Opposite Regulation of Gax Homeobox Expression by Angiotensin II and C-Type Natriuretic Peptide

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Abstract  Growth arrest-specific homeobox (Gax) gene was isolated from rat aorta cDNA library and its expression was largely confined to the cardiovascular tissues. Gax gene was rapidly downregulated by platelet-derived growth factor in vascular smooth muscle cells (VSMCs) and overexpressed Gax was reported to reduce the neointimal thickening after balloon injury in vivo. We have demonstrated that angiotensin II (Ang II) stimulates vascular growth. In contrast, we also reported that C-type natriuretic peptide (CNP) is secreted from vascular endothelial cells to act as a novel endothelium-derived relaxing peptide and inhibits vascular growth via cGMP cascade. In the present study, we examined the effects of Ang II and CNP on Gax gene expression in VSMCs. In quiescent rat aortic VSMCs, Gax mRNA (2.3 kb) level became negligible 6 hours after the addition of Ang II (10^{-6} mol/L). The inhibitory action of Ang II on Gax mRNA expression (ED_{50} 10^{-11} mol/L) was almost completely blocked by an AT,R antagonist, CV11974. In contrast, CNP 10^{-6} mol/L augmented Gax mRNA expression to exhibit 1.8-fold increase of the control 12 hours after the stimulation. Thus effect of CNP was mimicked by the addition of 8-bromoadenosine 3’5’-cyclic monophosphate. The addition of C-ANF[4-23], an atrial natriuretic peptide-C receptor-specific agonist and devoid of stimulating cGMP production, exhibited no effect on Gax mRNA expression. Simultaneous administration of Ang II and CNP revealed that CNP (10^{-6} mol/L) significantly attenuated the inhibitory action of Ang II (10^{-6} mol/L) on Gax mRNA expression. These results suggest that Gax is a common transcription factor involved in the signaling pathway of vascular growth for Ang II and CNP and regulates the cell cycle and/or phenotype of VSMCs for vascular remodeling in hypertension and atherosclerosis (Hypertension. 1997;29[part 2]:381-387.)

Key Words  natriuretic peptides  angiotensin II  growth arrest-specific homeobox  homeobox  growth  gene regulation  vascular smooth muscle cells

Migration and proliferation of VSMCs in neointima are key events for the development of hypertensive vascular complication, atherosclerosis and coronary restenosis. In contrast to VSMCs with the contractile phenotype present in the intima-media of the vascular wall, in the proliferative neointimal lesion, VSMCs exhibit the synthetic or embryonic phenotype which is characterized by enhanced proliferative and synthetic activities, reorganization of the cytoskeleton, and suppressed contractile activity. Such alternation of phenotypic and proliferative features of VSMCs is thought to be modulated by various growth factors and cytokines through the convergence of their signaling pathways into the network of transcription factors which govern the gene expressions and phenotypic diversity of the cells. However, until now the interaction between growth signals and transcription factors concerning the proliferation and differentiation of VSMCs has not been well understood.

Gax gene was isolated from the adult rat aorta cDNA library and its expression is largely confined to the cardiovascular tissues such as the aorta, heart, kidney and lung. Gax gene was rapidly downregulated during G0/G1 transition caused by PDGF or serum in cultured VSMCs and also downregulated in the rat carotid artery after balloon injury. Recently, microinjected recombinant Gax peptide was reported to inhibit mitogen-induced entry into the S phase in VSMCs and also demonstrated that overexpressed Gax reduced neointimal thickening after balloon injury. These findings all together suggest that Gax possesses the critical role in the growth inhibition of VSMCs.

We have been investigating the growth control of VSMCs by several vasoactive substances. We and others have demonstrated that Ang II stimulates the growth of VSMCs via the activation of PDGF, transforming growth factor-β, and basic fibroblast growth factor. On the other hand, we and others reported that natriuretic peptides inhibit the VSMC proliferation via guanosine 3’5’-cyclic monophosphate (cGMP) cascade triggered by the activation of the particulate guanylate cyclase which is the natriuretic peptide receptor itself. We also demonstrated that CNP, the third member of the natriuretic peptide family, is secreted from vascular endothelial cells and possesses vascular growth inhibitory action via CNP-specific receptor, ANP-B receptor, highly expressed on VSMCs with the synthetic phenotype. We, thus, indicated that CNP is a novel endothelium-derived relaxing peptide involved in vascular remodeling in hypertension and other cardiovascular diseases, and proposed the existence of the vascular NPS similar to the vascular RAS. Previously, we have demonstrated that the antagonistic relationship between the NPS and RAS in blood pressure control and blood fluid homeostasis, both as an endocrine system and as a neuropeptide system. We also reported that the vascular NPS is activated...
Selected Abbreviations and Acronyms

