Angiotensin II Type 2 Receptor Gene Transfer Downregulates Angiotensin II Type 1a Receptor in Vascular Smooth Muscle Cells

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Abstract—Two distinct subtypes of angiotensin (Ang) II receptors, type 1 (AT₁) and type 2 (AT₂), have been identified. Vascular smooth muscle cells (VSMCs) usually express AT₁ receptor. To elucidate the direct effects of the AT₂ receptor on the AT₁ receptor in VSMCs, we transfected AT₂ receptor gene into cultured rat VSMCs. Overexpression of AT₂ receptor significantly decreased expression of AT₁a receptor at both the mRNA and protein levels in the presence and absence of Ang II in VSMCs. Overexpression of AT₂ receptor increased expression of bradykinin and inducible NO in the presence and absence of Ang II in VSMCs. Bradykinin B₂ receptor antagonist HOE–140 and NO synthase inhibitor N’-nitro-L-arginine methyl ester (L-NAME) inhibited the decreases in AT₁a receptor expression by the overexpression of AT₂ receptor in VSMCs. L-Arginine augmented the decrease in AT₁a receptor expression. Overexpression of AT₂ receptor suppressed basal DNA synthesis and proliferation of VSMCs and abolished response of DNA synthesis to Ang II in VSMCs. Our results demonstrate that overexpression of the AT₂ receptor downregulates AT₁a receptor expression in rat VSMCs in a ligand-independent manner that is mediated by the bradykinin/NO pathway. Downregulation of AT₁a receptor is a novel mechanism by which the AT₂ receptor regulates growth and metabolism of VSMCs. (Hypertension. 2002;39:1021-1027.)

Key Words: receptors, angiotensin II || angiotensin II || muscle, smooth, vascular || nitric oxide || bradykinin

Angiotensin (Ang) II plays an important role in regulation of cardiovascular hemodynamics and growth. At least 2 distinct subtypes of Ang II receptors have been identified on the basis of their differential pharmacological and biochemical properties, and they are designated type 1 (AT₁) receptor and type 2 (AT₂) receptor.1-2 Both the AT₁ and AT₂ receptors belong to the family of seven-transmembrane G protein–coupled receptors.3 To date, most of the known effects of Ang II in adult tissues have been attributed to the AT₁ receptor. Less is known about the AT₂ receptor.

AT₁ receptor mRNA is expressed at very low levels in the aorta during early embryonic development, but it is expressed at high levels during later stages of development and in the neonate.4 After birth, AT₂ receptor levels decline rapidly.5 The significance of the increased expression of AT₂ receptor in many pathological conditions remains unclear. It has been reported that AT₂ receptor has antihypertrophic, antiproliferative, and proapoptotic effects.4,6,7 Thus, the function and signaling of the AT₁ and AT₂ receptors are quite different, and these receptors have opposing actions in terms of cell growth and blood pressure regulation.4 AT₁ receptor blockades are used clinically as antihypertensive therapies. It has been reported that during chronic AT₁ receptor blockade, the actions of Ang II, which increases in concentration, may be mediated by the AT₂ receptor, thereby contributing to the cardioprotective effects.4,6,7

Vascular smooth muscle cells (VSMCs) usually express only AT₁ receptor.4 In the present study, we investigated the effect of AT₂ receptor gene transfer on expression of the AT₁a receptor and growth of rat VSMCs.

Methods

Our investigation conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

Vectors

A 2.6-kb genomic DNA fragment containing the entire coding region of rat AT₂ receptor was cloned into the mammalian expression vector pcDNA3 (Invitrogen Japan KK). pcDNA3 alone was used as control.

Cell Culture

Cultured VSMCs were grown from explants of aortic media of Wistar-Kyoto/Lzumo rats (SHR Corp, Funabashi, Japan) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% calf serum (GIBCO Life Technologies Inc), 100 U/mL penicillin, and 100 mg/mL streptomycin. The hill-and-valley pattern was...
observed as typical of cultured smooth muscle cells when cells reached confluence. The purity of the VSMCs was further confirmed by immunofluorescence with an anti-a-smooth muscle actin antibody that showed >95% positive staining of the cultured cells. VSMCs were passaged by trypsinization with 0.02% EDTA and 0.05% trypsin (GIBCO) in Ca2+- and Mg2+-free Dulbecco’s phosphate-buffered saline (PBS) and incubated in 75-cm2 tissue culture flasks at a density of 104 cells/mL. Experiments were performed using 3 to 5 passages.

