Expression and control of C-type natriuretic peptide in rat vascular smooth muscle cells

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Woodard, Geoffrey E., Juan A. Rosado, and John Brown. Expression and control of C-type natriuretic peptide in rat vascular smooth muscle cells. Am J Physiol Regulatory Integrative Comp Physiol 282: R156–R165, 2002.—C-type natriuretic peptide (CNP) is a member of the natriuretic peptide family mainly distributed in the central nervous system. CNP is also produced and secreted by the endothelium and inhibits vascular smooth muscle cell proliferation. We have reported that endothelial damage stimulates only transiently vascular smooth muscle cell proliferation in arteries due to the development of an autocrine neointimal system for CNP that modulates neointimal growth. The present study demonstrates the production and secretion of CNP in rat vascular smooth muscle cells in the absence of endothelium. In addition, these cells express atrial natriuretic peptide (ANP) and the natriuretic peptide receptors A, B, and C. The production and secretion of CNP in vascular smooth muscle cells is stimulated by transforming growth factor-β, whereas basic fibroblast growth factor plays an inhibitory role. These data show that ANP and mainly CNP are coexpressed with the natriuretic peptide receptors in rat vascular smooth muscle cells. This provides evidence for a vascular natriuretic peptide autocrine system of physiological relevance in these cells.

atrial natriuretic peptide; brain natriuretic peptide; transforming growth factor-β; vascular smooth muscle

NATRIURETIC PEPTIDES have been shown to modulate several physiological functions. Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) have been shown to elicit renal and cardiovascular effects (1, 23). The third type, C-type natriuretic peptide (CNP), elicits mainly vasodilatory effects rather than regulation of body fluid homeostasis (33). The different physiological effects of these peptides could be attributed to the existence of three different receptor subtypes, natriuretic peptide receptor-A, -B, and -C [NPR-A, NPR-B, and NPR-C, respectively (25)]. NPR-A is a guanylate cyclase-coupled receptor activated by ANP and BNP (25). NPR-B is also a guanylate cyclase-coupled receptor, which specifically binds CNP (18). Finally, the NPR-C is devoid of kinase and guanylate cyclase activity and activates G proteins (24). NPR-C binds with high affinity to all three natriuretic peptides, and its ability to recycle rapidly suggests that it might also act as a clearance receptor to internalize and degrade circulating natriuretic peptides (21, 25).

Proliferation of vascular smooth muscle cells is an important response of arteries to several vascular injuries, such as atherosclerosis or restenosis after angioplasties. Cytokines and growth factors, released by the injured vascular wall and activated platelets, stimulate proliferation of the vascular smooth muscle cells (6); however, little is known about factors that limit proliferation of these cells once initiated. Natriuretic peptides have been reported to inhibit cell proliferation in several cell types, such as vascular smooth muscle cells through the generation of cGMP (11). In support of this hypothesis, nitric oxide, a cGMP-elevating agent, elicits antiproliferative effects in vascular smooth muscle cells (11). In vascular smooth muscle cells, CNP, which is commonly referred as an endothelium-derived relaxing factor (3, 4, 11), has been presented as the most potent inhibitor of growth and proliferation, an effect mediated by the occupation of the guanylate cyclase-coupled NPR-B (27). Consistent with this, CNP has been shown to inhibit arterial intimal thickening in vivo, most likely through inhibiting vascular smooth muscle proliferation induced by vascular injury (2, 3, 10).

Here we show for the first time that CNP and also ANP and their receptors are expressed in vascular smooth muscle cells in the absence of endothelium and independently of endothelial regulation. In addition, we investigated the regulation of CNP expression by cytokines and growth factors.

MATERIALS AND METHODS

Materials. Rats were from Charles River (Margate, UK). Stroke-prone spontaneously hypertensive rats (SPSHR) were from the Animal Facility of the University of Birmingham. Collagenase, dextran, fluorescein-conjugated anti-rabbit IgG antibody, rhodamine-conjugated anti-rabbit IgG antibody, rabbit monoclonal anti-α-smooth muscle actin antibody, and elastase were from Sigma (Poole, Dorset, UK). Rabbit antivon Willebrand factor (vWF) polyclonal antibody was from Dako (High Wycombe, UK). Penicillin, fetal calf serum, medium M199, SuperScript II preamplification kit, and Hanks' balanced salt solution (HBSS) were from Gibco-BRL (Paisley, UK). Sep-Pak C18 cartridges were from Waters (Watford, UK). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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UK). CNP radioimmunoassay and rabbit polyclonal CNP antibody were from Peninsula Laboratories (Merseyside, UK). Hybond-N, \[^{32}P\]CTP, and rapid-hyb buffer were from Amersham. Stratagene random-primer labeling kit was from Stratagene (Cambridge, UK). Geneclean II kit was from Bio101 (Vista, CA). MiniMessage Maker kit, transforming growth factor-\(\beta\) (TGF-\(\beta\)), tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), interleukin-1\(\beta\) (IL-1\(\beta\)), interferon-\(\gamma\) (INF-\(\gamma\)), and basic fibroblast growth factor (bFGF) were from R & D Systems (Abingdon, UK). Plasmid Midi kit was from Qiagen (Dorking, UK). Fuji RX film was from Stuart Basset (Nottingham, UK). All other reagents were of analytic grade.

