Phenotype-Related Alteration in Expression of Natriuretic Peptide Receptors in Aortic Smooth Muscle Cells

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To elucidate the physiological and pathophysiological roles of the natriuretic peptide family in vascular smooth muscle cells, in which the natriuretic peptide family is implicated in growth inhibition as well as vasorelaxation, we have examined the phenotype-related expression of three kinds of natriuretic peptide receptors in rat aortic smooth muscle cells. The expression of natriuretic peptide receptors at the mRNA level was studied by Northern blot hybridization, and the expression at the protein level was determined by the cGMP production method and receptor binding assay. In intact aortic media, atrial natriuretic peptide (ANP)-A receptor mRNA and ANP-B receptor mRNA were detected, and the potency of cGMP production by ANP was at least two orders of magnitude stronger than that by C-type natriuretic peptide. Clearance receptor mRNA was undetectable, and only a small amount of the clearance receptor was detected by the binding assay in intact aortic media. By contrast, in cultured aortic smooth muscle cells at the first, fifth, and 17th passages, the ANP-B receptor mRNA level markedly increased; meanwhile, the expression of the ANP-A receptor mRNA became undetectable. C-type natriuretic peptide was one order of magnitude more potent than ANP in cGMP production in cultured aortic smooth muscle cells. The clearance receptor density and its mRNA level increased tremendously in these cultured cells. These results demonstrate that the marked phenotype-related alteration occurs in the expression of natriuretic peptide receptors in rat aortic smooth muscle cells. (Circulation Research 1992;71:34-39)

Key Words • natriuretic peptide • natriuretic peptide receptors • phenotypic modulation • smooth muscle cells

After the discovery of atrial natriuretic peptide (ANP) from the heart,\(^1\)-\(^3\) brain natriuretic peptide (BNP) was isolated from the porcine brain\(^4\) and subsequently from the heart.\(^5\) Since then, ANP and BNP have been considered to form a natriuretic peptide family. Recently, the third member of the natriuretic peptide family, C-type natriuretic peptide (CNP), has been isolated from the porcine brain.\(^6\)

As for natriuretic peptide receptors, two distinct types of receptor have been identified. One type of receptor is a particulate guanylate cyclase\(^7,8\) and is designated as the biologically active receptor. Molecular cloning has revealed the existence of two subtypes of the biologically active receptor, named the ANP-A receptor or guanylate cyclase A (GC-A) and the ANP-B receptor or guanylate cyclase B (GC-B).\(^9,10\) The other type of receptor is not coupled to guanylate cyclase\(^11-14\) and is proposed to have a major role in the clearance of natriuretic peptides.\(^15\) Therefore, this receptor is termed the clearance receptor (C receptor). Recently, we\(^16\) and others\(^17\) have demonstrated that the ANP-A receptor has a ligand selectivity with the rank order of potency for cGMP production, ANP ≥ BNP >> CNP, and that the rank order of the ligand selectivity for the ANP-B receptor is CNP > ANP ≥ BNP. We have also revealed that the rank order of the binding affinity for the C receptor is ANP > CNP > BNP.\(^16\) Thus, the natriuretic peptide system is a complex system consisting of at least three endogenous ligands (ANP, BNP, and CNP) and three receptors (ANP-A receptor, ANP-B receptor, and the C receptor) and is involved in body fluid homeostasis and blood pressure control as cardiac hormones and as neuropeptides.\(^1-6,18-20\)

Vascular smooth muscle is one of the major target tissues of natriuretic peptides, and the natriuretic peptide system is implicated in relaxation and inhibitory regulation of proliferation and hypertrophy of vascular smooth muscle cells (SMCs).\(^21-23\) Cultured vascular SMCs possess a large number of natriuretic peptide receptors,\(^1,2,11,13,14\) and various factors have been reported to regulate the expression of natriuretic peptide receptors in cultured
vascular SMCs. Vascular SMCs are also known to undergo a process of phenotypic modulation under certain pathological conditions.