8-Br-cGMP = 8-bromo-adenosine-cGMP
Ang II = angiotensin II
ANP = atrial natriuretic peptide
CNP = cyclic-natriuretic peptide
DSF = defined serum-free
Gax = growth arrest-specific homeobox gene
MAPK = mitogen-activated protein kinase
NPS = natriuretic peptide system
PCR = polymerase chain reaction
PDGF = platelet-derived growth factor
RAS = renin-angiotensin system
VSMC = vascular smooth muscle cell

in proliferative vascular lesions, suppressing further proliferation of VSMCs by antagonizing the action of the vascular RAS.23

In the present study, to elucidate the role of Gax on vascular growth control regulated by the antagonism between the RAS and NPS, we have examined the regulation of the Gax gene expression by Ang II and CNP in VSMCs.

Methods

Cell Culture

Cultured aortic VSMCs were derived from the explants of the thoracic aorta of male Wistar rats as previously reported24 and were grown in Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories, Inc., Irvine, UK) supplemented with 10% fetal calf serum (FCS) (Hazleton Biologics, Inc., Lenexa, Kan.), 100 units/ml penicillin, and 100 μg/ml streptomycin. VSMCs from 6th to 12th passages were used in the present study.

Stimulation of VSMCs

VSMCs were plated into 10-cm-diameter dishes for collection of RNA. At about 70% confluent, cells were made quiescent by the incubation for 48 hours in DMEM with 0.5% FCS. Cells were made quiescent by the incubation for 48 hours in DMEM with 0.5% FCS. Cells were then incubated with Ang II 10^{-6} mol/L, transferrin (5 μg/ml), and ascorbate (0.2 mmol/L). Then, the medium was replaced with fresh DMEM medium and Ang II was added. Cells were incubated for the various times and harvested for RNA isolation. DMEM with 0.5% FCS was used instead of DMF medium in the experiments in which either CNP (Peptide Institute Inc., Osaka, Japan) or 8-Br-cGMP (Sigma Chemical Co) was added.

Northern Blot Analysis

Total RNA from cultured VSMCs was prepared by the acid guanidium thiocyanate-phenol-chloroform method using TRIzol Reagent (Life Technologies, Inc., Gaithersburg, MD.). Forty μg of total RNA per lane was electrophoresed on a formamide/1.2% agarose gel and transferred to a nylon membrane filter. Gax cDNA probe was obtained by cDNA synthesis and the polymerase chain reaction method using the total RNA of mesangial cells derived from Wistar-Kyoto Rats with the sense, 5'-AGA-TGTTCTCCCCCGCAAGCC-3', and antisense, 5'-TCTCGGGGATCTGAAGCTGTC-3', primers, corresponding to nucleotides 480-499 and 701-720 of rat Gax cDNA.25 The probe was labeled by random-primed synthesis to the specific activity of approximately 1 X 10^{6} cpm/μg. The filters were hybridized with the 32P-labeled probe at 42°C in 50% formamide, 5X SSC, 5X Denhardt's reagent, 50 mmol/L sodium phosphate buffer (pH 6.8), 0.1% SDS and 100 mg/ml heat-denatured salmon testis DNA, and washed at 55°C in 0.1X SSC and 0.1% SDS. Imaging and estimation of Gax mRNA expression were performed using image analyzer BAS2000 (Fuji Photo Film Co. Ltd., Tokyo, Japan). As a control, glyceraldehyde-3-phosphate dehydrogenase mRNA level was determined as previously reported.26

Statistical Analysis

All results were expressed as mean±SD with n=3 to 6. Statistical analysis of the data was performed using ANOVA, P<.05 was considered significant. The experiments presented are the representatives of at least two separate experiments.