Cell culture. Aortic smooth muscle cell (ASMC) preparation was performed as described previously (12). Briefly, young male Wistar rats (180–200 g) were killed by carbon dioxide asphyxiation and the aortas were removed. The aortas were incubated in 3 mg/ml collagenase in medium M199 supplemented with 100 U/ml penicillin for 30 min at 37°C in a shaker bath, and the tunica media was dissected from the adventitia and endothelium. The suspension was then incubated in 1 mg/ml elastase for 10 min at 37°C and for a further 2 h in 0.5 mg/ml elastase and 1.5 mg/ml collagenase in M199 at 37°C. The suspension was centrifuged at 900 g for 4 min, and cells were then resuspended into 10 ml M199 + 10% fetal calf serum. The cells were plated at 4 \times 10^5 cells/ml and incubated at 37°C in 5% carbon dioxide.

Cerebral vascular smooth muscle (CVSM) cells were isolated as previously described (7). Briefly, a total of 10 preparations was obtained from groups of 12 or 24 male Wistar rats weighing 120 g. Rat brains devoid of cerebellum were removed and placed in cold 4°C HBSS and centrifuged again at 3,000 g. The pial membranes were removed, and the cerebral cortices cleaned of white matter were homogenized. The homogenate was centrifuged, and the pellet was resuspended in HBSS containing 15% dextran and 5% fetal bovine serum and centrifuged again at 3,000 g. The microvesel pellet was resuspended in HBSS and filtered through a 150-μm nylon mesh sieve. The purity of the preparation was assessed by both phase-contrast and electron microscopy along with immunostaining with FITC-IgG to α-smooth muscle actin.

All studies were performed in cultured vascular smooth muscle cells at passage 3, because at further passages a certain degree of dedifferentiation was observed (data not shown) as described previously (15).

**RT-PCR.** The procedures were as we described previously (40). Briefly, poly(A)+ RNA was obtained from vascular smooth muscle cells, rat embryos, and heart using a MiniMessage Maker kit. RNA was reverse transcribed with the SuperScript II preamplification kit and subjected to PCR with rat gene-specific intron-spanning primers described in Table 1. Target sequences were amplified at the profile 94°C 4 min, 94°C 30 s, 58°C 30 s, 72°C 1 min, and 72°C 7 min by using the same amount of cDNA for all primer sets. The RT-PCR products were all of expected size. Negative controls were performed by omitting the RT step or cDNA template from PCR amplification. To confirm the identity of size-fractionated PCR products further, Southern blots were performed as described previously (28). Briefly, gels were denatured, neutralized, and transferred onto Hybond-N. Blots were fixed by ultraviolet transillumination for 4 min and hybridized to a rat cDNA fragment (internal to amplified region) being labeled with \[^{32}P\]CTP using a Stratagene random-primer labeling kit. Hybridization was performed in rapid-hyb buffer for 2 h at 65°C. Blots were washed twice in 2× standard saline citrate (SSC)-0.1% sodium dodecyl sulfate (SDS) for 15 min at 20°C and then in 0.1× SSC-0.1% SDS for 15 min at 65°C. Blots were then exposed to Fuji type RX film for 20 min at −80°C. Finally, RT-PCR products were purified from agarose gels after electrophoresis using a Geneclean II kit and subcloned into the pSPORT vector. The plasmid containing the cDNA insert was prepared using a Plasmid Midi kit and sequenced on an ABI 3T3 automated sequencer.

For semiquantitative RT-PCR, the cycle number was adjusted between 20 and 35 cycles to yield visible products.

