C-Type Natriuretic Peptide Inhibits Thrombin- and Angiotensin II–Stimulated Endothelin Release via Cyclic Guanosine 3',5'-Monophosphate

Masakazu Kohno, Takeshi Horio, Koji Yokokawa, Naotsugu Kurihara, and Tadanao Takeda

We examined the inhibitory effect of porcine C-type natriuretic peptide (CNP) on endothelin-1 secretion stimulated by thrombin and angiotensin II (Ang II) in cultured porcine endothelial cells. The results were compared with the effects of atrial (ANP) and brain (BNP) natriuretic peptides. Thrombin and Ang II produced a concentration-dependent stimulation of immunoreactive endothelin-1 secretion, and porcine CNP-22 potently inhibited this stimulated secretion in a concentration-dependent manner. CNP-22 had a stronger inhibitory effect than either porcine ANP(1-28) or porcine BNP-26. In addition, CNP potently increased the cellular level of cyclic guanosine 3',5'-monophosphate (GMP), with the inhibition of immunoreactive endothelin-1 secretion in response to thrombin and Ang II being paralleled by the increase in the cyclic GMP level. The increase of cyclic GMP produced by CNP was also greater than that due to porcine ANP(1-28) or porcine BNP-26. The immunoreactive endothelin-1 in the culture medium had two components on high-performance liquid chromatography; the major one corresponded to endothelin-1 (1-21) and the minor one to big endothelin-1 (porcine 1-39). Treatment with CNP did not affect this profile. Our results suggest that CNP probably inhibits the endothelin-1 secretion stimulated by thrombin and Ang II through a cyclic GMP–dependent process. The increase of cyclic GMP levels and the inhibition of immunoreactive endothelin-1 secretion produced by CNP appear to be greater than those produced by ANP or BNP. (Hypertension 1992;19:320-325)

KEY WORDS • peptides • atrial natriuretic peptides • endothelins • angiotensin II • thrombin • endothelium

Atrial natriuretic peptide (ANP) is a diuretic, natriuretic, and vasodilatory peptide hormone that was originally isolated from mammalian hearts.1,2 Subsequently, brain natriuretic peptide (BNP), which elicits a spectrum of diuretic, natriuretic, and vasodilatory effects very similar to those produced by ANP, was identified in the porcine brain3 and also isolated from mammalian hearts.4 Recently, a third type of natriuretic peptide, designated as C-type natriuretic peptide (CNP), was identified in the porcine brain.5 Porcine CNP has an amino acid sequence homologous to both ANP and BNP in the ring flanked by a disulfide linkage, but it also has five unique N-terminal amino acids and ends at the second cysteine with no C-terminal tail. Porcine CNP increases the level of cyclic guanosine 3',5'-monophosphate (GMP) in rat vascular smooth muscle cells, with a potency greater than that of rat ANP(1-28).6

Endothelin-1 is a 21-amino acid peptide that is produced by vascular endothelial cells and has a contractile effect.1-9 This peptide is present in human plasma10-13 and circulates at high levels in patients with acute myocardial infarction,10 certain types of severe hypertension,11,12 and subarachnoid hemorrhage.13 Cultured endothelial cells14,15 and aortic strips with intact endothelium16,17 secrete immunoreactive (ir) endothelin-1 in a time-dependent manner. The clotting enzyme thrombin and the potent vasoconstrictor angiotensin II (Ang II) both stimulate endothelin-1 secretion from endothelial cells.14-17 Recent studies by Saisionmaa et al18 and ourselves19 have shown that ANP and BNP inhibit the secretion of endothelin-1 by cultured human endothelial cells, probably via a cyclic GMP–dependent process. In the present study, we examined the effect of porcine CNP on endothelin-1 secretion and cyclic GMP levels in cultured porcine endothelial cells treated with thrombin and Ang II. The results were compared with those produced by porcine ANP(1-28) and porcine BNP-26. In addition, we examined the forms of ir-endothelin-1 in extracts of the medium obtained from cultures treated with porcine CNP by using reverse-phase, high-performance liquid chromatography (HPLC) coupled with a radioimmunoassay.

