Selective Silencing of Angiotensin Receptor Subtype 1a (AT\textsubscript{1a}R) by RNA Interference

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Abstract—Angiotensin II exerts its physiological effects by activating multiple subtypes of its receptor such as AT\textsubscript{1a}-, AT\textsubscript{1b}-, and AT\textsubscript{2}-receptors. Because of a high degree of similarity among these G-protein–coupled receptors, it has been difficult to assign diverse physiological actions of angiotensin II through these receptor subtypes. We have developed small interfering RNAs to selectively inhibit the expression of the AT\textsubscript{1a} receptor (AT\textsubscript{1a}R) subtype. A dsRNA, AT \textsubscript{47}, was found to be highly selective and efficient in reducing the levels of AT\textsubscript{1a}R subtype. Transfection of AT\textsubscript{1a}R-expressing CHO cells with dsRNA AT \textsubscript{47} resulted in an 80% decrease in the AT\textsubscript{1a}R expression. In contrast, dsRNA AT \textsubscript{47} showed no significant effects on both AT\textsubscript{1b}R and AT\textsubscript{2}R subtypes. Thus, AT \textsubscript{47} provides us with a powerful tool to selectively silence this subtype of receptor to investigate its role in cardiovascular physiology. (Hypertension. 2005;45:115-119.)

Key Words: receptors, angiotensin II ■ gene therapy ■ receptors, angiotensin

Angiotensin II (Ang II) exerts profound physiological effects on the cardiovascular system by regulating such diverse functions as increases in blood pressure (BP), extracellular fluid volume, hormone secretion, vascular and cardiac remodeling, stimulation of sympathetic nerve activity, and damping of baroreflexes. Most, if not all, of these effects are mediated by activation of a single angiotensin receptor subtype (AT\textsubscript{1}R). Since the discovery of 2 subtypes of Ang II, AT\textsubscript{1a}R, AT\textsubscript{1b}R, and AT\textsubscript{2}R in rodents, it has been proposed that this diversity of Ang II actions resides in these 2 subtypes. Because AT\textsubscript{1a}R and AT\textsubscript{1b}R share 94% sequence similarity, it has been difficult to develop subtype specific antagonists to target AT\textsubscript{1a}R and AT\textsubscript{1b}R to distinct cardiovascular effects of Ang II. Thus, investigators have relied on the use of genetic targeting of these receptors in mouse models to link them to various phenotypes. For example, AT\textsubscript{1a}R knockout studies have indicated the role of this receptor in BP regulation, sodium handling, and central dipsogenic responses. However, knockout studies indicate that AT\textsubscript{1b}R can partially replace the BP regulatory functions of AT\textsubscript{1a}R. These observations indicate that although knockout technology enables us to delineate an overall physiological perspective, it is limited because of the possible expression of compensatory mechanisms during development. Therefore, new and more selective alternatives need to be developed in which the expression of AT\textsubscript{1a}R and AT\textsubscript{1b}R could be attenuated in adult animals after development.

We have taken advantage of recent advances in RNA interference (RNAi) technology to determine whether it can be used to selectively silence the expression of these receptor subtypes. Thus, our objective in this study was to determine whether we could identify a double stranded RNA (dsRNA) sequence that is highly effective and selective for the AT\textsubscript{1a}R subtype.

Materials and Methods

Complete coding sequence of Rattus rattus AT\textsubscript{1}R (Gen Bank accession number X62295) was used for the selection of target sequences. The sequence was used in the Target Finder and Design Tool provided by Ambion Inc (Austin, Tex). For screening, 3 target sequences spaced throughout the gene were chosen from the list of candidate siRNAs. Selected sequences did not show near-exact matches to any other known sequence on a BLAST search, confirming their sequence specificity to the AT\textsubscript{1a}R. Synthetic duplexed-deprotected siRNAs were purchased from Dharmacon Research, Inc (Lafayette, Colo). Three 21-bp dsRNA sequences were designated as AT \textsubscript{9}, AT \textsubscript{39}, and AT \textsubscript{47} directed to nucleotides 880 to 901, 135 to 156, and 115 to 135 to 156, AT \textsubscript{9} (5'-AAACAGCCTTGTGGTGGTGATGTG-3') directed to nucleotides 135 to 156, AT \textsubscript{39} (5'-AAACACTGCTGAA-CCCTCTG-3') directed to nucleotides 880 to 901, and AT \textsubscript{47} (5'-AAAGGCACAGTCCCCACTCAAG-3') directed to nucleotides 966 to 987. In addition, a scrambled sequence with no near-exact match to any known sequence, Scr (5'-AATGACTCAGTCG-3') was designed as control for AT \textsubscript{9}, AT \textsubscript{39}, and AT \textsubscript{47} dsRNAs.

