Regulation of Natriuretic Peptide Receptor A and B Expression by Transforming Growth Factor-\(\beta_1\) in Cultured Aortic Smooth Muscle Cells

Nobuaki Fujio, Francis Gossard, Francis Bayard, Johanne Tremblay

Abstract Two types of natriuretic peptide receptors (NPR-A and NPR-B) are membrane guanylate cyclases whose relative expression varies in different tissues. Because natriuretic peptides have been shown to inhibit aortic smooth muscle proliferation, we investigated the regulation of NPR-A and NPR-B in these cells under different proliferative conditions. NPR-B subtype mRNA levels were measured by our newly developed quantitative reverse transcription-polymerase chain reaction assay using mutated NPR-A and NPR-B cRNA as internal standards. The functional impact of their expression was determined by atrial natriuretic peptide (ANP)- and C-type natriuretic peptide (CNP)-induced stimulation of cyclic GMP production. In the intact aorta, NPR-B mRNA levels were found to be 10-fold higher than those of NPR-A. This dominance was further amplified (1000-fold) in long-term cultures (10 to 15 passages) of aortic smooth muscle cells (ASMC). Higher cyclic GMP production with CNP than with ANP was observed in cultured ASMC from Wistar-Kyoto (WKY) rats. Similar stimulation by the two agonists was noted in spontaneously hypertensive rat (SHR) cells, paralleled by a 10-fold increase in NPR-A mRNA levels and ANP stimulation of cyclic GMP in hypertensive cells. The present study also evaluated NPR-A and NPR-B mRNA control by transforming growth factor-\(\beta_1\) (TGF-\(\beta_1\)), an important regulator of cell proliferation that is overexpressed in SHR ASMC. TGF-\(\beta_1\) decreased both NPR-A and NPR-B mRNA levels with a predominant effect in SHR cells at high cell density. This effect was paralleled by a fall in CNP- and ANP-induced cyclic GMP levels after preincubation with TGF-\(\beta_1\). Our study demonstrates the increase of NPR-B expression in contrast to proliferative phenotypes of ASMC and presents the first evidence of cytokine regulation of the two NPR subtypes at the mRNA level with the potential for an integrated role of these two systems in the control of vascular smooth muscle growth. (Hypertension. 1994;23[part 2]:908-913.)

Key Words • receptors, atrial natriuretic peptide • transforming growth factor beta • muscle, smooth, vascular • cyclic guanosine monophosphate • hypertension, essential

The natriuretic peptide family consists of at least three members: atrial natriuretic peptide (ANP),1,2 brain natriuretic peptide (BNP),3,4 and C-type natriuretic peptide (CNP).5,6 Three types of receptors of this natriuretic peptide family have been identified and cloned. Natriuretic peptide receptor-C (NPR-C) is considered to have mainly a clearance function,7,8 while the other two, natriuretic peptide receptor-A (NPR-A) and natriuretic peptide receptor-B (NPR-B), also known as guanylate cyclase-A and guanylate cyclase-B, respectively,9,11 are believed to be biologically active binding sites possessing intrinsic particulate guanylate cyclase activity.

Several studies have shown that NPR-A and NPR-B have different ligand selectivity. NPR-A mainly binds ANP and BNP, whereas NPR-B mainly binds CNP.9,12,13 This differential selectivity suggests that they play distinct physiological roles. Suga et al14 recently reported that NPR-B is highly expressed in vascular smooth muscle and that CNP is synthesized in and secreted from endothelial cells in response to various secretagogues, including transforming growth factor-\(\beta_1\) (TGF-\(\beta_1\)). They postulated the existence of a "vascular natriuretic system."

