ml	51.0±4.8*	5.80±0.17*

Cultured EC were incubated in the presence of various agents or vehicle for 24 h. Values are means±SEM of four separate experiments.
* Significantly different from vehicle ($P < 0.05$).

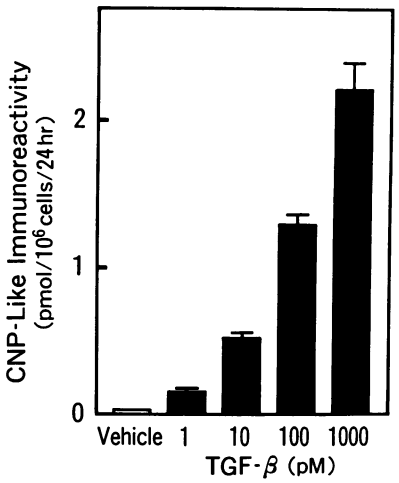

Figure 2. Effects of TGF-β on CNP-LI secretion from cultured bovine EC. Cells were incubated with different concentrations of TGF-β for 24 h. Each point represents mean±SEM of four separate experiments.

Detection of CNPmRNA and regulation of its expression by TGF-β in EC. Northern blot analysis detected CNPmRNA with a size of 1.2 kb in a limited quantity in EC (Fig. 3 *A*, lane *1*), which has the same size as that from the rat brain (Fig. 3 *A*, lane *3*) (10). The exposure to TGF-β (10^{-10} M) for 6 h markedly enhanced the expression of CNPmRNA (Fig. 3 *A*, lane *2*). The intensity of the hybridizing band for CNPmRNA in TGF-β–treated EC was comparable to that in the rat brain (Fig. 3 *A*, lane *3*). Actin mRNA levels were essentially equivalent among different RNA samples.

Detection of CNP transcript in aorta. To examine CNP gene expression in vivo, total RNA from the bovine aorta was reverse-transcribed into cDNA and the RT-PCR was performed. As shown in Fig. 3 *B*, lane *1*, the CNP transcript, which has the identical size to the predicted one (384 bp), was amplified in intact bovine aorta.

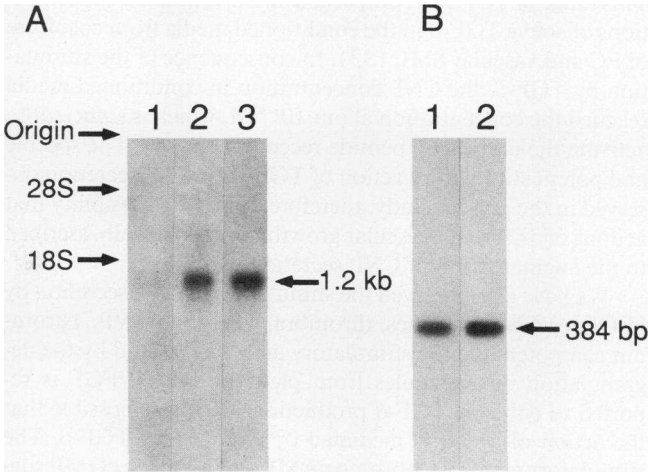

Figure 3. (*A*) Northern blot analysis on CNPmRNA in bovine EC and rat brain. Confluent bovine EC were exposed to vehicle (lane *1*) or TGF-β (10^{-10} M) (lane *2*). Expression of CNPmRNA in the rat brain is shown as a positive control (lane *3*). Poly $(A)^+$ RNA (5 μg) was used for analysis. (*B*) Southern blot analysis on RT-PCR amplified product of CNP transcript in intact bovine aorta (lane *1*). As a positive control, rat CNPcDNA subcloned into Bluescript (Stratagene Inc., La Jolla, CA) was used as a template for PCR (lane *2*).