**Establishment of Quiescence**

Trypsinized cells were plated into 24- or 6-well culture dishes (Corning Inc) at a density of 105 cells/cm2. Cells were allowed to grow in DMEM containing 10% calf serum for 24 hours, and the culture medium was then changed to DMEM with 0.2% calf serum. Cells were then incubated in this medium for 48 to 72 hours to establish quiescence.

**AT2 Receptor Gene Transfer**

Quiescent cells were transfected with the AT2 receptor gene expression vector by the lipofectin (GIBCO) method, according to manufacturer’s instructions. Briefly, 2 μg of plasmid DNA was diluted into 100 μL of serum-free DMEM as solution A, and 2 μL of lipofectin reagent was diluted into 100 μL of serum-free DMEM as solution B. Solutions A and B were allowed to stand at room temperature for 45 minutes. Solution A and solution B were then combined, mixed gently, and incubated for an additional 15 minutes at room temperature. For each transfection, 0.8 mL of serum-free medium was added to each tube containing the lipofectin reagent-DNA complexes, mixed gently, and then overlaid onto the cells. Cells were incubated for 24 hours at 37°C in an incubator.

**Reverse-Transcription Polymerase Chain Reaction Analysis**

Quiescent VSMCs were washed with PBS and lysed in 800 μL of RNAzol B (Biocentre Laboratories Inc). Each sample was mixed with 80 μL of chloroform by vortexing for 15 seconds, kept on ice for 15 minutes, and centrifuged at 12,000g for 15 minutes to extract total RNA. The colorless upper aqueous phase was mixed with an equal volume of isopropanol, allowed to stand at -20°C for 45 minutes, and centrifuged at 12,000g for 15 minutes at 4°C to precipitate the RNA. The RNA pellet was washed twice with 500 μL of 75% ethanol and centrifuged at 12,000g for 8 minutes at 4°C, dried, and dissolved in 10 μL of 10 mmol/L Tris-HCl (pH 8.0) and 1 mL of EDTA buffer. After denaturation at 65°C for 15 minutes, the RNA sample was treated with 0.5 U of DNase (GIBCO) in 0.5 μL of DNase buffer (20 mmol/L Tris-HCl pH 8.3, 50 mmol/L KCl, and 2.5 mmol/L MgCl2) at room temperature for 45 minutes. Then the DNase was inactivated by addition of 0.5 μL of 20 mmol/L EDTA and heating at 98°C for 10 minutes.

Reverse transcription–polymerase chain reaction (RT-PCR) was performed as described previously. Briefly, aliquots of RNA (1 μg/20 μL) were reverse-transcribed into single-stranded cDNA with 0.25 U/μL avian myeloblastoma virus reverse transcriptase (Life Sciences Inc) in 10 mmol/L Tris-HCl pH 8.3, 50 mmol/L MgCl2, 50 mmol/L KCl, 1 μmol/L deoxy-NTP, and 2.5 μmol/L random hexamers. Five microliters of the diluted cDNA product was mixed with 10 mmol/L Tris-HCl pH 8.3, 50 mmol/L KCl, 4 mmol/L MgCl2, 0.025 U/mL Taq DNA polymerase (Takara Biochemicals), and 0.2 mmol/L of each of the upstream sense primer and downstream antisense primer in a total volume of 25 μL. Sense primer (5'-CTGATCCTCATGACTGTTAATTC-3') and antisense primer (5'-GGGCAATCAATGGCACTGTG-3') were used for PCR amplification of AT2 receptor to yield a 183-bp product. Sense primer (5'-GTGACCCCTGCAATCCTT-3') and antisense primer (5'-CTCCCTAATCTCCTTAGTT-3') were used and specific for PCR amplification of AT1 receptor to yield a 198-bp product. Sense primer (5'-CTCGGCTGCTGCATTTAAGG-3') and antisense primer (5'-CTGAGCTTCCCCACAGGACT-3') were used for PCR amplification of AT1 receptor to yield a 306-bp product. Sense primer (5'-CGACGACCACCATGAACTTCT-3') and antisense primer (5'-GCTATTGAGCATGAACTCGG-3') were used for PCR amplification of 18S ribosomal RNA served as an internal control to yield a 312-bp product. After initial denaturation at 96°C for 5 minutes, PCR amplification was performed as 35 cycles of 94°C for 1 minute, 64°C for 1 minute, and 72°C for 1 minute for the AT2 receptor and as 30 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes for the AT1 receptor. PCR using primers for 18S ribosomal RNA was incubated in each reaction as an internal control. To confirm that no genomic DNA was co-amplified by PCR, control RT-PCR experiments without reverse transcriptase were performed. In all cases, no product was amplified. PCR was performed using a DNA Thermal Cycler (Perkin-Elmer Cetus). For semiquantitative analysis of mRNA levels, the kinetics of the PCR reaction were monitored; the number of cycles at which each PCR product became visible on the gel was compared between the different samples. Serial 10-fold dilutions of cDNA (100, 10, and 1 ng) were amplified; the PCR products were visible after a fewer number of cycles with increasing amounts of cDNA. PCR products were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and visualized by UV illumination.