Abnormal vascular smooth muscle proliferation is considered to be one of the factors contributing to increased peripheral vascular resistance in hypertension. Several studies, including our own, have demonstrated that proliferation of cultured aortic smooth muscle cells (ASMC) from spontaneously hypertensive rats (SHR) is increased in response to several growth factors.15-17

We previously reported that the accumulation of TGF-\(\beta_1\) mRNA levels is higher in SHR smooth muscle cells than in normotensive Wistar-Kyoto (WKY) rats and that DNA synthesis is enhanced in response to exogenous TGF-\(\beta_1\) in SHR at high cell densities without any effect in WKY rats.18

Because ANP and CNP have been shown to inhibit vascular cell proliferation in vitro,19 the present study was designed to investigate the influence of TGF-\(\beta_1\) on NPR gene expression in ASMC of WKY and SHR origin at the synthetic proliferative stage. For this purpose, we have developed a quantitative polymerase chain reaction (PCR) method to measure absolute NPR-A and NPR-B mRNA levels. The functional consequences of this expression were assessed by determination of agonist-induced cyclic GMP (cGMP) production.
Methods

Cell Culture

Cultured ASM cells were obtained by an explant method from aortas of 10-week-old male SHR and WKY rats as described previously. The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) plus 100 U/mL penicillin and 100 μg/mL streptomycin in the presence of 10% calf serum. At confluency, they exhibited a hill-and-valley pattern typical of smooth muscle cells in culture. The cells were passaged by trypsinization with 0.05% trypsin in Ca2+–Mg2+-free Dulbecco’s phosphate-buffered saline. They were counted in a Coulter counter (Coulter Electric Inc) and used for experimentation between passages 5 and 15.

Cyclic GMP Measurements

The cell culture dishes, washed with DMEM, to which the test agents were added, were kept in a 95% air/5% CO2-humidified incubator at 37°C for appropriate time periods. At the end of incubation, the reaction was arrested by removal of the medium and the addition of 500 μmol/L 1-methyl-3-isobutylxanthine (MIX) as well as 10 mmol/L EDTA. cGMP levels were measured with a previously developed radioimmunoassay. cGMP accumulation was quantitated in the extracellular medium of ASM after 90 minutes of incubation with increasing concentrations of ANP or CNP. Previous experiments have shown that inclusion of the CNP analogue C-ANP (102-121) (5x10^-6 mol/L) blocks C-receptors, thus enhancing cGMP stimulation induced by ANP or CNP (data not shown).

Five minutes of preincubation with this analogue as well as MIX, a phosphodiesterase inhibitor, were undertaken here.

RNA Extraction and Quantitative PCR

Measurement of NPR-A and NPR-B mRNA Levels

Total cellular RNA extracts were obtained by the acid guanidinium-thiocyanate-phenol-chloroform method, and the quality of the preparations was verified by gel electrophoresis. RNA concentrations were measured by UV spectrophotometry. Extracted RNA preparations were then stored at −80°C until they were used. NPR-A cDNA was processed as described elsewhere. A mutated NPR-A cDNA fragment was obtained by PCR with oligonucleotides, sense 5’-GGTTGGGAACCGATGAGGAGGCC-3’ and antisense 5’-GAGTGCTACATCCCCG-3’. NPR-B cDNA (data not shown). A reaction volume of 50 μL containing 50 mmol/L Tris-HCl buffer (pH 8.4), 75 mmol/L KCl, 3 mmol/L MgCl2, 10 mmol/L 1,4-dithiothreitol, 0.5 mmol/L deoxynucleotides, and 50 pmol random hexamers for a total volume of 10 μL. After 1 hour of incubation at 37°C, the reaction products were subjected to PCR amplification with the following primers: NPR-A: sense primer 5’-GGTTGGGAACCGATGAGGAGGCC-3’ and antisense primer 5’-GAGTGCTACATCCCCG-3’; NPR-B: sense primer 5’-GGTTGGGAACCGATGAGGAGGCC-3’ and antisense primer 5’-GAGTGCTACATCCCCG-3’. These primers did not reveal cross amplification between NPR-A and NPR-B cDNA (data not shown). A reaction volume of 50 μL containing 50 mmol/L Tris-HCl buffer (pH 8.4), 50 mmol/L KCl, 1.5 mmol/L MgCl2, 0.2 mmol/L deoxynucleotides, and 40 pmol of each primer, 2.5 U of Taq DNA polymerase (BRL Gibco Laboratories), and 10 μCi [3P]-deoxyribocytosine triphosphate was incubated for 30 cycles of 1-minute denaturation at 94°C, 1-minute annealing at 65°C, and 1.5-minute polymerization at 72°C. Ten microliters of each sample was digested at 37°C for 1 hour with 10 U of EcoRI, resulting in complete digestion of the mutated DNA. The digested samples were electrophoresed on 1.5% agarose gel, and radioactive bands were quantified with a PhosphorImager (Molecular Dynamics). The principle of this method is illustrated in Fig 1.