**Table 1. List and sequence of primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position</th>
<th>Sequence</th>
<th>Product Size</th>
<th>Reference</th>
</tr>
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<tr>
<td>ANP</td>
<td>bp 23-45</td>
<td>5'-CCGAGACAGCAAAAATCGATCG-3'</td>
<td>762 bp</td>
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<tr>
<td>BNP</td>
<td>bp 783-785</td>
<td>5'-CCTGGTGTCAGGAAGTTTATTG-3'</td>
<td>402 bp</td>
<td>16</td>
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<tr>
<td>CNP</td>
<td>bp 43-61</td>
<td>5'-CATCAGCTGCAGCAGG-3'</td>
<td>703 bp</td>
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<tr>
<td>NPR-A</td>
<td>bp 676-699</td>
<td>5'-GCAGCTGCAGCCAG-3'</td>
<td>965 bp</td>
<td>5</td>
</tr>
<tr>
<td>NPR-B</td>
<td>bp 1857-1877</td>
<td>5'-GCCAGTGCTGCTGCTG-3'</td>
<td>366 bp</td>
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<tr>
<td>NPR-C</td>
<td>bp 2214-2234</td>
<td>5'-AGCCACGCTGAACCTTTC-3'</td>
<td>491 bp</td>
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</tr>
<tr>
<td>VEGF-R2</td>
<td>bp 309-329</td>
<td>5'-CTGGCTGCTGCCTGCTG-3'</td>
<td>407 bp</td>
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<td>GAPDH</td>
<td>bp 204-222</td>
<td>5'-TCAGTCATGTACCTACCC-3'</td>
<td>398 bp</td>
<td>37</td>
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</table>

NPR-A, atrial natriuretic peptide (ANP) receptor; NPR-B, brain natriuretic peptide (BNP) receptor; NPR-C, C-type natriuretic peptide (CNP) receptor; VEGF-R2, vascular endothelial growth factor receptor 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
within the range of linear amplification and also a calibration curve was done by varying quantities of cDNA template for a fixed cycle number (35 cycles). Resulting RT-PCR products were run on agarose gel and visualized by staining with ethidium bromide. Their densities were quantified by using a phosphorimager (Molecular Dynamics, Sunnyvale, CA) and normalized to that of RT-PCR product of the housekeeping GAPDH gene in the same sample.

**Immunocytochemistry.** Cells were seeded onto poly-L-lysine-coated glass coverslips in 24-well plates and grown in M199 with 10% fetal calf serum. Monolayers were washed three times in PBS, fixed for 5 min in 70% ethanol and 1% glacial acetic acid at room temperature, washed twice with PBS for 3 min each, and blocked with PBS containing 3% BSA. Cells were then incubated for 2 h with either rabbit polyclonal CNP antibody, rabbit anti-α-smooth muscle actin monoclonal, or rabbit anti-vWF polyclonal antibody diluted 1:100, 1:100, and 1:200, respectively, in PBS containing 3% BSA. After three washes in PBS for 3 min each, cells were incubated with fluorescein-conjugated or rhodamine-conjugated anti-rabbit IgG secondary antibody diluted 1:200 for 2 h at room temperature. The cells were then washed three times for 3 min each as before. Slides were mounted with 50% glycerol-50% PBS and visualized with an immunofluorescence microscope (Olympus, London, UK).

**Radioimmunoassay.** Radioimmunoassays were done on extracts of cultured ASMC and conditioned media using a commercial CNP radioimmunoassay. Briefly, treated cells were harvested and studied under paired conditions with cells treated by vehicle alone. After being cooled on ice, glacial acetic acid was added (final concentration 1 M), and the cells were homogenized. The homogenates were centrifuged at 10,000 g for 10 min, and the supernatants were extracted using Sep-Pak C 18 cartridges previously activated by addition of 4 ml of acetonitrile followed by 4 ml of 0.1% (vol/vol) trifluoroacetic acid (TFA). The cartridges were loaded with sample and then washed with 4 ml of 0.1% TFA. CNP was eluted with 3 ml of acetonitrile-water-TFA (60:40:0.1 vol/vol/vol). The eluates were lyophilized and dissolved in 0.1 vol/vol/vol). The eluates were lyophilized and dissolved in

**RESULTS**

**Examination of the primary culture of Wistar rat ASMC.** Freshly dispersed ASMC from healthy adult Wistar rats were prepared and plated out in cell culture as described previously (12). To verify that the primary culture of ASMC was free of contamination with endothelial cells, we analyzed several samples with an antibody that specifically recognizes α-smooth muscle actin, which is a differentiation marker of smooth muscle cells and thus is suitable to distinguish smooth muscle cells from other cells (31). As shown in Fig. 1A, immunofluorescence studies of primary culture of ASMC incubated with α-smooth muscle actin antibody revealed that all the cells in the culture expressed α-smooth muscle actin (n = 8). To further investigate the absence of endothelial contamination in the primary culture of ASMC, we investigated the expression of VEGF-R2/Flk-1 receptor, which is expressed in endothelial cells (40), in the primary culture of ASMC. As a positive control for the expression of VEGF-R2/Flk-1, rat embryos were used as an endothelium-rich tissue. As expected, expression of VEGF-R2/Flk-1 was clearly found in homogenates of rat embryos (Fig. 1B; n = 6); however, it was undetectable in the

![Fig. 1](https://www.ajpregu.org/fig1.png)
C-TYPE NATRIURETIC PEPTIDE IN VASCULAR SMOOTH MUSCLE

R159

Effect of various agents on CNP mRNA expression in rat ASMC. We examined the effects of different agents that affect vascular tone and growth, such as the cytokines IFN-γ, TNF-α, and IL-1β, or the growth factors TGF-β and bFGF, on the regulation of CNP expression in ASMC. Primary culture of Wistar rat ASMC at passage 3 was treated with IFN-γ (100 ng/ml), TNF-α (100 ng/ml), IL-1β (100 ng/ml), bFGF (100 ng/ml), or TGF-β (500 pM), and CNP mRNA expression was evaluated with RT-PCR. GAPDH was PCR amplified simultaneously with CNP and used as an internal control.

