C-Type Natriuretic Peptide Upregulates Vascular Endothelin Type B Receptors

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_Endothelin is a potent vasoconstrictor peptide originally isolated from cultured vascular endothelial cells_.

Abstract Cultured rat vascular smooth muscle cells (VSMCs) possess receptors for potent vasoconstrictor endothelin-1 (ET-1) as well as potent vasodilator natriuretic peptides (atrial, brain, and C-type natriuretic peptides [ANP, BNP, and CNP, respectively]). However, little is known about molecular interactions between endothelin receptors and natriuretic peptides in VSMCs. To elucidate whether natriuretic peptides regulate vascular endothelin receptors, we studied the effects of three natriuretic peptides on the capacity of 125I-ET-1 binding and expression of endothelin type A (ET_A) and type B (ET_B) receptor mRNAs in cultured rat VSMCs. CNP (10^(-6) mol/L) increased 125I-ET-1 binding capacity in a time-dependent manner (6 to 48 hours) and stimulated cyclic GMP (cGMP) generation in a dose-dependent manner (10^(-8) to 10^(-6) mol/L). Pretreatment with CNP (10^(-6) to 10^(-4) mol/L) and 8-bromo-cGMP (10^(-2) to 10^(-3) mol/L) for 24 hours resulted in dose-dependent increases in 125I-ET-1 binding in VSMCs. The three natriuretic peptides at the highest concentration (10^(-6) mol/L) increased 125I-ET-1 binding and stimulated cGMP generation with almost the same rank order of efficacy (CNP>BNP>ANP). Scatchard analysis of binding studies revealed that CNP (10^(-6) mol/L) and 8-bromo-cGMP (10^(-3) mol/L) increased vascular endothelin receptor number by 28% and 88%, respectively, without changing its affinity. Pretreatment with both CNP and 8-bromo-cGMP increased ET-1-stimulated inositol 1,4,5-trisphosphate formation. Up-regulation of endothelin receptors induced by CNP was completely blocked by inhibitors for guanylyl cyclase-linked natriuretic peptide receptors (HS-142-1) and cGMP-dependent protein kinase (KT-5823). Northern blot analysis using cDNAs for rat ET_A and ET_B as probes demonstrated that pretreatment with both CNP and 8-bromo-cGMP increased steady-state levels of ET_A receptor mRNA but not ET_B receptor mRNA in rat VSMCs. These data suggest that CNP upregulates vascular ET_B receptors via a cGMP-dependent mechanism. (Hypertension. 1994;23[part 2]:936-940.)

Key Words: natriuretic peptides, C-type • endothelins • receptors, endothelin • muscle, smooth, vascular

Methods

Compounds

Synthetic rat ANP(1-28) (rANP), rat BNP(1-32) (rBNP), CNP(1-22), and ET-1 were purchased from Peptide Institute Inc; 8-bromo-cGMP and 3-isobutyl-1-methylxanthine (IBMX) from Sigma Chemical Co; the cGMP-dependent protein kinase inhibitor KT-5823 from Kyowa Hakko Kogyo Co, Ltd; and 125I-ET-1 (specific activity, 2000 Ci/mmol) and deoxyxystidine 5'-[32P]triphosphate ([32P]dCTP) (specific activity, 3000 Ci/mmol) from Amersham International. HS-142-1, a guanylyl cyclase-linked natriuretic peptide receptor antagonist, was a generous gift from Dr Y. Matsuda, Kyowa Hakko Research Laboratory, Tokyo, Japan.

Cell Culture

VSMCs were prepared from the thoracic aorta of 15-week-old male Wistar rats by the explant method and were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum at 37°C in a humidified atmosphere of 95%
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Fig 1. Line graph shows effect of C-type natriuretic peptide (CNP) on 123I-endothelin-1 binding to cultured rat vascular smooth muscle cells as a function of time. After pretreatment with CNP (10^-6 mol/L) for indicated times, 123I-endothelin-1 binding study was performed. Each point represents mean percentage of specific binding to basal binding (n=3); bars show SEM. *P<.05 vs control.

Binding Experiments
Confluent VSMCs (5×10^5 cells) were incubated in serum-free DMEM for 24 hours, after which cells were usually pretreated with three natriuretic peptides (rANP, rBNP, CNP) and 8-bromo-cGMP for 24 hours unless otherwise stated. Cells were incubated with 6 pmol/L 123I-ET-1 at 37°C for 60 minutes in Hanks’ balanced salt solution (HBSS) containing 0.1% bovine serum albumin in the same manner as reported. After completion, cells were extensively washed and solubilized in 0.5N NaOH, and cell-bound radioactivity was determined. Specific binding was obtained by subtracting nonspecific binding in the presence of excess (4×10^-7 mol/L) unlabeled ET-1 from total binding.