Reagents

Human Ang II and human CNP were purchased from Peptide Institute Inc. 8-Bromo-cGMP was purchased from Sigma Chemical Co. (St. Louis, Mo.). CV11974, an active metabolite of TCV-116, (2S)-1-(cyclohexyloxycarbonyl) ethyl 2-ethoxy-1-[2'-(1H-tetrazol-5-yl) biphenyl-4-yl] methyl]-1H-benzimidazole-7-carboxylate, which is an AT, R-selective non-peptide antagonist,27 was obtained from Takeda Chemical Industries, Ltd. (Osaka, Japan). des-Gln^{9},Ser^{10},Gly^{12},Leu^{13},Gly^{20}[ANP[4-23]]-NH_{2}(C-ANF[4-23]) was purchased from Peninsula Laboratories Inc. Other reagents were purchased from standard commercial suppliers.

Results

Regulation of Gax mRNA Expression by Ang II

The expression of Gax mRNA was analyzed in rat VSMCs by Northern blot analysis. Gax mRNA of approximately 2.3 kilobases was detected in a single band in quiescent VSMCs using the PCR-synthesized probe (see "Methods"), being consistent with the reported size of Gax mRNA, 2244 bases.24 The expression of Gax mRNA was analyzed in rat VSMCs by Northern blot analysis. Gax mRNA of approximately 2.3 kilobases was detected in a single band in quiescent VSMCs using the PCR-synthesized probe (see "Methods"), being consistent with the reported size of Gax mRNA, 2244 bases.24 The expression of Gax mRNA was analyzed in rat VSMCs by Northern blot analysis. Gax mRNA of approximately 2.3 kilobases was detected in a single band in quiescent VSMCs using the PCR-synthesized probe (see "Methods"), being consistent with the reported size of Gax mRNA, 2244 bases.24 FIG 1 shows the time course of the effect of Ang II 10^{-6} mol/L on Gax mRNA expression. Rapid and transient downregulation of Gax mRNA was observed after Ang II stimulation. The downregulation occurred within 1 hour after the stimulation and maximal at 6 hours when Gax mRNA became almost negligible. Gax mRNA level was revealed to recover towards the baseline from 12 hours to 24 hours. FIG 2 depicts the dose-dependent effect of Ang II on Gax mRNA expression. The left panel shows the...
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Gax mRNA

![Typical profile of Gax gene transcript obtained by Northern blot analysis, and quantitative measurements of Gax mRNA levels are demonstrated on the right panel. Ang II 10^{-11} to 10^{-8} mol/L suppressed Gax mRNA in the dose-dependent fashion. The 50% effective dose for Gax downregulation 3 hours after the stimulation was approximately 10^{-10} mol/L of Ang II. Suppression of Gax mRNA expression was observed from Ang II 10^{-11} mol/L, and maximally 98% decrease of Gax gene transcript compared with the control occurred by Ang II 10^{-7} mol/L. To confirm the receptor subtype specificity of the inhibitory action of Ang II on Gax mRNA expression, we examined the effect of angiotensin II type 1 receptor (AT_{1}-R) antagonist, CV11974. As shown in Fig 3, the effect of Ang II-induced Gax mRNA downregulation was almost completely blocked by CV11974 10^{-6} mol/L.](fig3)

![Regulation of Gax mRNA Expression by CNP and 8-Br-cGMP](fig4)

Fig 4 shows the time course of Gax mRNA expression after the administration of CNP 10^{-6} mol/L and 8-Br-cGMP 10^{-6} mol/L. CNP 10^{-6} mol/L upregulated Gax mRNA expression 12 hours after the stimulation, and the level of Gax mRNA tended to decrease 24 hours after the stimulation, however, the level of Gax mRNA was still higher than the control. The administration of 8-Br-cGMP 10^{-6} mol/L mimicked the effect of CNP on Gax mRNA expression. Gax mRNA increased from 3 hours after the stimulation, reaching a maximal level at 12 hours and also showed the tendency to decrease 24 hours after the stimulation. The effects of CNP and 8-Br-cGMP on Gax mRNA augmentation were dose dependent (Fig 5).

Twelve hours after the administration of CNP or 8-Br-cGMP, significant increase of Gax mRNA expression was observed by 10^{-6} mol/L of CNP and 10^{-7} to 10^{-6} mol/L of 8-Br-cGMP, respectively. The maximal augmentation of Gax mRNA expression compared with the vehicle administration was 1.8-fold and 2.5-fold increases by 10^{-6} mol/L of CNP and 10^{-6} to 10^{-7} mol/L of 8-Br-cGMP (n=6), respectively.