Table 2. CNP mRNA expression in ASMC following treatment with different agents

<table>
<thead>
<tr>
<th>Stimulatory Agent</th>
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<th>TGF-β</th>
<th>IFN-γ</th>
<th>TNF-α</th>
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<tr>
<td></td>
<td>100 ± 0</td>
<td>35 ± 22</td>
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<td>137 ± 26</td>
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Data are presented as means ± SE of 7 separate determinations. Aortic smooth muscle cells (ASMC) were treated for 24 h in the presence of basic fibroblast growth factor (bFGF; 100 ng/ml), transforming growth factor (TGF-β; 500 pM), interferon (IFN-γ; 100 ng/ml), tumor necrosis factor (TNF-α; 100 ng/ml), interleukin (IL-1β; 100 ng/ml) or the vehicles (Control) as indicated. mRNA preparation, RT-PCR with GAPDH internal positive control, Southern blot hybridization, and radiolabeled probing were performed as described in MATERIALS AND METHODS. Values represent CNP mRNA expression as a percentage of that for GAPDH and presented as percentage of Control (vehicle was added). *P < 0.01 compared with CNP mRNA expression in Control (nontreated cells).

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Treatment of cultured ASMC for 24 h with the cytokines IFN-γ, TNF-α, or IL-1β did not significantly alter CNP mRNA expression (Table 2 and Fig. 3; n = 6). In contrast, treatment for 24 h with the growth factors TGF-β or bFGF significantly modified CNP mRNA expression in cultured ASMC. As shown in Table 2 and Fig. 3, incubation with TGF-β (500 pM) for 24 h significantly increases CNP mRNA expression compared with control (nontreated cells; n = 6; P < 0.01); however, bFGF had opposite effects. Treatment with 100 ng/ml bFGF for 24 h resulted in a significant reduction in CNP mRNA expression in ASMC compared with vehicle-treated cells (Table 2 and Fig. 3; n = 6; P < 0.01). The effect of TGF-β on CNP expression in cultured Wistar rat ASMC was concentration dependent. Treatment of ASMC for 24 h with TGF-β increases CNP expression, reaching a maximal effect at 500 pM with 4.3 ± 0.4-fold increase. We found that incubation of ASMC with higher concentrations of TGF-β induced smaller increases in CNP expression (Fig. 4).

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The effect of TGF-\(\beta\) on CNP expression in ASMC was confirmed by immunocytochemistry. Cultured Wistar rat ASMC were treated with TGF-\(\beta\) (500 or 2,000 pM) for 24 h, and CNP was detected using an anti-CNP polyclonal antibody. In agreement with the results reported above, fluorescent microscopy of ASMC revealed that treatment with TGF-\(\beta\) for 24 h induced a biphasic increase in fluorescence compared with untreated cells, indicating that TGF-\(\beta\) stimulated CNP expression in ASMC (Fig. 5; \(n = 8\)). The negative controls (cells incubated with anti-CNP polyclonal antibody saturated with CNP 1 \(\mu\)M) reveal that fluorescence intensity was indicative of CNP expression (Fig. 5).

**Effect of various agents on CNP mRNA expression in rat CVSM cells.** To further investigate the effect of TGF-\(\beta\), bFGF, IFN-\(\gamma\), TNF-\(\alpha\), and IL-1\(\beta\) on CNP expression in vascular smooth muscle cells, we examined their effects in cultured CVSM cells from Wistar rats.

CVSM cells at passage 3 in culture were treated for 24 h with bFGF (100 ng/ml), TGF-\(\beta\) (500 pM), IFN-\(\gamma\) (100 ng/ml), TNF-\(\alpha\) (100 ng/ml), or IL-1\(\beta\) (100 ng/ml), and CNP mRNA expression was evaluated as described in MATERIALS AND METHODS. GAPDH was PCR amplified simultaneously with CNP and used as an internal control. Consistent with the results obtained in ASMC, treatment of CVSM cells for 24 h with TGF-\(\beta\) (500 pM) significantly increases CNP mRNA expression (Table 3 and Fig. 3; \(n = 6\); \(P < 0.01\)). In addition, incubation with bFGF (100 ng/ml) significantly decreases CNP mRNA expression (Table 3 and Fig. 3; \(n = 6\); \(P < 0.01\)). No significant modifications were detected when CVSM were treated with IFN-\(\gamma\), TNF-\(\alpha\), or IL-1\(\beta\) as reported for ASMC (Table 3 and Fig. 3; \(n = 6\)).