Measurement of Inositol 1,4,5-Trisphosphate
Confluent VSMCs (5×10^5 cells) pretreated with 10^-6 mol/L CNP and 10^-3 mol/L 8-bromo-cGMP for 24 hours were incubated at 37°C for 30 seconds in HBSS containing 10 mmol/L LiCl. Incubation was terminated by the addition of perchloric acid. Inositol 1,4,5-trisphosphate (IP_3) was measured by a radioreceptor assay kit (Du Pont) as reported.

Measurement of Intracellular cGMP
Confluent VSMCs (5×10^5 cells per well) were incubated with or without 10^-6 mol/L each of rANP, rBNP, and CNP at 37°C for 10 minutes in HBSS containing 0.5 mmol/L IBMX. Intracellular cGMP was determined by a radioimmunoassay kit (New England Nuclear) as reported.

Northern Blot Analysis
Total RNAs from VSMCs were extracted by the acid guanidinium thiocyanate–phenol–chloroform method. Northern blot analysis using cDNAs for rat ET_4 and ET_6 receptors as probes was performed as recently described. Briefly, total RNAs (15 µg) from cultured VSMCs were separated by formaldehyde/1.1% agarose gel electrophoresis and transferred to a nylon membrane. cDNA probes for rat ET_A and ET_B receptors were labeled with [32P]dCTP by the random-primed method and incubated at 42°C for 16 hours with the membranes in the presence of 50% formamide. The membranes were washed with 0.3 mol/L NaCl, 30 mmol/L sodium citrate, and 1% sodium dodecyl sulfate at 60°C and were autoradiographed on X-Omat AR film (Eastman Kodak Co) at -80°C for 3 days.

Statistical Analysis
Data are expressed as mean±SEM. Statistical analysis was performed by unpaired Student’s t test. A probability value less than .05 was considered statistically significant.

Results
As shown in Fig 1, CNP (10^-6 mol/L) increased specific binding of 123I-ET-1 to VSMCs in a time-dependent (6 to 48 hours) manner, reaching apparent equilibrium between 24 and 48 hours. CNP (10^-6 to 10^-3 mol/L) and 8-bromo-cGMP increased 125I-ET-1 binding to VSMCs in a dose-dependent (10^-8 to 10^-3 mol/L) manner (Fig 2A). Approximately 28% and 47% of the increases in 125I-ET-1 binding over control levels were induced with 10^-6 mol/L CNP and 10^-3 mol/L 8-bromo-cGMP, respectively. CNP also stimulated cGMP formation in VSMCs in a dose-dependent (10^-6 to 10^-3 mol/L) manner (Fig 2B). However, neither rANP nor rBNP (10^-7 mol/L) affected 125I-ET-1 binding (data not shown).

We also examined the effects of maximal concentrations (10^-6 mol/L) of rANP, rBNP, and CNP on the binding capacities of 125I-ET-1 and cGMP generation (Fig 3). The three natriuretic peptides increased...
The effects of pretreatment with CNP and 8-bromo-cGMP on ET-1-stimulated IP3 formation are shown in Table 4. Pretreatment with CNP (10^(-6) mol/L) and 8-bromo-cGMP (10^(-3) mol/L) significantly (P<.05) increased ET-1-stimulated IP3 production by 21% and 40%, respectively, compared with control cells.

To determine which endothelin subtype (ET1, or ET2) is responsible for CNP- and cGMP-induced upregulation of endothelin receptors, we performed Northern blot analysis using cDNAs for rat ET1, and ET2 receptors as probes (Fig 6). Pretreatment with CNP (10^(-6) mol/L) and 8-bromo-cGMP (10^(-3) mol/L) for 24 hours increased steady-state levels of ET1 receptor mRNA (4.8 kb), whereas ET2 receptor mRNA (5.2 and 4.2 kb) remained unchanged.

**Discussion**

VSMCs are major target cells for ET-1 as a potent vasoconstrictor and mitogen as well as for natriuretic peptides as potent vasorelaxant and anti-growth-promoting factors. However, little information is available about molecular interactions between natriuretic peptides and ET-1 in VSMCs. Regulation of vascular endothelin receptors is influenced by a variety of humoral factors. It has been shown that endothelin receptors in cultured rat VSMCs are homologously downregulated by ET1, and that the ET1 receptor is heterologously upregulated by cyclic AMP but downregulated by glucocorticoids. In the present study we have demonstrated for the first time that CNP heterologously upregulates ETB receptors in cultured rat VSMCs.

It has been shown that cultured rat VSMCs possess ANP-B (GC-B) receptors, through which CNP stimulates cGMP production more potently than ANP. In the present study we have confirmed that CNP dose-dependently stimulates cGMP generation and further demonstrated that natriuretic peptides upregulate endothelin receptors with the same rank order of efficacy (CNP > BNP > ANP).

Competitive binding of [125I]-ET-1 with various concentrations of unlabeled ET-1 to rat VSMCs pretreated or without 10^(-8) mol/L each of rat atrial natriuretic peptide (rANP), rat brain natriuretic peptide (rBNP), and C-type natriuretic peptide (CNP) for 24 hours; then [125I]-ET-1 binding studies were performed. Open columns represent mean percentage of specific binding to basal binding (n=3); bars show SEM. SEM.