Statistical Analysis

Results are expressed as mean±SEM when three to five experiments were performed. Comparisons between experi-
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Fig 2. Representative Phosphorimager blots of polymerase chain reaction products after EcoRI digestion in the aorta and cultured aortic smooth muscle cells (ASMC). One microgram of total RNA was titrated with an increasing number (1, 5, 20, 100, 500, 2000, and 10,000 x 10^8) of mutated natriuretic peptide receptor-A (NPR-A) or natriuretic peptide receptor-B (NPR-B) cRNA or 50, 200, and 1000 x 10^8 of NPR-B cRNA molecules.

Results

mRNA Levels of NPR-A and NPR-B in Aorta and Cultured ASMC Measured by Quantitative RT-PCR Assay

Fig 2 provides representative blots of PCR products after EcoRI digestion and gel electrophoresis in the aorta and in cultured ASMC. One microgram of total RNA was titrated with an increasing number (1, 5, 20, 100, 500, 2000, and 10,000 x 10^8) of mutated NPR-A or NPR-B cRNA or 50, 200, and 1000 x 10^8 of NPR-B cRNA molecules. Upper nondigested bands were derived from endogenous mRNA (NPR-A, 677 bp; NPR-B, 762 bp). Lower digested bands were derived from mutated cRNA (NPR-A, comigrating 373- and 304-bp bands; NPR-B, comigrating 385- and 377-bp bands). ASMC (10 to 15 passages) required a large number of mutated cRNA molecules for titration of NPR-B mRNA (Fig 2, right panel). The mean amount of mRNA (in number of molecules per microgram of total RNA) from three different experiments was 0.5 ± 0.3 x 10^8 for NPR-A and 5.1 ± 3.3 x 10^8 for NPR-B in the intact aorta. In cultured ASMC, the NPR-A mRNA levels in early passed cells were 1.05 ± 0.15 x 10^8, and in later passages (10 to 15) they were 0.2 ± 0.04 x 10^8; those of NPR-B increased from 12.4 ± 1.6 x 10^8 in early passed cells to 310 ± 130 x 10^8 at later passages. Thus, NPR-B mRNA levels were approximately 10 times higher than those of NPR-A in the aorta and in early cultures, increasing to approximately 1000 times in long-term cultured ASMC.

Stimulation of cGMP Production in WKY and SHR ASMC by ANP and CNP

Basal cGMP levels were similar between WKY and SHR cells. In WKY ASMC, 10^-7 mol/L CNP produced a 10-fold higher cGMP response than did the same dose of ANP, whereas in SHR a similar response was noted with both agonists (Fig 3). On the other hand, a near eightfold difference in ANP stimulation was observed between WKY rats and SHR, whereas the difference between the two strains was less than twofold with CNP.

Effect of TGF-β on CNP-Induced Increases of cGMP Levels in WKY and SHR ASMC

TGF-β acts as a bifunctional modulator of vascular smooth muscle cell growth. It inhibits serum- or platelet-derived growth factor-induced proliferation at low cell density and stimulates it at high cell density. We recently reported that in WKY ASMC, TGF-β has no such growth effect at either low or high cell density but stimulates DNA synthesis and cell proliferation in SHR ASMC at high cell density. We therefore examined the effect of TGF-β on CNP-induced cGMP production in WKY and SHR ASMC at low and high cell densities. Twenty hours after inoculation at low (2 x 10^4 cells per square centimeter) or high (10^5 cells per square centimeter) cell density, the medium was removed and the cells were replenished with fresh medium without (control) or with 10 ng/mL TGF-β, and incubated for 6 to 9 hours before stimulation with a maximal dose of CNP. Fig 4 shows that TGF-β inhibited the CNP-induced cGMP elevations in both WKY and SHR ASMC with a greater effect at high cell density. Addition of TGF-β before stimulation for 90 minutes with 10^-7 mol/L CNP.
Effect of Transforming Growth Factor-β, on Atrial Natriuretic Peptide- and C-Type Natriuretic Peptide-Induced Cyclic GMP Production In Wistar-Kyoto and Spontaneously Hypertensive Rat Aortic Smooth Muscle Cells

