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C-TYPE NATRIURETIC PEPTIDE INHIBITS INTIMAL THICKENING AFTER VASCULAR INJURY

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SUMMARY : Recently we have found that C-type natriuretic peptide (CNP) inhibits proliferation of cultured rat vascular smooth muscle cells through an elevation of cGMP. We have now tested whether administration of CNP inhibits the development of intimal lesions induced by air-drying injury in rat common carotid arteries in vivo. CNP treatment (1μg/kg/min, i.v. infusion) for either 14 or 5 days resulted in 70% or 60% reduction, respectively, of intimal cross-section area 14 days after injury as compared with control rats. We also found that CNP potently stimulated cGMP production in injured carotid arteries with intimal thickening, but not in intact ones. These results indicate that GC-B, CNP specific receptor/guanylyl cyclase, is expressed at the sites of vascular injury, and that CNP might be efficacious in the prevention of restenosis caused by intimal thickening following coronary angioplasty.

The natriuretic peptides (NPs) are a family of three related polypeptide hormones termed A-type natriuretic peptide (atrial natriuretic peptide, ANP), B-type natriuretic peptide (brain natriuretic peptide, BNP) and C-type natriuretic peptide (CNP). NPs exert natriuretic/diuretic and hypotensive effects in vivo through the activation of cell surface receptor guanylyl cyclases (1, 2). Molecular cloning studies have so far identified two types of the natriuretic peptide receptor guanylyl cyclases, which were designated as GC-A and GC-B (2), and it has recently been reported that both ANP and BNP are selective ligands for GC-A, whereas CNP is a selective ligand for GC-B (3). Physiological functions of ANP and BNP have been well investigated. They are now known to play important roles in the regulation of body fluid and blood pressure homeostasis, however, little is known about those of CNP. We have previously shown that CNP has considerably weak diuretic and vasorelaxant activities as compared with ANP (less than 1/20 to ANP) (4). In contrast, it has been found that CNP

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Abbreviations: CNP, C-type natriuretic peptide, prepro-CNP[105-126]; ANP, atrial natriuretic peptide (A-type natriuretic peptide); α-hANP, human prepro-ANP[124-151]; BNP, brain natriuretic peptide (B-type natriuretic peptide); HPLC, high performance liquid chromatography; TGF-β, transforming growth factor-β.

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exhibits more potent activities for stimulation of cGMP accumulation and inhibition of DNA synthesis than ANP in cultured rat vascular smooth muscle cells, while ANP exerts more potent activities for elevation of cGMP and vasorelaxation than CNP in intact vascular tissue (5-7). These results suggest that functional GC-B and GC-A are dominantly expressed in cultured vascular smooth muscle cells and intact vascular tissue, respectively, and that CNP may function as a regulator of the proliferation of vascular smooth muscle cells.

The proliferation of vascular smooth muscle cells is recognized to be a central event in the pathogenesis of restenosis after coronary angioplasty (8, 9). After vascular injury induced by coronary angioplasty, the vascular smooth muscle cells are known to undergo a process of phenotypic modulation from contractile state to synthetic state, and this leads to intimal thickening (8). In the present study, we have investigated which subtype of natriuretic peptide receptors is predominantly expressed on the smooth muscle cells after vascular injury, and we have also examined whether administration of CNP inhibits intimal thickening after vascular injury in vivo.

MATERIALS AND METHODS

Peptides : CNP with 22 amino acids and α-human ANP (α-hANP) were produced in E. coli by using recombinant DNA method and purified (to be published). The peptides were characterized by HPLC and amino acid analysis.

Intimal thickening model : Male Sprague-Dawley rats, weighing 300-350g (Charles River Japan), were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). Endothelial injury of the right common carotid artery was performed by the air-drying method (10). Briefly, the carotid artery was isolated in a length of approximately 1 cm from surrounding connective tissues, and both ends of the isolated segment were clipped. After blood in the isolated segment was rinsed out with saline, a gentle stream of air (25 ml/min) was passed through the lumen for 3 min to produce endothelial injury. The rats were sacrificed 14 days after the operation and the carotid arteries were removed for determination of particulate guanylyl cyclase activity and for morphometric analysis as described below.