Methods

Endothelial Cell Culture

Endothelial cells were isolated from adult porcine thoracic aortas by a standard scraping technique and were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), penicillin (100 μg/ml), and streptomycin (50 μg/ml). These cells were identified by their typical "cob-
blestone appearance on phase-contrast microscopy and by their immunofluorescence after staining for factor VIII antigen. Contamination by cells with the morphological features of smooth muscle cells was not observed. Cultures were maintained at 37°C with atmospheric air and 5% CO₂, and subcultures were carried out after the treatment with Versene (Gibco Laboratories, Grand Island, N.Y.) followed by trypsin.

**Materials and Apparatus**

Ang II, 3-isobutyl-1-methylxanthine (IBMX), and the endothelial cell growth supplement were purchased from Sigma Chemical Co., St. Louis, Mo. DMEM, trypsin, Versene, and FCS were purchased from Gibco Laboratories, Grand Island, N.Y. Purified human thrombin was the gift of Sankyo Co., Tokyo, Japan. Flasks and polypropylene tubes were purchased from Becton Dickinson & Co., Lincoln Park, N.J. Synthetic endothelin-1, endothelin-2, endothelin-3, big endothelin-1 (porcine 1–39), somatostatin, β-endorphin, human secretin, porcine ANP (1–28), rat ANP (5–25), human BNP-32, porcine BNP-26, and porcine CNP-22 were purchased from the Peptide Institute, Inc., Osaka, Japan. Endothelin-1 antiserum was purchased from Yamasa Shoyu Co., Ltd., Chiba, Japan. 20

**Pharmacological Treatment**

Contained confluent cultures of endothelial cells in tissue culture flasks (3–5×10⁶ cells per well) were used in their third through fifth passages. The culture medium was removed, and the cell monolayers were washed twice with serum-free DMEM. Ang II, thrombin, ANP, BNP, or CNP was added to the replacement medium at a volume of less than 1.0%, and both the control and treated cultures were incubated for 4 hours at 37°C. All cultures were done in 2 ml serum-free DMEM. After an incubation, the medium was aspirated, centrifuged at 3,000g for 15 minutes, and stored at −80°C for use in the radioimmunoassay. A 1.5 ml sample was used for the measurement of ir-endothelin-1.

**Extraction of Immunoreactive Endothelin-1**

ir-Endothelin-1 was extracted as previously described. 21 Briefly, each sample was diluted with 2 volumes of 4% acetic acid and centrifuged. Then the solution was pumped at 1 ml/min through a Sep-Pak C₁₈ cartridge (Millipore Corp., Milford, Mass.). After the cartridge was acetic acid and centrifuged. Then the solution was dissolved in the assay buffer described below.

**Radioimmunoassay of Endothelin-1**

The ir-endothelin-1 concentration was assayed using endothelin-1 antiserum and ¹²⁵I-endothelin-1 as a tracer, as described previously. 21 This antibody showed a 100% reaction with endothelin-1 and cross-reactions of 7% with endothelin-2, 7% with endothelin-3, and 35% with big endothelin-1 (porcine 1–39). It did not cross-react with porcine ANP (1–28), rat ANP (5–25), human BNP-32, porcine BNP-26, porcine CNP, somatostatin, β-endorphin, human secretin, Ang II, or vasopressin.

The radioimmunoassay was done in an assay buffer of 0.01 M sodium phosphate (pH 7.4), containing 0.05 M NaCl, 0.1% bovine serum albumin, 0.1% Nonidet P-40, and 0.01% NaN₃. In brief, rehydrated antiserum (100 µl) was added to 100 µl sample or 100 µl standard endothelin-1 dissolved in the assay buffer, and the mixture was incubated for 24 hours at 4°C. Then approximately 15,000 cpm of ¹²⁵I-endothelin-1 was added to each reaction mixture, and incubation was continued for an additional 24 hours. Next, 100 µl diluted normal rabbit serum and 100 µl diluted goat anti-rabbit immunoglobulin G were added, and the mixture was again incubated for 24 hours. After the third incubation, the precipitate was collected by centrifugation at 1,700g for 30 minutes. The supernatant was removed by aspiration, and the pellet was counted for ¹²⁵I with a gamma counter. The effective range of the standard curve was 0.5–100 pg endothelin-1 per tube. Interassay variation was 13%, and intra-assay variation was 7%. ANP, BNP, CNP, thrombin, and Ang II did not interfere with the radioimmunoassay.