<table>
<thead>
<tr>
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<th>WKY</th>
<th>SHR</th>
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<tr>
<td>−TGF-β_1</td>
<td>16.3</td>
<td>9.1</td>
</tr>
<tr>
<td>+TGF-β_1</td>
<td>45.5</td>
<td>195.4</td>
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<tr>
<td>−TGF-β_1</td>
<td>4.8</td>
<td>300.1</td>
</tr>
<tr>
<td>+TGF-β_1</td>
<td>195.4</td>
<td>157.8</td>
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WKY indicates Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; TGF-β_1, transforming growth factor-β_1; ANP, atrial natriuretic peptide; and CNP, C-type natriuretic peptide. Aortic smooth muscle cells were plated at high cell density (10^6 cells per square centimeter). Twenty hours after incubation, serum was removed and TGF-β_1 (10 ng/mL) was added for 6 hours before stimulation for 90 minutes with 10^-7 mol/L ANP or 10^-7 mol/L CNP. The effect of TGF-β_1 analyzed by the nonparametric Wilcoxon test was significant at the level of P<.05.

Fig 5 includes a representative PhosphorImager blot of PCR products after EcoRl digestion and gel electrophoresis. Increasing amounts of total RNA from WKY ASMC were titrated with 5x10^6 molecules of mutated NPR-A cRNA or 1x10^6 molecules of mutated NPR-B cRNA. The upper band represents endogenous nondigestable mRNA, and the lower digested bands represent mutated cRNAs that have been reverse transcribed and coamplified with total ASMC RNA. As shown in Fig 2, ASMC required a large number of mutated cRNA molecules for NPR-B titration, indicating that these cells predominantly express NPR-B mRNA. Standard curves shown in the lower part of Fig 5 were used to quantify the amount of NPR-A and NPR-B mRNA in ASMC. Total RNA was extracted from WKY and SHR ASMC (10 to 15 passages) inoculated at low or high cell density. Then, 5x10^6 NPR-A cRNA or 1x10^6 NPR-B cRNA (representing a 50% titration point) was added to 1 μg of cellular total RNA before reverse transcription and PCR, as shown in the upper part of Fig 6. NPR-B mRNA levels were approximately 1000-fold higher than those of NPR-A in WKY ASMC at low or high cell density. In SHR ASMC inoculated at high cell density, NPR-A mRNA levels were increased by approximately 10-fold compared with SHR cells inoculated at low density, whereas NPR-B mRNA levels were only slightly elevated. At high but not at low cell density, a significant difference of NPR-A mRNA levels between WKY rats and SHR was in agreement with the higher ANP-induced cGMP increase depicted in Fig 3. Analysis of the TGF-β_1 action by the nonparametric Wilcoxon test revealed a significant (P<.01) reduction of NPR-A and NPR-B mRNA levels in both WKY rats and SHR cells at low and high cell densities, but a major effect was evident at high cell density for both NPR-A and NPR-B in SHR. This effect of TGF-β_1 on NPR-B mRNA appeared to be reflected at the level of the expressed protein because TGF-β_1 inhibited the CNP- and ANP-induced cGMP increment under all test conditions (Fig 4 and Table).