Guanylyl cyclase activity : Both injured and contralateral intact carotid arteries were homogenized in 50mM Tris-HCl, pH 7.4, containing 1mM EDTA, 1mM phenylmethylsulfonyl fluoride, 10μg/ml soybean trypsin inhibitor and 1mM dithiothreitol. The homogenates were centrifuged at 105,000xg for 1hr at 4°C. The particulate fraction was solubilized by resuspending the pellet into 50mM Tris-HCl, pH 7.4, containing 1mM EDTA, 1mM dithiothreitol and 0.5(w/v)% Triton X-100 and incubating for 2 hr at 4°C. The solubilized membrane fraction was obtained after centrifugation at 10,000xg for 30 min (11). The activity of guanylyl cyclase in solubilized membrane was determined in 50mM Tris-HCl, pH 7.4, containing 1mM GTP, 0.5mM ATP, 5mM $MgCl_2$, 7.5mM phosphocreatine, 13U/ml creatine kinase and 1mM 1-methyl-3-isobutylxanthine in the presence of various concentrations of CNP or α-hANP. Concentration of cGMP generated during 5 min incubation at 37°C was determined by radioimmunoassay (Yamasa Shoyu).

CNP treatment and morphometric analysis : The rats received CNP at 1.0 μg/kg/min by a constant i.v. infusion. The infusion of CNP was started 30 min after air-drying vascular injury and continued for 14 or 5 days. The carotid arteries were dissected, fixed and sectioned for morphometric analysis 14 days after the injury. Intimal and media areas were determined in 6 to 8 different cross-sections of each of the vessel using computerized digitizing system (IBAS II, Carl Zeiss), and the maximal intimal/media (I/M) ratio was calculated. Blood was collected on day 14 from 8 rats which received CNP for 14 days. Plasma concentration of CNP was determined by radioimmunoassay (12).

RESULTS

Normal rat carotid arteries have virtually no detectable intimal smooth muscle layer (I/M = 0) (Figure 1A). Compared to uninjured arteries, injured carotid arteries developed marked