Next, we examined the effect of ANP-C receptor-specific agonist, C-ANF [4-23]. ANP-C receptor is not coupled to guanylate cyclase and is proposed to have a major role in the clearance of natriuretic peptide. As shown in Fig 6, 10^{-7} mol/L of C-ANF [4-23] exerted no significant effect on Gax mRNA expression 12 hours after the stimulation.

Interaction of Ang II and CNP on Gax mRNA Expression

To confirm the opposite action of Ang II and CNP on Gax mRNA expression, we examined the effect of the simultaneous administration of Ang II and CNP. As shown in Fig 7, 10^{-6} mol/L of CNP attenuated the inhibitory action of Ang II 10^{-12} to 10^{-8} mol/L on Gax mRNA expression 6 hours after the stimulation. The suppression of Gax mRNA expression caused by 10^{-10} mol/L of Ang II was reversed approximately 60% by the administration of CNP 10^{-6} mol/L.

Discussion

The present study demonstrates that Ang II, which possesses the promoting action on proliferation and hypertrophy of VSMCs, potently induced rapid and transient downregulation of Gax mRNA expression. The temporal profile of the downregulation of Gax mRNA expression caused by Ang II was similar to that induced by PDGF and serum.

The present study also showed that AT_{1}-R antagonist CV11974 almost completely blocked the inhibitory action of Ang II on Gax mRNA expression, suggesting that the effect of Ang II on Gax gene expression is mediated via AT_{1}-R. Ang II exhibits proliferative and hypertrophic effects on VSMCs via G-protein-coupled AT_{1}-R with multiple signal transduction pathways, including the activation of phospholipase C, calcium mobilization, and the activation of protein ki-
nase C,\textsuperscript{29} the induction of proto-oncogenes,\textsuperscript{31,32} protein tyrosine kinase phosphorylation,\textsuperscript{33} the activation of MAPK,\textsuperscript{34,35} and the stimulation of Janus kinase/STAT (signal transducers and activators of transcription) pathway.\textsuperscript{36,37} On the other hand, PDGF exerts the proliferative action mainly via the activation of the receptor intrinsic protein tyrosine kinase,\textsuperscript{38} and other pathways similar to AT\textsubscript{1}-R.\textsuperscript{39} Recent study revealed that AT\textsubscript{1}-R signal transduction pathway converges into the PDGF-\(\beta\) receptor signaling cascades and results in the activation of proteins upstream of p21\textsuperscript{29} which initiates the phosphorylation cascades towards the MAPK activation.\textsuperscript{39} Similar action on Gax mRNA expression of Ang II and PDGF suggests that the gene regulation of Gax expression is under control of the molecules existed in the downstream after the convergence of Ang II and PDGF receptor signaling pathways, such as MAPK.

Ang II exerted quite potent effect on Gax mRNA suppression. Ang II 10\textsuperscript{-11} mol/L and Ang II 10\textsuperscript{-10} mol/L caused 50\% and 98\% reduction of Gax mRNA expression 3 hours after the stimulation, respectively. Previously, we have demonstrated that Ang II induced the growth of VSMCs,\textsuperscript{16} but significant growth stimulation was observed by more than 10\textsuperscript{-9} mol/L of Ang II.\textsuperscript{16} The reason for the difference of sensitivity to Ang II between Gax expression and DNA synthesis is not known at present. The present and previous studies demonstrated that the suppression of Gax mRNA expression is immediately early event in the cell cycle progression, that is, that occurred at least from 3 hours to 12 hours after the mitogen stimulation, corresponding to the period from G0/G1 transition to G1/S boundary.\textsuperscript{40} To achieve further progression of the cell cycle and initiation of DNA synthesis, the participation of other factors should be necessary in addition to the suppression of Gax mRNA expression mechanism. It can be, however, at least said that the suppression of Gax mRNA expression is a highly sensitive marker of growth stimulation of VSMCs.