**Reverse-Phase High-Performance Liquid Chromatography**

Reverse-phase HPLC was performed using an octadecylsilica column (4.6×250 mm, Gasukuro Kogyo, Inc., Tokyo) eluted at 1 ml/min with a linear gradient of acetonitrile (33–51%) in 0.09% trifluoroacetic acid and 0.01 M NaCl. 18 Fractions of 0.5 ml were collected and assayed by radioimmunoassay. For chromatographic analysis of ir-endothelin-1, 30 ml pooled medium was separated and treated by reverse-phase HPLC.

**Cyclic GMP Measurement**

After preincubation, the cell monolayers were washed twice with serum-free DMEM and then stimulated for 30 minutes with different concentrations of ANP, BNP, or CNP dissolved in DMEM containing 0.5 mM IBMX. 18 Rapid aspiration and the addition of 2 ml ice-cold 65% ethanol stopped the reaction. Cyclic GMP levels were then measured with a cyclic GMP assay kit (Yamasa Shoyu Co., Ltd., Chiba, Japan).

**Statistical Analysis**

The statistical significance of differences was evaluated by one-way analysis of variance, and probability values were calculated by Scheffe’s method. 22 Data are given as the mean±SD.

**Results**

**Effects of Thrombin and Angiotensin II on Immunoreactive Endothelin-1 Secretion by Cultured Porcine Endothelial Cells**

Confluent cultured cells secreted ir-endothelin-1 into the medium in a time-dependent manner. The effects of thrombin and Ang II on ir-endothelin-1 secretion were concentration-dependent (Figures 1A and 1B). ir-Endothelin-1 secretion stimulated by thrombin and Ang II increased during the initial 4 hours of incubation, and then the rate of increase showed a slight but significant decline. Therefore, the subsequent experiments on ir-
FIGURE 1. Bar graphs show effects of thrombin (panel A) and angiotensin II (Ang II) (panel B) on immunoreactive (ir)-endothelin-1 secretion by cultured porcine endothelial cells. Each value is mean ± SD for six cell cultures. *Significant difference vs. baseline level (p<0.05). †Significant difference vs. treatment with 0.1 unit/ml thrombin (p<0.05). ‡Significant difference vs. treatment with 10^-8 M Ang II (p<0.05).

endothelin-1 secretion were performed with cells incubated for 4 hours.

Effect of Porcine C-type Natriuretic Peptide on Immunoreactive Endothelin-1 Secretion Before and After Stimulation With Thrombin and on Cellular Cyclic GMP Levels

The basal secretion of ir-endothelin-1 was not affected by porcine CNP-22 at concentrations between 10^-12 and 10^-6 M (Figure 2A).

The effects of porcine CNP-22, porcine ANP(1-28), and porcine BNP-26 on ir-endothelin-1 secretion by cells treated with 1.0 unit/ml thrombin are shown in Figure 2A. CNP, ANP, and BNP all potently inhibited the secretion of ir-endothelin-1 in a concentration-dependent manner, and the inhibition by CNP was greater than that produced by either ANP or BNP at concentrations between 10^-10 and 10^-6 M.

In parallel with the inhibition of ir-endothelin-1 secretion, cellular cyclic GMP levels increased after treatment with ANP, BNP, and CNP (Figure 2B). The stimulatory effect of CNP on the cyclic GMP level was also greater than the effects of ANP and BNP. There was a significant inverse correlation between the percent decrease in ir-endothelin-1 secretion and the percent increase in the cellular cyclic GMP level (Figure 3).