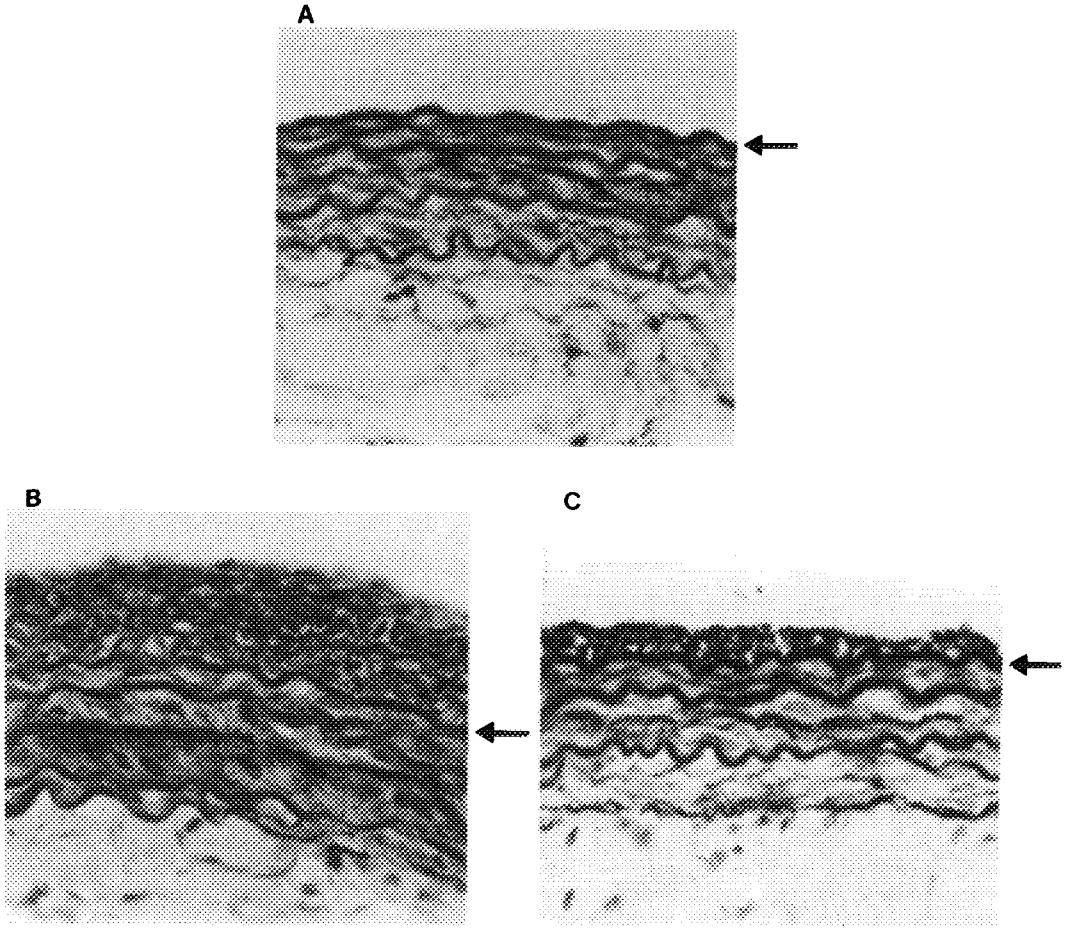

Figure 1. Cross-sections of rat carotid arteries (Elastica-van Geison stained). Photographs show intact rat carotid artery (A), artery 14 days after air-drying injury of control rat (B) and that from a rat treated with CNP for 14 days (C). Arrows indicate the internal elastic lamina between intima and media (x370). Typical examples in each group are represented.

intimal thickening (Figure 1B). Morphometric analysis of the arteries 14 days after vascular injury revealed an average I/M ratio of 37.5 %. The thickened intima was primarily composed of smooth muscle cells and extracellular matrix.

We first intended to clarify which subtype of natriuretic peptide receptors was expressed in carotid arteries after injury. For this purpose, the particulate fractions prepared from injured and contralateral intact carotid arteries 14 days after the injury were treated with CNP or α-hANP, and analyzed for cGMP production. In the injured tissues, both CNP and α-hANP increased cGMP production with a similar potency (Figure 2B). In contrast, a marked elevation of cGMP production was only evoked by α-hANP in intact tissues (Figure 2A).

We next examined whether exogenous CNP could inhibit the development of intimal lesions. The rats were treated with CNP by continuous i.v. infusion at 1μg/kg/min after vascular injury. The plasma concentration of CNP on day 14 was 12.6±2.7 ng/ml (ca. $0.6 \times 10^{-}$

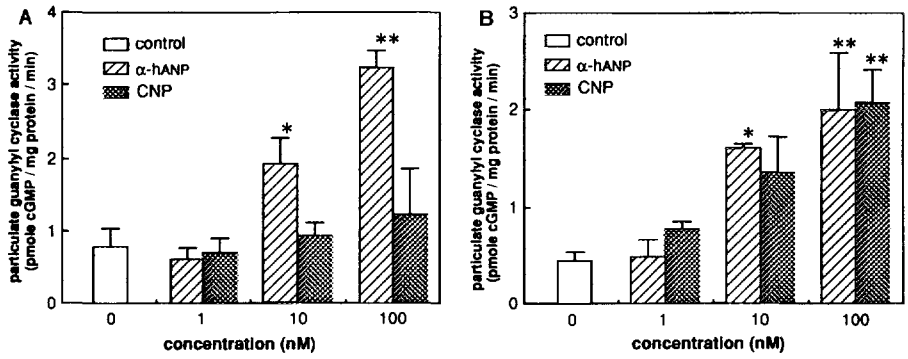

Figure 2. Effect of CNP and α-hANP on guanylyl cyclase activity in solubilized membrane fractions of intact (A) and injured (B) rat carotid arteries. The data represent means±S.E. of 3 determinations. *, ** Significant difference from control at $P<0.05$, or 0.01, respectively (Duncan new multiple range test).