In the present study, to elucidate the physiological and pathophysiological implications of the natriuretic peptide system in vascular SMCs, we have examined the phenotype-related expression of natriuretic peptide receptors in intact rat aortic media and cultured rat aortic SMCs.

Materials and Methods
Preparation of Tissue and Cells
Intact aortic media. Thoracic aortas were excised from male Wistar rats (250–300 g). The intact aortic media was obtained by isolating the media from the adventitia and intima as previously reported.

Cultured SMCs. Cultured aortic SMCs were derived from the explants of the thoracic aortas of male Wistar rats (250–300 g) by the method of Ross and were grown in Dulbecco’s modified Eagle’s medium (DMEM, Flow Laboratories, Inc., Irvine, UK) supplemented with 10% fetal calf serum (Hazleton Biologics, Inc., Lenexa, Kan.). 100 units/ml penicillin, and 100 μg/ml streptomycin. SMCs at the first, fifth, and 17th passages were used in the present study.

Characterization of Natriuretic Peptide Receptor Proteins
Preparation of receptors. Crude receptor preparations were obtained by solubilizing the membranes from intact aortic media and confluent aortic SMCs in culture with Triton X-100 according to the procedure of Shimomaka et al. Protein content was determined by use of a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.).

Analysis of the C Receptor by binding assay. The C receptor has been reported not to be coupled to guanylate cyclase nor to affect cGMP production by ANP. Therefore, the expression of the C receptor was examined by the competitive binding study of [125I]ANP by ANP and a selective ligand for the C receptor, des-[Gln18,Ser9,Gly20,Leu21,Gly22]ANP-[4-23]-NH2 (C-ANF-[4-23]), as we previously reported. C-ANF-[4-23] binds with at least three orders higher affinity to the C receptor than to the biologically active receptor, and [α-14C]cAMP (α-14CAMP, Peptide Institute, Inc., Minoh, Japan) was radioiodinated by the chloramine T method. Specific activity was 700–1,400 mCi/μmol. Crude receptor preparations (5–30 μg protein) were incubated for 48 hours at 4°C with [125I]α-14CAMP and various concentrations of C-ANF-[4-23] (donated by Professor T. Maack, Cornell University Medical College, New York) or unlabeled α-14CAMP. Nonspecific binding was determined using 0.1 μM unlabeled α-14CAMP and was less than 10% of total binding.

Expression of the C Receptor by cGMP production. The expression of two subtypes of the biologically active receptor was determined by the effects of ANP and CNP on cGMP production in intact aortic media and cultured aortic SMCs, on the basis of the observation that ANP is at least 1,000-fold stronger than CNP in cGMP production via ANP-A receptor and that CNP is more potent than ANP in cGMP production via ANP-B receptor. Cultured aortic SMCs, grown to confluence in six-well plates, and segments of the intact aortic media, weighing 15–25 mg, were preincubated at 37°C for 10 minutes with 0.9 ml DMEM containing 0.1% bovine serum albumin and 0.5 mM isobutyl-methylxanthine (Sigma Chemical Co., St. Louis, Mo.). ANP or CNP (0.1 ml, generously provided by Dr. K. Inouye, Shionogi Research Laboratories, Shionogi & Co., Ltd., Osaka, Japan) was added to the medium. Then, the mixtures were further incubated at 37°C for 30 minutes. After the incubation, the medium was rapidly removed and added to tubes containing 1 ml ice-cold 12% trichloroacetic acid (TCA). The tissue segments were frozen in liquid nitrogen and mechanically homogenized in 6% TCA. To each well containing the cells, 1 ml ice-cold 6% TCA was added. TCA was removed from the samples by extracting three times with water-saturated ether. The cGMP concentration was determined by radioimmunoassay, as we previously reported.

Characterization of Natriuretic Peptide Receptor mRNA and Actin mRNA
RNA extraction. Total RNA was extracted from intact aortic media and confluent aortic SMCs in culture by homogenization in guanidinium thiocyanate buffer and centrifugation in cesium chloride solution as we previously reported.