**Western Blot Analysis**

Quiescent VSMCs at a density of 105 cells/cm2 in 6-well culture dishes were transfected with pcDNA3 containing AT2 receptor cDNA as described above for 24 hours in serum-free DMEM, and then incubated with 0.1 μmol/L Ang II for 24 hours. Cells were washed with PBS and incubated in lysis buffer (50 mmol/L Tris-HCl pH 8.0, 150 mmol/L NaCl, 0.02% sodium azide, 100 μg/mL phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, and 1% Triton X-100). Samples were dissolved in 20 μL of sample buffer, boiled, and subjected to 10% polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membranes. After blocking with 100% Block Ace (Daipinpur Pharm Chemical Co) at 4°C overnight, the membranes were incubated with mouse monoclonal antibodies specific for the AT1a and AT2 receptors (Alpha Diagnostic International), rabbit polyclonal antibody specific for bradykinin (Biogenex Ltd), or rabbit polyclonal antibody specific for inducible NO synthase (Biotrend Chemikalien GmbH) diluted in 200 vol TBST solution (10 mmol/L Tris-HCl pH 8.0, 150 mmol/L NaCl, and 0.05% Tween 20) containing 15% Block Ace at room temperature for 3 hours. After washing with TBST twice for 10 minutes, the membranes were incubated with goat anti-mouse IgG (BioRad Laboratories) diluted in 3000 vol in TBST containing 15% Block Ace at room temperature for 1 hour, washed with TBST for 10 minutes for 3 times, then visualized with ECL method. Membranes were reprobed with mouse monoclonal antibody specific for α-tubulin (Sigma) as an internal control. Antisera to AT1 receptor and AT2 receptor showed no reactivity with other G protein–coupled receptors. Antisera to bradykinin showed 57% cross-reactivity with kallidin. Antisera to human inducible NO synthase (iNOS) showed 50% cross-reactivity with rat iNOS and no cross-reactivity with neuronal and endothelial NO synthase.

**Determination of DNA Synthesis**

[3H]Thymidine incorporation into newly synthesized DNA was determined as described previously. Transfected cells were incubated with 0.10 to 1.0 μmol/L Ang II for 24 hours. The medium was then changed to DMEM containing [3H]thymidine (0.5 μCi/mL) (NEN Research Products), and cells were incubated for 2 hours. Each well was then washed with 1 mL of 150 mmol/L NaCl to remove excess [3H]thymidine, and the cells were fixed in 1 mL of 2% glutaraldehyde, 1% acetic acid for 10 minutes. The perchloric acid containing solubilized DNA was transferred to a scintillation vial, and the radioactivity was measured with a liquid scintillation spectrometer.
Expression of Bradykinin and iNOS Proteins After Transfection of the AT₂ Receptor Gene

Expression of bradykinin and iNOS proteins after transfection of the AT₂ receptor gene in the absence and presence of Ang II are shown in Figure 3. Ang II (0.1 µmol/L) significantly (P<0.05) increased amounts of bradykinin and iNOS proteins in VSMCs. Furthermore, levels of bradykinin and iNOS proteins were increased significantly (P<0.01) after transfection of the AT₂ receptor gene in the presence and absence of Ang II.

Effect of Bradykinin B2 Receptor Antagonist, NO Synthase Inhibitor, or L-Arginine on Expression of AT₁a Receptor in VSMCs After Transfection of the AT₂ Receptor Gene

To assess contributions of bradykinin and NO on the decreases in expression of AT₁a receptor after transfection of the AT₂ receptor gene, we elucidated effect of a bradykinin B₂ receptor antagonist, HOE–140, or a NO synthase inhibitor, N⁴-nitro-L-arginine methyl ester (L-NAME), on the of AT₁a receptor suppression. A delivery agent lipofection alone had no effect on amounts of AT₁a receptor protein. HOE–140 (10 mmol/L) blocked the decreases in AT₁a receptor preen levels after transfection of the AT₂ receptor gene (Figure 4A). L-NAME (10 mmol/L) blocked the decreases in AT₁a receptor mRNA levels after transfection of the AT₂ receptor gene (Figure 4B). L-Arginine (150 mmol/L) alone did not affect AT₁a receptor mRNA levels in VSMCs. However, the same dose of L-arginine augmented significantly (P<0.05) the decreases in AT₁a receptor mRNA levels after the transfection of AT₂ receptor gene in VSMCs (Figure 4C).