^{8}M, mean±S.E. of 8 rats). CNP treatment for 14 days resulted in a marked reduction of intimal cross-section area 14 days after the injury as compared with control rats (Figure 1C) ; in other words, the I/M ratio of the group was only 30% of those in control rats (Table 1). We also examined the infusion period of CNP, and as shown in Table 1, the treatment with CNP for only initial 5 days after the injury also inhibited the intimal thickening by 60%, which was almost the same extent to that obtained by the treatment for 14 days.

DISCUSSION

In the previous paper, we showed that functional GC-B and GC-A are dominantly expressed in cultured vascular smooth muscle cells and intact aortic media, respectively (5, 6). This was recently confirmed at mRNA levels (13). The present study demonstrated that GC-B was expressed in injured arteries with intimal thickening, but not in intact ones. CNP potently stimulated cGMP production in injured arteries, but not in intact ones, while α-hANP stimulated cGMP production in both intact and injured arteries to the same extent (Figure 2A

Table 1. Effect of CNP on intimal thickening of rat carotid arteries after vascular injury induced by air-drying

treatment	dose	period	N	I/M ratio (%)
none (control)	-	-	16	37.5 ± 6.7
CNP	1 μg/kg/min	14 days	14	11.5 ± 5.0**
CNP	1 μg/kg/min	5 days	9	14.7 ± 7.9*

CNP (1μg/kg/min) was administered by constant i.v. infusion into rats for 14 days or 5 days after air-drying injury of the carotid arteries. Control rats received no treatment after the injury. The rats were sacrificed 14 days after vascular injury, and the intimal/media (I/M) ratio was determined by morphometric analysis. *, ** Significant difference from control rats at $P<0.05$, or 0.01, respectively (Mann-Whitney U-test).

and 2B). Since injured arteries are composed of both medial and intimal smooth muscle cells (Figure 1B), it is likely that α-hANP enhanced cGMP production in both intact and injured arteries through the activation of GC-A in the medial smooth muscle cells, and that CNP activated GC-B expressed in the newly developed intimal smooth muscle cells of injured arteries. To confirm this in more detail, we are now investigating the expression of mRNAs for GC-A and GC-B in injured carotid arteries by in situ hybridization.

The existence of GC-B at the sites of injured carotid arteries with intimal thickening and our previous finding that CNP has antiproliferative effect on cultured vascular smooth muscle cells have prompted us to investigate the inhibitory effect of CNP on the formation of intimal thickening after vascular injury. CNP was administered into rats after vascular injury by constant i.v. infusion, since intravenously injected CNP had quite a short half-life (less than 2 min, unpublished data). The plasma concentration of CNP was approximately 10^{-8}M during constant infusion of 1μg/kg/min of CNP, which was comparable to the concentration of CNP required for the activation of the guanylyl cyclase and for the antimitogenic effect in cultured vascular smooth muscle cells in vitro (5, 6). As shown in Table 1, CNP treatment for 14 days led to a significant reduction of intimal cross-section area 14 days after the injury. Inhibition of intimal thickening was also observed when CNP was administered for only initial 5 days after the injury. Since it has been shown that the mitogenesis of the intimal smooth muscle cells reached to the maximum within 7 days (10), CNP is supposed to inhibit replication of intimal smooth muscle cells in early stage after vascular injury, and this leads to an inhibition of the formation of the intimal lesion.

Although CNP has been thought to localize only in central nervous system (12, 14), we have recently found that human monocytic cell line, THP-1, produced CNP when stimulated by phorbor ester (15). It has also been reported that CNP is produced in cultured endothelial cells, and the production is markedly stimulated by TGF-β (16). These results suggest that CNP/GC-B system physiologically plays an important role in the regulation of the proliferation of vascular smooth muscle cells. It is of interest whether CNP is secreted from activated macrophages or endothelium after vascular injury.

In conclusion, we showed that GC-B, CNP specific receptor, was expressed in injured arteries with intimal thickening and that exogenously administered CNP could inhibit proliferation of vascular smooth muscle cells in vivo. These results show the possibility that CNP might be useful to prevent the progression of restenosis after coronary angioplasty.

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