In agreement with the results reported above, the effect of TGF-\(\beta\) on CNP expression was confirmed by immunocytochemistry. Treatment of CVSM cells with TGF-\(\beta\) (500 or 2,000 pM) for 24 h induced a biphasic increase in CNP immunodetected compared with untreated cells, indicating that TGF-\(\beta\) stimulated CNP expression in CVSM cells (Fig. 6; \(n = 8\)). The negative controls (CVSM cells immunostained with anti-CNP polyclonal antibody saturated with CNP 1 \(\mu\)M) reveal that fluorescence was indicative of CNP expression (Fig. 6). These findings reveal for the first time the expression of CNP in smooth muscle cells from rat aorta or cerebral vasculature.

**Regulation of CNP secretion by TGF-\(\beta\) and bFGF in Wistar and SPSPHR rat ASMC and CVSM.** The effect of TGF-\(\beta\) on CNP mRNA expression was confirmed by radioimmunoassay analysis of CNP in ASMC and CVSM cells in culture. As reported above, TGF-\(\beta\) induced a biphasic increase in CNP expression in ASMC. Stimulation with maximal concentration of TGF-\(\beta\) (500 pM) induced a significant increase in the level of CNP expressed, whereas incubation with a supramaximal concentration (2,000 pM) did not induce any detectable increase over the basal level (Table 4; \(n = 8\)). Similar results were observed in CVSM (Table 4; \(n = 8\)). Consistent with the effect of bFGF on CNP mRNA expression reported above, treatment of ASMC or CVSM with 100 ng/ml bFGF induced a slight decrease in CNP-like immunoreactivity and significantly reduced CNP expression stimulated by 500 pM TGF-\(\beta\) (Table 4; \(n = 8\)).

TGF-\(\beta\) has been shown to stimulate CNP secretion in endothelial cells in a concentration-dependent manner (36). Hence the effect of TGF-\(\beta\) on CNP secretion in ASMC and CVSM was investigated. Table 4 shows the effect of incubation for 24 h with 500 and 2,000 pM TGF-\(\beta\) on CNP concentration in ASMC- and CVSM-conditioned media. As reported in endothelial cells (36), TGF-\(\beta\) stimulated accumulation of CNP-like im-

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**Fig. 3.** C-type natriuretic peptide (CNP) mRNA expression in ASMC (A) or cerebral smooth muscle cells (CVSM; B) following treatment with different agents. ASMC or CVSM cells were treated for 24 h in the presence of basic fibroblast growth factor (bFGF; 100 ng/ml), transforming growth factor (TGF)-\(\beta\) (500 pM), interferon (IFN)-\(\gamma\) (100 ng/ml), tumor necrosis factor (TNF)-\(\alpha\) (100 ng/ml), interleukin (IL)-1\(\beta\) (100 ng/ml), or the vehicles (Control) as indicated. mRNA preparation, RT-PCR with GAPDH internal positive control, Southern blot hybridization, and radiolabeled probing were performed as described in MATERIALS AND METHODS. Each autoradiogram is representative of 6 separate determinations.
from in normotensive Wistar rats, treatment with 100 ng/ml bFGF for 24 h resulted in a significant increase in CNP mRNA expression in both ASMC and CVSM from SPSHR compared with vehicle-treated cells (Table 5; n = 6; P < 0.01). As shown in Table 4, in ASMC and CVSM cells from SPSHR, TGF-β induced a concentration-dependent increase in CNP production. Consistent with the results obtained when CNP mRNA was examined, we found that TGF-β increases CNP protein level in the cells without the biphasic effect observed in ASMC from normotensive rats at the same range of concentrations. bFGF induced a significant increase in CNP protein level in ASMC and CVSM from SPSHR and potentiated the effect of TGF-β (500 pM) when incubated simultaneously (Table 4; n = 8). ASMC and CVSM cells from SPSHR showed a higher basal level of CNP-like immunoreactivity in the media. As in normotensive Wistar rat smooth muscle cells, treatment for 24 h with TGF-β induced a significant increase in CNP-like immunoreactivity in ASMC- and CVSM-conditioned media (Table 4; n = 8). In contrast, treatment with bFGF (100 ng/ml) did not induce either increased CNP secretion (Table 4; n = 8) or reduction of TGF-β-stimulated increase in CNP-like immunoreactivity (Table 4; n = 8).