Growth of VSMCs After Transfection of the AT₂ Receptor Gene

Increasing doses (0.01 to 1 µmol/L) of Ang II increased DNA synthesis in VSMCs in a dose-dependent manner. Basal DNA synthesis was decreased significantly (P<0.05), and the response of DNA synthesis to Ang II was abolished after transfection of the AT₂ receptor gene (Figure 5A).

Proliferation of VSMCs after transfection of the AT₂ receptor gene in the presence of 5% calf serum is shown in Figure 5B. Transfection of the AT₂ receptor gene inhibited significantly (P<0.05) cell proliferation.

Discussion

The present study demonstrates that overexpression of the AT₂ receptor gene downregulates AT₁a receptor and inhibits proliferation of rat VSMCs. An increasing body of evidence indicates that the AT₂ receptor exerts antigrowth, anti hypertrophic, and proapototic effects that may counteract the growth stimulation mediated by AT₁ receptor. Several recent studies have demonstrated that there is negative crosstalk between AT₁ receptor and AT₂ receptor signaling. It was
reported that AT₁ receptor expression is significantly higher in AT₂ receptor knockout mice than in control animals. Horiuchi et al. observed that transfection of the AT₂ gene in rat VSMCs inhibited AT₁ receptor-mediated tyrosine phosphorylation of signal transducers and activators of transcription (STAT). AbdAlla et al. demonstrated that the AT₂ receptor binds directly to the AT₁ receptor by heterodimerization and inhibits AT₁ receptor function, which is indepen-

Figure 2. A, Expression of AT₁ (AT1aR) and AT₁b (AT1bR) receptor mRNAs after transfection of AT₂ receptor gene in rat VSMCs in the absence and presence of Ang II. Quiescent VSMCs were incubated with pcDNA2 containing or lacking the AT₂ receptor genes for 24 hours in the presence of 0.1 μmol/L Ang II. B, levels of AT₁ receptor and 18S ribosomal RNA mRNAs were then determined by RT-PCR analysis. The ratio of AT₁a receptor mRNA to 18S ribosomal RNA mRNA was determined by densitometric analysis. Values are mean±SEM (n=4). *P<0.01 vs no transfection.

Figure 3. Expression of AT₂ receptor (AT2R), AT₁a receptor (AT1aR), bradykinin, and iNOS protein in rat VSMCs after transfection of the AT₂ receptor gene in the absence or presence of Ang II. Quiescent VSMCs were incubated with pcDNA2 containing the AT₂ receptor gene for 24 hours in the presence of lipofectin and in the absence or presence of 0.1 μmol/L Ang II. Levels of AT₂ receptor, AT₁a receptor, bradykinin, iNOS, and α-tubulin proteins were determined by Western blot analysis. Molecular weight of AT₂ receptor, AT₁a receptor, bradykinin, and iNOS proteins is 45, 45, 110, and 120 kDa, respectively. The ratio of the level of each test protein to α-tubulin was determined by densitometric analysis. Values are mean±SEM (n=4). *P<0.05, **P<0.01 vs no transfection.
dent of AT₂ receptor activation and signaling. These studies, however, did not demonstrate the downregulation of AT₁a receptor by transfection of the AT₂ receptor gene. We showed that AT₁a receptor expression was downregulated by overexpression the AT₂ receptor in rat VSMCs and that AT₁a receptor levels were decreased in the absence and presence of Ang II, indicating that overexpression of the AT₂ receptor downregulates AT₁a receptor in a ligand-independent manner. It is possible that AT₂ receptor alone seems to act as one of intracellular signalings. Miura et al. also observed that overexpression of the AT₂ receptor induces apoptosis of fibroblasts in the absence of Ang II, which is in the transfection efficiency-dependent manner, suggesting that overexpression of AT₂ receptor induces apoptosis in a ligand-independent manner. Thus, the downregulation of AT₁a receptor by the AT₂ receptor gene transfer in these experiments may be results by the state of overexpression of AT₂ receptor in VSMCs. AT₂ receptor is expressed at a high level during later stages of development, in neonates, and in the neointima of artery after angioplasty. AT₂ receptor may thus have effects to regulate AT₁a receptor in cells and tissues. However, the AT₁a receptor downregulation was not reported in neointima after the vascular injury in which expression of AT₂ receptor is increased. It is possible that the AT₁a receptor downregulation can be seen only by the overexpression of AT₂ receptor.