Cell Culture and Transfection

Chinese hamster ovary cells (CHO) stably expressing either rat AT\textsubscript{1a}R, AT\textsubscript{1b}R, or AT\textsubscript{2}R were maintained at 37°C in Ham F12 medium supplemented with 10% fetal bovine serum. Cells were seeded in 12-well plates at a cell density of 1×10\textsuperscript{4} cells/well, 24 hours before their use in transfection. Oligofectamine-mediated (Invitrogen Corporation) transfection of the dsRNA was performed...
after manufacturer's instructions. Briefly, 2 μL of oligofectamine was mixed with 8 μL/well of OptiMEM (Invitrogen Corporation); similarly, the dsRNA was diluted a final volume of 90 μL/well in OptiMEM. Both solutions were incubated at room temperature for 10 minutes, mixed, and further incubated for 10 to 15 minutes. Cells were washed with phosphate-buffered saline (PBS) and 400 μL/well of serum-free Ham F12 media added. After transfection complexes were formed, the transfection mixture was added to the cells and incubation continued for 4 hours at 37°C. This was followed by addition of 250 μL/well of Ham F12 to 30%. Cells were maintained for 3 days and used for measurements of Ang II binding activity.

Measurement of $^{[125]}$I-Sar$^\text{-Ile}^8$ Ang II Binding to AT$_1$R and AT$_2$R

Cell cultures were rinsed with PBS (pH 7.2) and incubated with the Binding Buffer (PBS; pH 7.2; 0.16% heat-inactivated bovine serum albumin) containing 10 nmol/L $^{[125]}$I-Sar$^\text{-Ile}^8$ Ang II for 1 hour at room temperature in the absence and presence of 1 μmol/L of either losartan (AT$_1$R-specific antagonist) or PD-123319 (AT$_2$R-specific antagonist). Unbound radioligand was removed by rinsing the cells 3 times with ice-cold PBS pH 7.2. Cells were removed from the dish by dissolving with NaOH–SDS (0.1 mol/L NaOH and 10% SDS solution) for 2 hours at room temperature. Bound radioactivity was measured in a DP5500 gamma counter (Beckman Coulter, Fullerton, Calif). Specific binding was calculated by subtracting the $^{[125]}$I-Sar$^\text{-Ile}^8$ Ang II bound in the presence of either losartan or PD123319 from that in its absence. Scatchard analysis was performed from saturation binding experiments with increasing Ang II concentration for the calculation of $K_d$ and $B_{max}$ values.

Semi-Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated using 4-PCR RNA isolation kit (Ambion, Austin, Tex) according to the manufacturer’s instructions. Polymerase chain reaction (PCR) specific primers for AT$_1$R and AT$_2$R sequence were obtained from GenoMechanix LLC (Gainesville, Fla). Sequences for these primers were as follows: AT$_1$R: sense, 5′-TACGCTATGCAGATGGTGATGGG-3′; antisense, 5′-CTG-GCTGATGGCTGGATGGG-3′; AT$_2$R: sense, 5′-GCACACATTGAGTCCGCATTTA-3′; antisense, 5′-CAGAAAAGGGTGTAAGCAGCATGTTG-3′. We used as internal control the amplification of 18S rRNA using the primer set provided by on the TaqMan Ribosomal RNA Control Reagents kit (Applied Biosystems, Foster City, Calif). Reverse transcription reactions were performed using 2 μg of total RNA in a volume of 100 μL. The PCR conditions were: 95°C for 5 minutes for 50 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute, with a final extension step of 72°C for 10 minutes. The resulting PCR products were subjected to a 2% agarose gel electrophoresis. Bands representing PCR products for the AT$_1$R and AT$_2$R were quantitated and data normalized with PCR bands for 18S rRNA.