**DISCUSSION**

The present study demonstrates the expression and secretion of CNP in vascular smooth muscle cells and their regulation by the growth factors TGF-β and bFGF. CNP, a peptide of 22 or 53 amino acid residues initially isolated from the porcine brain (34), has also been shown to be expressed and secreted by endothelial cells, suggesting a significant role in the modula-

**Fig. 4.** Concentration-dependence relationship of CNP expression in response to TGF-β in cultured rat ASMC. A: rat ASMC were treated for 24 h in the presence of various concentrations of TGF-β (100–2,000 pM) or the vehicle as indicated. RT-PCR with GAPDH internal positive control, Southern blot hybridization, and radiolabeled probing were performed as described in MATERIALS AND METHODS. The autoradiograms are representative of 8 separate determinations. B: histograms indicating CNP mRNA expression after the treatment with different concentrations of TGF-β relative to that for GAPDH and represented as percentage of control (vehicle was added). Values are means ± SE of 8 representative experiments.

munoreactivity in ASMC- and CVSM-conditioned media in a concentration-dependent manner (Table 4; n = 8). In contrast, treatment with bFGF (100 ng/ml) did not induce any detectable increase in CNP-like immunoreactivity (Table 4; n = 8) and simultaneous stimulation with bFGF (100 ng/ml) and TGF-β (500 pM) induced an increase in CNP-like immunoreactivity significantly smaller than that achieved by TGF-β alone (Table 4; n = 8).

The effect of TGF-β and bFGF on CNP production and secretion was also examined in ASMC and CVSM cells from SPSHR, which present a higher level of TGF-β mRNA than in normotensive smooth muscle cells (14). ASMC from SPSHR were obtained and cultured following the same protocol used for ASMC from Wistar rats. Treatment for 24 h with the growth factors TGF-β or bFGF significantly modifies CNP mRNA expression in cultured ASMC or CVSM from SPSHR. As shown in Table 5, incubation with TGF-β (500 or 2,000 pM) for 24 h significantly increases CNP mRNA expression compared with control in a linear concentration-dependent manner (n = 6; P < 0.01). Different
Table 3. CNP mRNA expression in cerebral smooth muscle cells following treatment with different agents

<table>
<thead>
<tr>
<th>Stimulatory Agent</th>
<th>Control</th>
<th>bFGF</th>
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</table>

Data are presented as means ± SE of 6 separate determinations. Cerebral vascular smooth muscle (CVSM) were treated 24 h in the presence of bFGF (100 ng/ml), TGF-β (500 pM), IFN-γ (100 ng/ml), TNF-α (100 ng/ml), IL-1β (100 ng/ml) or the vehicles (Control) as indicated. mRNA preparation, RT-PCR with GAPDH internal positive control, Southern blot hybridization, and radiolabeled probing were performed as described in MATERIALS AND METHODS (23). CNP mRNA expression was modulated by growth factors such as TGF-β and bFGF.

Endothelium-secreted CNP has been reported to inhibit vascular smooth muscle cell proliferation (19), but after arterial injury, in regions stripped of endothelium, smooth muscle cell proliferation is only transiently stimulated, resulting in development of neointima, which consists mainly of modified vascular smooth muscle cells that become a new source of CNP in the absence of endothelium (3). Our results reporting the expression of CNP in cultured vascular smooth muscle cells support the hypothesis that these cells develop an autocrine system for CNP that regulates neointimal proliferation independently or in concert with the endothelial cells.

Proliferation and migration of vascular smooth muscle cells with subsequent intimal thickening is a major process in the development of restenosis after angioplasty or atherosclerosis. Several growth factors have been shown to be involved in these events. In agreement with this, TGF-β is a growth inhibitor of endothelial cells (32) and a potent stimulator of CNP secretion (36). In vascular smooth muscle cells TGF-β is a growth modulator (32), and, consistent with the effect on endothelial cells, our results indicate that TGF-β stimulates CNP expression and secretion in these cells. We found that the effect of TGF-β on CNP mRNA expression is concentration dependent, reaching a maximum at 500 pM. At higher concentrations, TGF-β induced smaller increases in CNP mRNA and protein level. These results suggest that supramaximal doses of TGF-β are less effective at stimulating CNP production, which highlights the significance of the concentration of TGF-β in the vascular tissues in the regulation of smooth muscle cell proliferation. On the other hand, the expression of vascular tone and proliferation in an antagonistic manner to the renin-angiotensin system (36). Our data show the presence of specific mRNAs coding for the ANF and predominantly CNP in aortic vascular smooth muscle cells from rat. In addition, the expression of NPR-A, -B, and -C was simultaneously detected in rat ASMC. The presence of NPR-B and CNP, believed to be the specific ligand for NPR-B (18), suggests a role for CNP as an autocrine and/or paracrine regulator of smooth muscle cell growth and vascular tone independently or in concert with endothelial cells.