Preparation of rat ANP-A receptor cDNA, rat ANP-B receptor cDNA, bovine C receptor cDNA, and human β-actin genome probes. The cDNA probes specific for rat ANP-A receptor and rat ANP-B receptor were prepared as previously described. Since cDNA of the rat C receptor has not been cloned, bovine C receptor cDNA probe was obtained by cDNA synthesis and the polymerase chain reaction method using the total RNA of bovine lung and the sequence, 5'-CTACTGAGGACAGTGAAAA-3', and antisense, 5'-CGAAAAGTGGAATGCTGAT-3', primers, corresponding to nucleotides 1423-1440 and 2053-2070 of bovine C receptor cDNA, respectively. The human β-actin genome probe was the 443-bp HindIII fragment of the human β-actin genome (Wako Pure Chemical, Osaka, Japan). The probes were labeled by random-primed synthesis to the specific activity of approximately 1×10⁶ cpm/μg.

Northern blot analysis. Northern blot analyses of ANP-A receptor, ANP-B receptor, C receptor, and actin mRNA were performed using 5 μg total RNA, as we previously reported. Measurement of the density of the hybridizing bands was done under the condition that the density shows a linear relation with the amount of RNA applied to the electrophoresis gel.

Results
Expression of the C Receptor
Competitive inhibition of [125I]α-14CAMP binding to the receptor preparation from intact aortic media by C-ANF-[4-23] revealed that C-ANF-[4-23] competed for 5% of the total ANP binding sites with a high affinity and that concentrations of C-ANF-[4-23] approximately four orders of magnitude higher were necessary for the displacement of [125I]α-14CAMP from the remaining binding sites (Figure 1A). The density of total ANP binding sites obtained by Scatchard analysis was 38 fmol/mg.
protein. Therefore, the density of the C receptor was estimated to be 2 fmol/mg protein in intact aortic media. In marked contrast, in cultured aortic SMCs at the fifth passage, C-ANF-[4-23] effectively competed for 96% of the total ANP binding sites with a high affinity (Figure 1B), and the density of total ANP binding sites was 870 fmol/mg protein. Similar results were obtained in cultured aortic SMCs at the first and 17th passages (data not shown). Thus, the expression of the C receptor is minimal in intact aortic media but tremendously augmented in cultured aortic SMCs.

Expression of Biologically Active Receptor Subtypes

In SMCs of intact aortic media, ANP was at least two orders of magnitude more potent than CNP in cGMP production (Figure 2A). The patterns of cGMP production by ANP and CNP in intact aortic media were similar to those via the ANP-A receptor.\(^{16,17}\) By contrast, CNP was found to be approximately one order of magnitude more potent than ANP in cGMP production in cultured aortic SMCs both at the first passage (Figure 2B) and at the fifth passage (Figure 2C). Similar results were obtained in cultured aortic SMCs at the 17th passage (data not shown). The patterns of cGMP production in cultured aortic SMCs at the first, fifth, and 17th passages were compatible with those via the ANP-B receptor.\(^{16,17}\)

Expression of Natriuretic Peptide Receptor mRNA

Figure 3 shows the results of Northern blot analysis of total RNA from intact aortic media and cultured aortic SMCs at the first, fifth, and 17th passages. As shown in the left panel of Figure 3, ANP-A receptor mRNA of approximately 4.0 kb was abundantly expressed in intact aortic media, but it became undetectable in cultured aortic SMCs at the first, fifth, and 17th passages. The middle panel of Figure 3 shows that a hybridizing band for ANP-B receptor mRNA of approximately 4.0 kb existed in intact aortic media and that the ANP-B receptor mRNA level was markedly increased in cultured aortic SMCs. C receptor mRNA (Figure 3, right panel) was undetectable in intact aortic media when 5 \(\mu\)g total RNA was used; however, in cultured aortic SMCs at the first, fifth, and 17th passages, the expression of C receptor mRNA was tremendously aug-

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**Figure 1.** Displacement curves of \(^{125}\)I-\(\alpha\)-rat atrial natriuretic peptide (\(^{125}\)I-\(\alpha\)-rANP on figure) by \(\alpha\)-rANP (○) and C-ANF-[4-23] (○) in the binding assay with crude receptor preparations from intact aortic media (panel A) and cultured aortic smooth muscle cells at the fifth passage (panel B). Each point represents the mean of three separate experiments.