Expression of AT₁a receptor is regulated by various vasoactive substances, growth factors, and glucocorticoids. Circulating Ang II does not affect AT₁ receptor expression. However, local Ang II has been reported to have a regulatory effect on its receptors. Ang II decreases AT₁ receptor expression in kidney, vascular and bladder smooth muscle cells, and in rat adrenal grand. There are tissue- and species-dependent differences in regulation of AT₁ receptor gene expression. AT₁a receptor is mainly expressed in heart, kidney, and VSMCs, whereas AT₁b receptor is mainly expressed in adrenal gland.

AT₂ receptor has been reported to mediate renal productions of bradykinin and NO. Tsutsumi et al. reported that overexpression of AT₂ receptor in aortic smooth muscle cells in AT₂ receptor transgenic mice blocks the amiloride-sensitive Na⁺–H⁺ exchange and promotes intracellular acidification that stimulates the production of bradykinin and NO in a paracrine manner to promote vasodilation. In addition, AT₁ receptor expression has been known to be transcriptionally suppressed by NO. It is therefore possible that increased production of NO by overexpression of AT₂ receptor could suppress AT₁a receptor expression by the inhibition of its transcription.

To evaluate the mechanisms underlying the downregulation of AT₁a receptor by overexpression of the AT₂ receptor, we investigated the influence of AT₂ receptor overexpression on levels of bradykinin and iNOS and found that overexpression of the AT₂ receptor significantly increased both bradykinin and iNOS protein levels. In addition, the bradykinin B₂ receptor...
and metabolism of VSMCs in cardiovascular developments and diseases.

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References
20. Matsubara H, Kanasaki M, Murasawa S, Tsukaguchi Y, Nio Y, Inada M. Differential gene expression and regulation of angiotensin II receptor antagonist HOE 140 and the NO synthase inhibitor L-NNAME ameliorate the decrease of AT1 receptor expression, and L-arginine as substrate for NO synthesis augmented the down regulation of AT1 receptor by overexpression of the AT2 receptor in VSMCs. These findings indicate that the downregulation of AT1 receptor is mediated through the bradykinin/NO pathway induced by overexpression of the AT2 receptor in VSMCs. The overexpression of the AT2 receptor suppressed the basal DNA synthesis and the proliferation of VSMCs in the present experiment. The growth inhibitory effect of the AT1 receptor has been reported to be mediated by the activation of protein tyrosine phosphatase which inhibits activation of MAP kinases.6–27 The AT2 receptor also mediates apoptosis of cells through dephosphorylation of Bcl-2 by activation of MAP kinase phosphatase-1.28 Thus, suppression of basal DNA synthesis and proliferation of VSMCs by overexpression of the AT2 receptor is consistent with the growth inhibitory effects of AT1 receptor described originally. In addition, transfection of the AT2 receptor gene abolished the response of DNA synthesis to Ang II in VSMCs. This phenomenon is considered to be a result by the downregulation of AT1 receptor by overexpression of the AT2 receptor in VSMCs. Thus, the growth inhibition by the AT2 receptor may not only be associated with suppression of MAP kinases and the proapoptotic effects, but also with the downregulation of AT1 receptor in VSMCs.

We demonstrated that overexpression of AT1 receptor gene downregulates AT1a receptor in rat VSMCs in a ligand-independent manner, which is mediated by the bradykinin/NO pathway. This downregulation of AT1a receptor is novel mechanisms by which AT2 receptor regulates growth

Figure 5. A, Change in DNA synthesis in rat VSMCs in response to Ang II with or without transfection of the AT2 receptor gene. Quiescent VSMCs were incubated with pcDNA3 containing the AT2 receptor gene for 24 hours in the presence of lipofectin and in the absence or presence of 0.01 to 1.0 μmol/L Ang II. [3H]Thymidine incorporation into DNA was then determined. Values are mean±SEM (n=4). *P<0.05, **P<0.01 vs no transfection.
B, Proliferation of rat VSMCs with or without transfection of the AT2 receptor gene. VSMCs were plated at a density of 104 cells/cm2 in DMEM containing 5% calf serum with pcDNA3 lacking or containing AT2 receptor gene. Cells were counted after treatment for 24, 48, or 72 hours. Values are mean±SEM (n=4). *P<0.05, **P<0.01 vs vector lacking the AT2 receptor gene.
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