Discussion

Several studies including ours have reported that ANP elicits natriuretic and vasodilator responses more...
potently in SHR than in WKY rats. Similarly, responsiveness to ANP is greater in hypertensive humans and monkeys than in normotensive controls. We previously demonstrated increased cGMP production in response to ANP in different organs of SHR compared with three normotensive rat strains. This phenotype appeared demonstrated in vivo, with a less striking difference at an older age. We suggested that the higher response of the cGMP system to ANP could be a primary event in the pathogenesis of hypertension. We report here a similar exaggerated pattern of cGMP production in cultured ASMC derived from 10-week-old SHR compared with age-matched normotensive WKY controls. In cultured cells derived from older animals (20 to 25 weeks old), a diminished response has been noted. In fact, previous reports demonstrated that the enhanced cGMP response to ANP in the aorta of young SHR evolves to normal cGMP production and blood vessel relaxation in older animals compared with age-matched normotensive controls. Our recent studies demonstrated that the heightened cGMP response to ANP in SHR is paralleled by increased mRNA levels of NPR-A but not of NPR-B. The present investigation extends these earlier observations to cultured ASMC. The difference between WKY and SHR ASMC was greater for NPR-A than for NPR-B mRNA. This was reflected functionally in that the greatest difference in cGMP increases between WKY rats and SHR was seen with ANP rather than with CNP. Surprisingly, although NPR-B mRNA levels were much higher than those of NPR-A, this was not quantitatively reflected by ANP and CNP stimulation of cGMP production. As expected, the CNP response was higher than that of ANP in WKY ASMC. However, for 1000-fold higher NPR-B than NPR-A mRNA levels, there was only 10-fold higher CNP- than ANP-induced cGMP synthesis. In SHR cells, where NPR-A mRNA levels were elevated by approximately 10-fold compared with WKY ASMC, the difference between ANP- and CNP-induced cGMP increases was almost completely lost. Thus, the mRNA and functional data are consistent qualitatively, but other factors such as translational efficiency, protein processing and turnover, transport, and insertion into the plasma membrane could contribute to the apparent lower activity of NPR-B. Interestingly, in vivo it has been demonstrated that the CNP effects on natriuresis, diuresis, and vasodilation are approximately 1% of those of ANP. This suggests that the biological activity of NPR-B is much weaker than that of NPR-A or that the two receptors mediate different functions. Alternatively, the best selective endogenous agonist of NPR-B may not yet have been uncovered.

We also observed induction of NPR-B gene expression from contractile to proliferative phenotypes of ASMC with little effect on NPR-A expression, amplifying the differential expression of the two receptors. Our study presents the first evidence of gene regulation of NPR by TGF-β. Exposure to TGF-β decreased the mRNA levels of the two receptors in all conditions tested. This was paralleled by a fall in ANP- as well as CNP-induced cGMP production in cells treated with TGF-β. Because natriuretic peptides also act as growth inhibitors and TGF-β, is a bifunctional growth regulator of vascular smooth muscle cells, the interactions of these two systems may be of great importance in the process of cell proliferation. On the other hand, regulation of the natriuretic peptide system by TGF-β appears to be rather complex since TGF-β has the potential to upregulate the expression and secretion of CNP from endothelial cells and downregulate the expression of its receptor, NPR-B. More studies are needed to better understand this complex system. In conclusion, the present investigation provides the first data on cytokine regulation of NPRs at their gene level. The use of the newly developed quantitative RT-PCR assay allows discrimination between NPR-A and NPR-B expression in the contractile and proliferative stages of ASMC and demonstrates the selectively increased expression of NPR-A in SHR ASMC. It remains to be explored whether both systems, TGF-β and natriuretic peptides, play an integrated role in the process of vascular smooth muscle proliferation.

Acknowledgments

This study was supported by a grant from the Medical Research Council of Canada (MT-11463). Dr Tremblay was a senior scholar from Fonds de la Recherche en Santé du Québec, and Dr Fujio was the recipient of a Canadian Hypertension Society–Medical Research Council of Canada fellowship. Dr Bayard was a visiting scientist from the Laboratory of Experimental Endocrinology, Centre Hospitalier Universitaire Rangueil, Toulouse, France. The authors wish to acknowledge the technical expertise of Suzanne Cossette, Carole Long, Gilles Corbeil, and Monique Poirier, the secretarial skills of Josée Bédard-Baker, and Ovid Da Silva for editing this manuscript. The advice of Dr Pavel Hamet throughout these studies and during the preparation of this manuscript was greatly appreciated.

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Regulation of natriuretic peptide receptor A and B expression by transforming growth factor-beta 1 in cultured aortic smooth muscle cells.
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Hypertension. 1994;23:908-913
doi: 10.1161/01.HYP.23.6.908

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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