In contrast to the effect of Ang II, CNP and 8-Br-cGMP upregulated Gax mRNA expression. It has been reported
that Gax mRNA expression was dependent on the cell quiescence, that is, Gax was expressed at low levels in subconfluent, but its mRNA level increased within 8 hours after the cells were placed in serum-poor medium. We demonstrated herein that CNP upregulated Gax mRNA expression in a similar time course with the serum starvation in a dose-dependent manner. The administration of 8-Br-cGMP mimicked the effect of CNP, and C-ANF [4-23], the specific agonist for ANP-C receptor which is not coupled with guanylate cyclase and exhibits no cGMP genesis by natriuretic peptides in VSMCs, exerted no significant effect on Gax mRNA expression. All these results suggest that CNP up-regulates Gax mRNA expression via the activation of cGMP cascade. This is, to our knowledge, the first report that growth inhibitory signals up-regulate the Gax mRNA expression. As we have previously demonstrated, CNP possesses the growth inhibitory effect on VSMCs via cGMP cascade. cGMP has been reported to suppress the proliferation of VSMCs by inhibiting the progression from the G1 into S phase of the cell cycle. Indeed, recently we demonstrated that overexpression of CNP in VSMCs by adenovirus system caused the G1 growth arrest of VSMCs, by flow cytometry analysis and Northern blot analysis for mRNA expressions of cyclins and cyclin-dependent kinases required for the cell cycle progression in G1/S boundary. The antiproliferative action via cGMP pathway may be mediated by Gax, which might control the expressions of the genes regulating G1/S boundary.

We have proposed the counterregulatory effects of NPS on RAS not only in blood pressure and body fluid homeostasis through vasorelaxation, diuresis and natriuresis, and inhibition of aldosterone secretion, but also in vascular growth control. The present study revealed that CNP also exerted the antagonistic effect on Ang II-induced suppression of Gax mRNA expression, suggesting that the antagonistic effect between Ang II and CNP on vascular growth is controlled via the regulation of Gax gene expression. Now we examine the involvement of Gax in the growth control of VSMCs by Ang II and CNP using the antisense oligonucleotide method for Gax gene, as we previously reported. Taking these results together, it is indicated that Gax can be involved in the signaling pathway of vascular growth control by Ang II and CNP as a common transcription factor and may act as an intracellular switch for controlling on and off of cell proliferation.

Homeobox gene family is well known to be important in the regulation of organogenesis, body plan formation in embryo and cell differentiation as well as cell proliferation. Some homeobox genes contribute to the regulation of both cell proliferation and differentiation. For example, an intestine-specific homeobox gene, Cdx-2, leads to arrest of proliferation and induces enterocyte-specific gene expressions in an undifferentiated intestine cell line, IEC-6 cells. Gax is a cardiovascular-specific homeobox gene that participates in the growth inhibition of VSMCs. VSMCs are known to dedifferentiate into the synthetic or embryonic phenotype when they re-enter the cell cycle by mitogen stimulation. Inferring from multiple functions of the homeobox gene family on cell proliferation and differentiation, Gax may take some roles on differentiation of VSMCs as well as the growth inhibitory effect. Indeed, the recent studies demonstrated that overexpression of catalytic domain of cGMP-dependent protein kinase, which is one of the major signaling molecules for the cGMP cascade, leads the alternation of morphology of cultured VSMCs into differentiated phenotype. In addition, CNP is also reported to stimulate the differentiation of osteoblastic cells via cGMP cascade. It is, therefore, suggested that CNP may promote not only the growth inhibition but also VSMC differentiation by upregulating Gax gene expression via cGMP cascade. Further study to find target genes of Gax for VSMC differentiation should be required.

Recently, Walsh et al reported that transfer of Gax gene into VSMCs after balloon injury has been shown to inhibit cell proliferation and neointima formation in vivo. For gene therapy against vascular proliferative diseases such...
as hypertensive vascular complication, atherosclerosis and coronary restenosis. Common intracellular molecules which affect the cell cycle progression such as retinoblastoma gene, p53 and p21CIP1 are recommended as good targets. The present study revealed that Gax can be a common regulator of VSMC growth control by many vascular growth promoting and inhibiting substances including Ang II and CNP, and may be involved in VSMC differentiation. Gax is, therefore, a suitable candidate for gene therapeutic target for vascular diseases.

In conclusion, we demonstrated that Ang II and CNP oppositely regulate Gax gene expression in VSMCs. The findings suggest that Gax is a common transcription factor involved in the signaling pathway of vascular growth for Ang II and CNP, and regulates the cell cycle and/or phenotype of VSMCs for vascular remodeling in hypertension and atherosclerosis.

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