**Figure 2.** cGMP production by \(\alpha\)-rat atrial natriuretic peptide (○) and C-type natriuretic peptide (○) in intact aortic media (panel A) and cultured aortic smooth muscle cells at the first (panel B) and fifth (panel C) passages. Each point represents the mean of four separate experiments.

**Figure 3.** Northern blot analysis of atrial natriuretic peptide (ANP)-A receptor (guanylate cyclase [GC]-A), ANP-B receptor (GC-B), and clearance (C) receptor mRNA in intact aortic media and cultured aortic smooth muscle cells. Total RNA (5 \(\mu\)g) from intact aortic media (lane 1) and cultured aortic smooth muscle cells at the first (lane 2), fifth (lane 3), and 17th (lane 4) passages was used for analysis.
mRNA in intact aortic media and cultured aortic smooth muscle cells. Total RNA (5 μg) from intact aortic media (lane 1) and cultured aortic smooth muscle cells at the first (lane 2), fifth (lane 3), and 17th (lane 4) passages was used for analysis.

Expression of Actin Isoforms

The expression of actin isoforms in vascular SMCs has been regarded as one of the biological markers of the phenotypic modulation; i.e., vascular SMCs show a decrease in the expression of α-actin with a corresponding increase in the expression of β- and γ-actin in association with the modulation from the contractile phenotype to the synthetic phenotype. Therefore, Northern blot analysis of mRNA of three actin isoforms was performed in intact aortic media and cultured aortic SMCs with a human β-actin genome probe. The intact aortic media (Figure 4, lane 1) gave intense bands of 1.6 kb and 2.1 kb corresponding to α-actin mRNA and β- and γ-actin mRNA, respectively. By contrast, in cultured aortic SMCs at the first, fifth, and 17th passages (Figure 4, lanes 2–4), α-actin mRNA levels decreased substantially along with the slight increase in β- and γ-actin mRNA levels. The decrease in α-actin mRNA and the corresponding increase in β- and γ-actin mRNA in cultured aortic SMCs are consistent with previous reports.

Discussion

The present study demonstrates the phenotype-related alteration in the expression of natriuretic peptide receptors in vascular SMCs. In intact aortic media, ANP-A receptor mRNA and ANP-B receptor mRNA were detected, but in cultured aortic SMCs at the first, fifth, and 17th passages, the ANP-A receptor mRNA level became undetectable, and the ANP-B receptor mRNA level markedly increased. The cGMP production method revealed that, in intact aortic media, the potency of ANP was at least two orders of magnitude stronger than that of CNP, which was similar to the patterns of cGMP production via the ANP-A receptor. By contrast, in cultured aortic SMCs, CNP was one order of magnitude more potent than ANP in cGMP production, which was consistent with the patterns of cGMP production via the ANP-B receptor. As for the C receptor, only a small amount of the receptor was detected by the binding assay, and C receptor mRNA was undetectable in intact aortic media; however, the C receptor density and its mRNA level increased tremendously in these cultured cells. Thus, the marked phenotype-related alteration in the expression of natriuretic peptide receptors occurs in rat aortic SMCs, as summarized in Table 1.

Table 1. Phenotype-Related Alteration in Expression of Natriuretic Peptide Receptors in Aortic Smooth Muscle Cells

<table>
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<tr>
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<th>ANP-A receptor (GC-A)</th>
<th>ANP-B receptor (GC-B)</th>
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<td>SMCs from intact media</td>
<td>+++</td>
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<td>Cultured aortic SMCs</td>
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ANP, atrial natriuretic peptide; GC, guanylate cyclase; C, clearance; SMCs, smooth muscle cells.