Effect of Porcine C-type Natriuretic Peptide on Immunoreactive Endothelin-1 Secretion After Stimulation With Angiotensin II and on Cellular Cyclic GMP Levels

Figure 4A shows the effects of porcine CNP-22, porcine ANP(1-28), and porcine BNP-26 on ir-endothelin-1 secretion by cells treated with 10^-8 M Ang II. The effect of CNP on these cells was essentially the same as that seen after thrombin stimulation. CNP,

FIGURE 2. Panel A: Line graph shows effects of porcine C-type natriuretic peptide (CNP)-22, porcine atrial natriuretic peptide (ANP)(1-28), and porcine brain natriuretic peptide (BNP)-26 on immunoreactive (ir) endothelin-1 secretion in cells stimulated with 1.0 unit/ml thrombin. Cells were exposed to different concentrations of porcine CNP-22 ( ), porcine ANP(1-28) ( ), and porcine BNP-26 ( ) for 4 hours at 37°C. Effect of porcine CNP-22 ( ) on the spontaneous secretion of ir-endothelin-1 is also shown. Panel B: Line graph shows effects of CNP-22, ANP(1-28), and BNP-26 on the cyclic GMP level in cells stimulated with 1.0 unit/ml thrombin. Cells were exposed to different concentrations of CNP-22 ( ), ANP(1-28) ( ), BNP-26 ( ) for 30 minutes at 37°C in the presence of 0.5 mM 3-isobutyl-1-methylxanthine. Each point is the mean of six measurements. *Significant difference vs. addition of thrombin alone (p<0.05).
ANP, and BNP all potently inhibited the secretion of ir-endothelin-1 in a concentration-dependent manner; inhibition by CNP was greater than that by both ANP and BNP from $10^{-8}$ to $10^{-6}$ M and was greater than that by ANP from $10^{-12}$ to $10^{-8}$ M. In parallel with the inhibition of ir-endothelin-1 secretion, the cellular cyclic GMP level increased (Figure 4B). Again, there was a significant inverse correlation between the percent decrease in ir-endothelin secretion and the percent increase in cyclic GMP ($n=12$, $r=-0.90$, $p<0.01$).

High-Performance Liquid Chromatography of Immunoreactive Endothelin-1 in Extracts of Medium From C-type Natriuretic Peptide-Treated Cultures

The reverse-phase HPLC elution profiles of extracts from the medium of cultures treated with 1.0 unit/ml thrombin or $10^{-8}$ M Ang II in the presence or absence of porcine CNP are shown in Figure 5. Two components of ir-endothelin-1 were observed, a major component eluted at the position of synthetic endothelin-1 (1–21) and a minor component eluted at the position of big endothelin-1 (porcine 1–39). Addition of CNP to the cultures did not affect this elution pattern.

Discussion

We confirmed that the clotting enzyme thrombin and the vasoconstrictive peptide Ang II stimulated ir-endothelin-1 secretion by cultured porcine endothelial cells in a concentration-dependent manner18 and that porcine CNP as well as ANP and BNP potently inhibited ir-endothelin-1 secretion and increased cyclic GMP levels in cells treated with thrombin and Ang II. In fact, CNP produced a more potent inhibition of ir-endothelin-1 secretion than either ANP or BNP. The inhibitory

![Figure 4](image-url)

**FIGURE 4.** Panel A: Line graph shows effect of porcine C-type natriuretic peptide (CNP)-22, porcine atrial natriuretic peptide (ANP)(1–28), and porcine brain natriuretic peptide (BNP)-26 on immunoreactive (ir) endothelin-1 secretion in cells stimulated with $10^{-8}$ M angiotensin II (Ang II). Cells were exposed to different concentrations of porcine CNP-22 (○), porcine ANP(1–28) (○), and porcine BNP-26 (○) for 4 hours at 37°C. Panel B: Line graph shows effects of CNP-22, ANP(1–28), and BNP-26 on the cellular cyclic GMP level in cells stimulated with $10^{-8}$ M Ang II. Cells were exposed to different concentrations of CNP-22 (○), ANP(1–28) (○), and BNP-26 (○) for 30 minutes at 37°C in the presence of 0.5 mM 3-isobutyl-1-methylxanthine. Each point is the mean of six measurements. *Significant difference vs. addition of Ang II alone ($p<0.05$).