Calcium Uptake

Cells were transfected with either dsRNA AT$_1$ or AT$_2$R or a dsRNA Scr, as described. Two days after transfection, Ang II-stimulated $[^{45}]Ca^{2+}$ uptake was tested as described elsewhere. Briefly, cells were washed 3 times with Tyrodes solution (137 mmol/L NaCl, 2.68 mmol/L KCl, 1.36 mmol/L CaCl$_2$, 0.46 mmol/L MgCl$_2$, 5.6 mmol/L glucose, 12 mmol/L NaHCO$_3$, 0.36 mmol/L NaH$_2$PO$_4$, pH 7.4) and equilibrated in this solution for 1 hour at 37°C. Cells were incubated for 20 seconds in Tyrode solution containing 490 mCi/mmol $[^{45}]Ca^{2+}$ in the presence or absence of 0.1 μmol/L Ang II. Immediately after incubation, the assay medium was removed and the cells were quickly rinsed twice with 1 mL of ice-cold Wash solution (100 mmol/L MgCl$_2$, 10 mmol/L HEPES, 10 mmol/L CdCl$_2$, 10 mmol/L MnCl$_2$) and then rinsed rapidly with 10 mL of ice-cold 100 mmol/L MgCl$_2$ containing 10 mmol/L HEPES. Cells were solubilized in 1 mL of 0.1 mol/L NaOH and $[^{45}]Ca^{2+}$ was quantified in a LS6500 liquid scintillation counter (Beckman Coulter, Fullerton, Calif).

Statistical Analysis

Data are shown as mean±SEM. Statistical differences between 2 means were determined by unpaired Student t test.

Results

In the first series of experiments, our objective was to identify target sequences that would trigger a highly efficient and specific silencing of the AT$_1$R. For this, we selected 3 dsRNA sequences targeting the 5′-middle and 3′-regions of AT$_1$R coding sequence.

Of these dsRNAs, 2 (AT$_1$R 9, AT$_1$R 47) significantly reduced specific Ang II binding in CHO cells that overexpress AT$_1$R (Figure 1A). These dsRNAs were without effect on Ang II binding in the CHO cells that overexpress AT$_2$R (Figure 1A). Whereas AT$_1$R 47 was able to decrease binding of...
Ang II to cells expressing AT\textsubscript{1R} by 95%, AT\textsubscript{1} caused a decrease of only 60% (Figure 1A). Scrambled dsRNA failed to influence the binding of Ang II. In contrast, the AT\textsubscript{1} sequence was found to be nonselective, decreasing the binding of Ang II in both AT\textsubscript{1R}- and AT\textsubscript{2R}-expressing cells (Figure 1A). These data indicate that AT\textsubscript{1} sequence is the most effective and selective for AT\textsubscript{1R} silencing. Next, we determined the AT\textsubscript{1R} subtype specificity of dsRNA AT\textsubscript{1}. For this, both AT\textsubscript{a} and AT\textsubscript{b} overexpressing cells were transfected with either AT\textsubscript{1} or Scr dsRNA. Figure 1B shows that AT\textsubscript{1} was able to completely silence AT\textsubscript{1R} with only modest effect on the AT\textsubscript{2R} subtype. Transfection with Scr dsRNA did not influence binding of either receptor (data not shown). This indicated that AT\textsubscript{1} was predominantly selective for the AT\textsubscript{1R} subtype.

Next, we characterized the effect of AT\textsubscript{1} on AT\textsubscript{1aR}. dsRNA AT\textsubscript{1} caused a dose-dependent decrease in the specific binding of [\textsuperscript{125}I]-Sar\textsuperscript{1}-Ile\textsuperscript{8} Ang II to AT\textsubscript{1aR}-expressing CHO cells, with a maximal inhibition of 90% observed at 50 nmol/L dsRNA (Figure 2A). The IC50 was calculated to be 1.2 nmol/L. No effect was seen on AT\textsubscript{1bR} cells with these concentrations of dsRNA. Incubation with 100 nmol/L dsRNA AT\textsubscript{1} caused a time-dependent silencing of AT\textsubscript{1aR} (Figure 2B). An 80% decrease in binding was observed within 1 day, followed by a 97% decrease at 2 days after transfection. This level of decrease was maintained for an additional 24 hours. The receptor levels returned to control levels 7 days after transfection.