The depressor action of CNP in vivo is approximately 100-fold weaker than that of ANP in rats, in spite of the higher potency of CNP than ANP for cGMP production in cultured rat aortic SMCs. The cause of this discrepancy was unclear. Recently, others have clarified the ligand selectivity of the natriuretic peptide receptors: the rank order of potency for cGMP production via the ANP-A receptor is ANP ≥ BNP > CNP; the rank order via the ANP-B receptor is CNP > ANP ≥ BNP. Thus, the discrepancy in the relative potency of CNP to ANP between the in vivo depressor action and the potency for cGMP production in cultured aortic SMCs can be explained by the marked phenotype-related alteration in the expression of natriuretic peptide receptors in rat aortic SMCs, demonstrated in the present study.

As for the C receptor, previous studies using cultured vascular SMCs raised the possibility that the vascular SMC could be one of the major clearance sites of natriuretic peptides. However, the expression of small amounts of the C receptor in intact aortic media demonstrated in the present study suggests that tissues such as lung or kidney, in which a high density of the C receptor is expressed in vivo, mainly serve as clearance binding sites. Therefore, although cultured vascular SMCs provide a useful system for in vitro studies of actions of natriuretic peptides, careful analysis should be necessary to elucidate the actions of natriuretic peptides on vascular SMCs in vivo. The augmented expression of the C receptor at the protein and mRNA levels and the existence of several discrete C receptor mRNA bands in cultured vascular SMCs are consistent with previous observations. The cause of the mRNA heterogeneity is not clear at present; however, these findings indicate the validity of our method for analysis.
uretic peptide receptors in rat aortic SMCs is not clear at present. Vascular SMCs in situ and in culture are known to express two phenotypes, depending on the conditions present.29-31 In intact media of adult arteries, SMCs contract in response to chemical and mechanical stimuli and synthesize only small amounts of extracellular matrix materials, and SMCs at this stage are referred to as being in a “contractile phenotype.” By contrast, when the intact media of adult arteries are placed in culture, the vascular SMCs undergo a process of phenotypic modulation that is characterized by increased proliferative and synthetic activity, reorganization of the cytoskeleton, and decreased contractile activity29-31,33; at this stage, they are referred to as being in a “synthetic phenotype.” The phenotypic modulation to the synthetic phenotype can also be triggered under certain pathological conditions in vivo such as atherosclerosis and hypertension.30-32 In the present study, the pattern of the expression of actin isoforms in intact aortic media is consistent with that of vascular SMCs in the contractile phenotype,30,33 whereas cultured aortic SMCs express actin isoforms compatible with those of vascular SMCs in the synthetic phenotype.29,33 Therefore, it is possible that the phenotype-related alteration in the expression of natriuretic peptide receptors occurs in vascular SMCs in atherosclerosis and hypertension.

Accumulating evidence indicates the growth-inhibitory and vasorelaxant actions of natriuretic peptides on vascular SMCs.1,4,6-21,23 These effects are thought to be mediated, at least in part, by cGMP produced via the biologically active receptor,1,3,22-23 We16 and others,17 have demonstrated that the ANP-A receptor is at least one order of magnitude more sensitive to ANP or BNP in cGMP production than the ANP-B receptor. Therefore, if the increase in the ANP-B receptor density and the decrease in the ANP-A receptor density take place in vascular SMCs in vivo as discussed above, this indicates that vascular SMCs are less sensitive to ANP and BNP in vasorelaxation or growth inhibition.

Since the C receptor is not coupled to guanylate cyclase,13,15,55 the increase in the expression of the C receptor is unlikely to contribute to the growth inhibition or relaxation of SMCs by the natriuretic peptide family through cGMP. However, recent evidence suggests that some actions of natriuretic peptides could be mediated through a non-guanylate cyclase–linked receptor system.42 Therefore, it is likely that the augmented expression of the C receptor in cultured aortic SMCs shown in the present study may have functional significance.

In conclusion, the present study demonstrates that the phenotype-related alteration in the expression of natriuretic peptide receptors occurs in rat aortic SMCs. Further studies are necessary to clarify the mechanism and functional significance of the alteration in the expression of natriuretic peptide receptors in vascular SMCs.

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