The production of CNP in vascular smooth muscle cells was corroborated in vascular smooth muscle cells from cerebral microvessels. In these cells, as well as in ASMC, we found that CNP mRNA expression is modulated by growth factors such as TGF-β and bFGF.

Table 4. Changes in CNP-like immunoreactivity in Wistar and SPSHR aortic and cerebral smooth muscle cells and medium in response to TGF-β and bFGF treatment as measured by radioimmunoassay

<table>
<thead>
<tr>
<th>Stimulatory Agent</th>
<th>Wistar ASMC</th>
<th>SPSHR ASMC</th>
<th>Wistar CVSM</th>
<th>SPSHR CVSM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.83 ± 0.12</td>
<td>0.88 ± 0.12</td>
<td>0.44 ± 0.07</td>
<td>0.48 ± 0.09</td>
</tr>
<tr>
<td>500 pM TGF-β</td>
<td>1.11 ± 0.23</td>
<td>1.14 ± 0.26</td>
<td>0.64 ± 0.03</td>
<td>0.69 ± 0.12</td>
</tr>
<tr>
<td>2000 pM TGF-β</td>
<td>0.75 ± 0.16</td>
<td>2.10 ± 0.32</td>
<td>0.41 ± 0.06</td>
<td>1.51 ± 0.22</td>
</tr>
<tr>
<td>100 ng/ml bFGF</td>
<td>0.70 ± 0.09</td>
<td>1.60 ± 0.24</td>
<td>0.40 ± 0.08</td>
<td>1.02 ± 0.13</td>
</tr>
<tr>
<td>bFGF + TGF-β</td>
<td>0.90 ± 0.15†</td>
<td>2.32 ± 0.20</td>
<td>0.52 ± 0.05‡</td>
<td>1.13 ± 0.20</td>
</tr>
<tr>
<td><strong>Medium</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.82 ± 0.20</td>
<td>2.60 ± 0.16</td>
<td>0.90 ± 0.20</td>
<td>1.31 ± 0.13</td>
</tr>
<tr>
<td>500 pM TGF-β</td>
<td>3.02 ± 0.10†</td>
<td>3.30 ± 0.19†</td>
<td>1.52 ± 0.10†</td>
<td>1.66 ± 0.11†</td>
</tr>
<tr>
<td>2000 pM TGF-β</td>
<td>3.28 ± 0.10†</td>
<td>3.28 ± 0.10†</td>
<td>1.60 ± 0.10†</td>
<td>1.62 ± 0.07†</td>
</tr>
<tr>
<td>100 ng/ml bFGF</td>
<td>1.79 ± 0.10</td>
<td>2.40 ± 0.52</td>
<td>0.93 ± 0.05</td>
<td>1.22 ± 0.09</td>
</tr>
<tr>
<td>bFGF + TGF-β</td>
<td>2.38 ± 0.23†</td>
<td>3.12 ± 0.14</td>
<td>1.28 ± 0.23†</td>
<td>1.52 ± 0.14</td>
</tr>
</tbody>
</table>

Values given are pg CNP/10⁶ cells and are presented as means ± SE of 8 separate determinations. ASMC and CVSM from Wistar and SPSHR aortic and cerebral smooth muscle cells were treated for 24 h in the presence of TGF-β (500 or 2000 pM), bFGF (100 ng/ml), TGF-β (500 pM) plus bFGF (100 ng/ml), or the vehicles (Control) as indicated. Radioimmunoassay of ASMC or CVSM and their respective medium was performed as described in MATERIALS AND METHODS. *P < 0.05 and †P < 0.01 compared with CNP content in nonstimulated conditions. ‡P < 0.05 compared with CNP content after treatment with 500 pM TGF-β.
response to exogenous TGF-β stimulates CNP secretion in a linear concentration-dependent manner. The fact that high doses of TGF-β stimulate CNP secretion while having little effect in production might explain why CNP protein levels in treated cells with TGF-β (2,000 pM) are smaller than in nonstimulated cells. In agreement with the effect reported in endothelial cells (36), the stimulatory effect of TGF-β on CNP production and secretion raises the possibility that the role of TGF-β on vascular smooth muscle growth might be mediated by increases in CNP secretion.

Other growth factors, such as bFGF, have been shown to stimulate CNP secretion in endothelial cells (35, 36). In contrast, our present observations clearly show that incubation with bFGF inhibited the expression of CNP mRNA in both aortic and CVSM cells. In addition, treatment of ASMC with bFGF reduced TGF-β-stimulated production and secretion of CNP.