Saturation analyses of [\textsuperscript{125}I]-Sar\textsuperscript{1}-Ile\textsuperscript{8} Ang II binding were performed in Scr and AT\textsubscript{1} transfected AT\textsubscript{1aR} CHO cells (Figure 3). Scatchard analyses revealed a 20-fold decrease in the B\textsubscript{max} for AT\textsubscript{1aR} in the AT\textsubscript{1} transfected cells when compared with Scr controls (0.46±0.01 versus 8.80±0.46 pmol/mg protein). In contrast, the K\textsubscript{d} values for both treatments remained comparable (0.9±0.09 versus 2.0±0.30 nmol/L). The observed decrease in the number of AT\textsubscript{1aR} was also reflected in a significant decrease in AT\textsubscript{1aR} mRNA levels (Figure 4A). An 80% decrease in the mRNA was observed 48 hours after transfection (Figure 4B).

Finally, we determined the effect of AT\textsubscript{1} on Ca\textsuperscript{2+} uptake. To find out if decreases in AT\textsubscript{1}-specific binding was associated to a reduction in functional AT\textsubscript{1R}, we determined the effects of dsRNA transfection on Ang II-stimulated calcium uptake. Ang II (100 nmol/L) caused a 54% increase in [\textsuperscript{45}Ca\textsuperscript{2+}] uptake in CHO cells expressing AT\textsubscript{1aR}. Transfection of these cells with dsRNA Scr did not influence this stimulation and it was comparable to that of nontransfected cells. In contrast, the Ang II-induced increase in [\textsuperscript{45}Ca\textsuperscript{2+}] uptake was completely abolished in by dsRNA AT\textsubscript{1} transfected cells (Figure 5).

**Discussion**

The most significant finding of our study is that it establishes, for the first time to our knowledge, that RNAi technology can be used to silence the AT\textsubscript{1R} subtype. This provides us with a powerful tool for elucidating the respective roles of AT\textsubscript{1R}...
and AT1bR subtypes in the diverse and profound physiological effects of Ang II in the cardiovascular system in normal animals. The observed reduction in [125I]-Sar1-Ile8 Ang II binding, AT1aR mRNA levels, and Ang II-stimulated [45Ca2+] uptake demonstrates that at least 1 of the 3 dsRNA targeted for the AT1aR subtype is selective and efficient in silencing the AT1aR subtype gene. The dsRNA AT1 47 is more efficient in reducing AT1aR numbers compared with its sister subtype, the AT1bR, despite the fact that these subtypes share 96% similarity. The dsRNA AT1 47 is highly efficient and produces AT1aR silencing that lasts for ~3 days. Until now, the popular technology to reduce angiotensin receptors has been the use of antisense oligonucleotides.13–18 RNAi technology not only is highly selective for the subtypes of AT1aR and AT1bR but also is more effective than antisense oligonucleotides. For example, a previous study 19 demonstrated a reduction of AT1R by 57% to 73% with 1 μmol/L concentration of AT1-specific antisense oligonucleotides. In contrast, in the present study, a concentration of 100 nmol/L of dsRNA was found to silence AT1R by 95% in 48 hours.

The efficacy of the dsRNA 47 to silence AT1R in a physiologically relevant cell was confirmed with the use of astroglia cells in primary cultures. Our observations indicated an 80% decrease in AT1 binding elicited by this dsRNA in these cultures, confirming the validity of the silencing data obtained for the CHO cells.

In summary, our observations demonstrate that RNAi technology can be successfully used to silence 2 closely related genes for Ang II receptors. This provides us with a powerful tool to selectively silence 1 or more subtypes of AT1R and to study their role in cardiovascular physiology and pathophysiology in a way that was not previously possible.

Perspectives

Delineating the roles of the AT1R subtypes in various cardiovascular functions has been difficult in the past because of lack of selective antagonists or antisense targeting. This study uses RNAi technology and successfully demonstrates the effectiveness of a dsRNA that selectively targets AT1aR subtype. Thus, our study provides a well-needed tool to dissect AT1R functions in vivo.

References


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