**Bar graph shows effect of three natriuretic peptides on [125I]-endothelin-1 ([125I]ET-1) binding and cyclic GMP (cGMP) generation in cultured rat vascular smooth muscle cells. For [125I]-ET-1 binding study, cells were pretreated with or without 10^(-8) mol/L each of rat atrial natriuretic peptide (rANP), rat brain natriuretic peptide (rBNP), and C-type natriuretic peptide (CNP) for 24 hours; then [125I]-ET-1 binding study was performed.**

**Choline chloride** 0.5 mmol/L, isobutylmethylxanthine. Closed columns represent mean of four samples; bars show SEM. *P<.05 vs control.
most effectively induces upregulation of endothelin receptors through a cGMP-dependent mechanism. Thus far, three distinct receptor subtypes for natriuretic peptides have been characterized: two guanylyl cyclase-containing receptors, termed ANP-A (GC-A) and ANP-B (GC-B), and a guanylyl cyclase–free receptor, ANP-C (clearance). CNP is a selective ligand for the ANP-B (GC-B) receptor, and ANP and BNP are selective ligands for the ANP-A (GC-A) receptor, both of which mediate biologic effects via cGMP-dependent mechanisms. In contrast, the ANP-C receptor is nonselective for the three natriuretic peptides, which mediate metabolism and clearance of ligands. Recently, a phenotypic change of natriuretic peptide receptors in vascular smooth muscle cells has been reported: ANP-A (GC-A) is predominant in intact aortic media, whereas GC-B and clearance receptors predominate during in vitro culture conditions. It has recently been reported that HS-142-1, an antagonist for guanylyl cyclase–linked natriuretic peptide receptors, competitively blocked CNP-induced cGMP production in cultured bovine VSMCs. Our results have shown that CNP-induced upregulation of endothelin receptors is inhibited by HS-142-1 and the cGMP-dependent protein kinase inhibitor KT-5823. Taken together, these results strongly suggest that CNP upregulates vascular endothelin receptors through ANP-B (GC-B) receptors via a cGMP-dependent mechanism.

At least two receptor subtypes exist for endothelin isopeptides: the ET-1–selective subtype (ET₃) and non–isopeptide-selective subtype (ET₄). Both receptor subtypes functionally coupled to GTP-binding protein (Gs), which stimulates phospholipase C–mediated phosphoinositide breakdown to generate IP₃ and diacylglycerol. The present study has shown that upregulation of endothelin receptors by CNP and 8-bromo-cGMP is concomitantly associated with enhanced IP₃ formation stimulated by ET-1, suggesting that modulation of vascular endothelin receptors is functionally linked to signal transduction.

It remains unanswered which vascular endothelin receptor subtype is modulated by CNP and 8-bromo-cGMP. The present Northern blot analysis using rat ETA and ETB receptor cDNAs as probes has clearly demonstrated that CNP increases steady-state levels of ETB receptor mRNA but not of ETA receptor mRNA in cultured rat VSMCs. Although CNP was localized mainly in the nervous system, it has recently been shown that CNP is also produced by the human monocytic cell line THP-1 and bovine vascular endothelial cells under stimulated conditions. Furthermore, the phenotypic change of cultured VSMCs induces subtype switching of natriuretic peptide receptors from ANP-A (GC-A) receptors to ANP-B (GC-B) receptors. Therefore, it is currently proposed that CNP locally produced by the vessel walls may participate in the antiproliferation of modulated VSMCs under pathologic conditions. We have recently demonstrated that cultured rat VSMCs acquire ETB receptors other than ETA receptors during phenotypic change, which potentiates mitogenic activity of ET-1 in VSMCs. Taken together, these results make it possible to speculate that CNP locally secreted from activated macrophages and/or injured endothelium may also induce upregulation of ETB receptors via ANP-B (GC-B) receptors in modulated VSMCs, thereby leading to enhanced vasoconstrictive and mitogenic effects by ET-1 via ETB receptors in vascular lesions associated with atherosclerosis and hypertension.
**ETα**  **ETβ**  **rRNA**

1  2  3  1  2  3  18S

**Fig. 6.** Northern blot shows analysis of endothelin subtype A (ETα) and subtype B (ETβ) mRNA in cultured rat vascular smooth muscle cells. Cells were incubated with or without C-type natriuretic peptide (CNP) (1×10⁻⁶ mol/L) or 8-bromo-cyclic GMP (cGMP) (1×10⁻⁴ mol/L) for 24 hours. Total cellular RNA (15 μg) was subjected to Northern blot analysis using cDNAs for rat ETα receptor (left) and ETβ receptor (middle) as probes. Ribosomal RNA (rRNA) loaded in each lane is shown as an internal control (right). Lane 1 shows control; 2, CNP; and 3, 8-bromo-cGMP.

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**References**


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