Abnormal vascular smooth muscle proliferation is considered to be one of the factors contributing to increased peripheral vascular resistance in hypertension. Proliferation of cultured ASMC from SPSHR has been reported to be increased in response to treatment with several growth factors (13, 30, 41). The accumulation of TGF-β mRNA levels is higher in SPSHR smooth muscle cells than in normotensive Wistar-Kyoto (WKY) rats, and DNA synthesis is enhanced in response to exogenous TGF-β in SPSHR, although it does not have any detectable effect in WKY rats (14). In the present study we show that the actions of TGF-β and bFGF on CNP production and secretion are altered in SPSHR compared with normotensive rats. Our results indicate that CNP mRNA expression and protein level in the cells are not reduced when supramaximal concentrations of TGF-β were used to stimulate ASMC or CVSM from SPSHR, whereas in normotensive rats, those concentrations had little effect in CNP production. Although we have not further investigated this event, the data presented suggest that the relationship between TGF-β and CNP production in vascular smooth muscle cells from SPSHR could be linear instead of the biphasic pattern observed in normotensive rats. In contrast to the inhibitory role of bFGF on CNP expression in normotensive rats, we found that bFGF stimulated CNP production in ASMC and CVSM cells from SPSHR. Consistent with this, the combined effect of TGF-β and bFGF was found to be additive. In addition, our data clearly show an elevated basal CNP secretion in ASMC and CVSM cells from SPSHR compared with normotensive rats, which might be a response to compensate for the hypertension. Although the cause of these differences is not well understood, the regulation of CNP production and secretion in ASMC and CVSM cells from SPSHR is altered and we do not exclude the possibility that the role of CNP in the regulation of vascular smooth muscle cell proliferation in these rats might be different from that in normotensive rats.

Cytokines have been reported to stimulate CNP production and secretion in endothelial cells (35). In contrast, we found that stimulation of ASMC or CVSM with either IFN-γ, TNF-α, or IL-1β did not significantly modify CNP mRNA expression in these cells, indicating that CNP secretion is not modulated by cytokines in rat vascular smooth muscle cells.

In conclusion, we have shown the presence of transcripts of ANP and, predominantly, CNP as well as mRNAs coding for all three natriuretic peptide receptors in cultured rat vascular smooth muscle cells. These data provide evidence for the existence of a vascular natriuretic peptide system that may serve as a local control to modulate vascular growth and tone, either alone or in concert with the endothelial cells.

**Perspectives**

It has been proposed that natriuretic peptides can act not only as vasodilators but also as growth inhibitors of vascular smooth muscle cells (36). In the present study we reported the expression of the natriuretic peptides ANP and CNP as well as the receptors NPR-A, -B, and -C in vascular smooth muscle cells. We found that production and secretion of CNP, which induces inhibition of vascular smooth muscle cell proliferation (39), is augmented by TGF-β, whereas opposite effects were exerted by bFGF. As well as in endothelial cells, TGF-β has been presented as a growth regulator of vascular smooth muscle cells (32). In endothelial cells, this effect has been suggested to be mediated by stimulation of CNP secretion (36). The findings presented here raise the possibility that the role of TGF-β in vascular smooth muscle growth might be mediated by the modulation of CNP production and secretion. Studies performed in SPSHR vascular smooth muscle cells have demonstrated that the regulation of CNP production and secretion by growth factors are altered and the basal levels of CNP production and secretion are higher than in normotensive rats. Although speculative, the different regulation of CNP in SPSHR might be a response to compensate for the factors contributing to increased peripheral vascular resistance in hypertension. Taken together these results support the existence of a vascular natriuretic peptide system where

<table>
<thead>
<tr>
<th>Stimulatory Agent</th>
<th>Control</th>
<th>100 ng/ml bFGF</th>
<th>500 pM TGF-β</th>
<th>2000 pM TGF-β</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASMC</td>
<td>100 ± 0</td>
<td>182 ± 15†</td>
<td>166 ± 22‡</td>
<td>281 ± 24†</td>
</tr>
<tr>
<td>CVSM</td>
<td>100 ± 0</td>
<td>205 ± 21†</td>
<td>159 ± 17‡</td>
<td>305 ± 25†</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE of 6 separate determinations. CSVM were treated 24 h in the presence of bFGF (100 ng/ml), TGF-β (500 pM), TGF-β (2,000 pM), or the vehicle (Control) as indicated. mRNA preparation, RT-PCR with GAPDH internal positive control, Southern blot hybridization, and radiolabeled probing were performed as described in MATERIALS AND METHODS. Values represent CNP mRNA expression as a percentage of that for GAPDH and presented as percentage of Control (vehicle was added). †*P < 0.05 and ‡P < 0.01 compared with CNP mRNA expression in Control (nonstimulated).

**Table 5. CNP mRNA expression in aortic and cerebral smooth muscle cells from SPSHR following treatment with bFGF and TGF-β**
endothelial and vascular CNP production might induce vascular relaxation and growth inhibition in a paracrine or autocrine manner. This work was supported by The British Heart Foundation.

REFERENCES


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