Discussion

The present study clearly demonstrates the production and secretion of CNP in EC and their marked augmentation by TGF-β. CNP secretion from EC is stimulated by several vasoactive substances. The present study also shows the gene expression of CNP in intact vascular wall, although precise production site of CNP in vascular wall is not elucidated at present. Since vascular SMC and EC are known to contain abundant natriuretic peptide receptors (2–4, 13, 16), these findings suggest the possible existence of the local natriuretic peptide system within vascular walls. So far, the natriuretic peptide family has been identified in the heart and in the central nervous system, and has been considered to serve as cardiac hormones and as neuropeptides, respectively. The present study demonstrates that CNP, the third member of the natriuretic peptide family, may act as a local regulator in vascular walls. The possible existence of "vascular natriuretic peptide system" suggested in the present study opens up a new aspect for the physiological and pathophysiological roles of the natriuretic peptide system.

It has been elucidated that natriuretic peptides can act not only as vasodilators but also as growth inhibitors of vascular SMC and EC (21, 30). Therefore, endothelial CNP could induce relaxation and/or growth inhibition of vascular SMC in a paracrine manner and also modulate endothelial proliferation and/or function in an autocrine way. The proliferation and migration of SMC with subsequent intimal thickening is a major event in the development of atherosclerotic lesion and restenosis after angioplasty (31), both of which are clinically serious matters. Several growth factors are thought to be involved in these processes. TGF-β, a potent growth inhibitor of EC and a bifunctional growth regulator of vascular SMC, is released from platelets and synthesized by vascular SMC and EC (32, 33). In addition, TGF-β has been shown to be abundant in neointima after vascular injury (34), suggesting its significant role in vascular remodeling. In the present study, 10^{-11}–10^{-10} M of TGF-β caused a 30–60-fold stimulation of CNP secretion. This range of TGF-β is comparable to the estimated concentrations of active TGF-β in the conditioned media from coculture of EC and vascular SMC (33). In consequence of the stimulation by TGF-β, the CNP concentration in conditioned media reached the concentration about 10^{-9} M, which is sufficient to activate the natriuretic peptide receptor (13, 14). The specific and potent stimulatory action of TGF-β on CNP secretion observed in the present study, therefore, raises the possibility that actions of TGF-β on vascular growth may be partially ascribed to the augmentation of CNP secretion.

We have also observed the stimulation of CNP secretion by bFGF, cyclic nucleotides, thrombin, TPA, and AVP. Thrombin can potentiate the stimulatory action of TGF-β by the degranulation of α-granules from platelets. Since bFGF is reported to enhance TGF-β production (35), it is possible that the action of bFGF is mediated by endogenous TGF-β. The stimulatory action of 8-bromo cAMP and 8-bromo cGMP suggests the involvement of vasodilators, including endothelium-derived relaxing factor, prostaglandin I_2, and natriuretic peptides per se in CNP secretion. Augmentation by AVP and TPA raises the possibility that the activation of protein kinase C or phospholipase C-mediated phosphoinositide breakdown modulates CNP secretion. The present study also suggests that CNP is released from the endothelium constitutively, like ET-1 (36), based on the observation that the ratios of intracellular contents to the conditioned media-concentrations of these peptides are similar. Further studies are required for the elucidation of molecular mechanisms responsible for CNP secretion.

The importance of the local RAS, especially cardiovascular RAS, for the blood pressure control and vascular remodeling has been recently suggested both by the experimental and clinical studies (17–20). Angiotensin converting enzyme (ACE) inhibition reduced the vascular hypertrophy in several experimental hypertensive animals and prevented neointima formation after vascular injury (19, 20, 37). Clinically, the use of an ACE inhibitor for the prevention of cardiac hypertrophy has been demonstrated (38). Since the natriuretic peptide system appears to show functional antagonism to the RAS, both in peripheral and central actions (3, 5, 21), a vascular natriuretic peptide system might have clinical significance for cardiovascular disorders.

In conclusion, the present study demonstrates the endothelial production of CNP and its marked augmentation by TGF-β, and that CNP transcript is expressed in intact aorta. Taken together with the presence of natriuretic peptide receptors in vascular SMC and EC, these findings suggest the possible existence of a vascular natriuretic peptide system that may exert local control over vascular tone and growth, either alone or in concert with the endocrine natriuretic peptide system.

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