![Figure 5](image-url)

**FIGURE 5.** Line graph shows reverse-phase high-performance liquid chromatography of immunoreactive (ir) endothelin-1 in extracts from control culture medium, medium from cultures treated with thrombin or angiotensin II (Ang II), and medium from cultures treated with porcine C-type natriuretic peptide (CNP)-22 plus thrombin or Ang II. Elution positions of standard endothelin-1, endothelin-2, endothelin-3, and big endothelin-1 (porcine 1–39) are shown by the arrows.
effects of these three natriuretic peptides on endothelin-1 secretion were paralleled by an increase of cyclic GMP in the cells treated with thrombin and Ang II, and there was a close correlation between the percent decrease in ir-endothelin-1 and the percent increase in cyclic GMP. These results suggest that CNP probably inhibits thrombin- and Ang II-stimulated endothelin-1 secretion via a cyclic GMP-dependent process, as is the case for ANP and BNP.16,19 This explanation seems to be consistent with the findings recently reported by Boulanger and Lüscher,17 Saijonmaa et al,18 and ourselves19 that a cyclic GMP analogue reduced ir-endothelin-1 secretion after the stimulation of cultured human endothelial cells and porcine aorta with thrombin or Ang II. Concerning the elevation of cyclic GMP, CNP was more potent than both ANP and BNP. One possible explanation is that the cultured endothelial cells expressed one different type of natriuretic peptide receptor that was preferentially bound and activated by CNP. Molecular cloning has defined two types of natriuretic peptide receptors that contain guanylate cyclase domain and are designated as the ANP-A and ANP-B receptors.23,24 CNP has been shown to bind to the ANP-B receptor with a very high affinity,25 so it is possible that the marked elevation of cyclic GMP produced in the cultured endothelial cells by CNP may have been mediated through binding to the ANP-B receptor. In addition, we found that the ir-endothelin-1 in the culture medium consisted of two components, with the major one corresponding to endothelin-1 (1–21) and the minor one (1–17) of endothelin-1. Treatment with CNP did not affect the ratio of big endothelin-1 to endothelin-1, suggesting that the mechanism of the changes due to CNP did not involve any effect on the activity of endothelin converting enzyme.

The basal secretion of endothelin-1 was not affected by CNP, so its spontaneous secretion by endothelial cells appears to be insensitive to modulation by CNP. This finding seems to be consistent with the report of Boulanger and Lüscher.17 Overall, our results suggest that CNP may act to reduce the excess secretion of endothelin-1 caused by activation of the coagulation cascade or the renin-angiotensin system and that it may work together with ANP and BNP in this respect. However, it remains uncertain whether CNP has a physiological role as a modulator of endothelin-1 secretion from vascular endothelial cells. For example, CNP has not yet been isolated from the circulating blood despite the wide distribution of its binding sites.6

Endothelin and its receptors are distributed throughout the vascular and nonvascular elements of the brain, including the neurons and the glial cells,26–28 and the intracerebroventricular administration of endothelin has been reported to increase the blood pressure and the heart rate.30 Accordingly, this peptide has been suggested to participate in the central control of cardiovascular functions. ANP and BNP are also present in the brain as neuropeptides as well as in the heart as cardiac hormones. CNP is assumed to be present at a higher concentration than ANP in the brain.4 It therefore seems possible that the inhibitory effect of CNP on endothelin-1 secretion in response to stimulation with thrombin and Ang II may represent an additional mechanism through which the endogenous brain CNP contributes to the central control of cardiovascular function. Thus, the ANP-BNP-CNP system may act as a modulator of endothelin-1 secretion, especially when the coagulation cascade or the renin-angiotensin system has been activated.

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References


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