Angiotensin-(1-7) Downregulates the Angiotensin II Type 1 Receptor in Vascular Smooth Muscle Cells

Michelle A. Clark, Debra I. Diz, E. Ann Tallant

Abstract—Angiotensin (Ang)-(1-7) is a biologically active peptide of the renin-angiotensin system that has both vasodilatory and antiproliferative activities that are opposite the constrictive and proliferative effects of angiotensin II (Ang II). We studied the actions of Ang-(1-7) on the Ang II type 1 (AT1) receptor in cultured rat aortic vascular smooth muscle cells to determine whether the effects of Ang-(1-7) are due to its regulation of the AT1 receptor. Ang-(1-7) competed poorly for [125I]Ang II binding to the AT1 receptor on vascular smooth muscle cells, with an IC50 of 2.0 μmol/L compared with 1.9 nmol/L for Ang II. The pretreatment of vascular smooth muscle cells with Ang-(1-7) followed by treatment with acidic glycine to remove surface-bound peptide resulted in a significant decrease in [125I]Ang II binding; however, reduced Ang II binding was observed only at micromolar concentrations of Ang-(1-7). Scatchard analysis of vascular smooth muscle cells pretreated with 1 μmol/L Ang-(1-7) showed that the reduction in Ang II binding resulted from a loss of the total number of binding sites [Bmax, 437.7±261.5 fmol/mg protein in Ang-(1-7)–pretreated cells compared with 607.5±301.2 fmol/mg protein in untreated cells, n=5, P<0.05] with no significant effect on the affinity of Ang II for the AT1 receptor. Pretreatment with the AT1 receptor antagonist L-158,809 blocked the reduction in [125I]Ang II binding by Ang-(1-7) or Ang II. Pretreatment of vascular smooth muscle cells with increasing concentrations of Ang-(1-7) reduced Ang II–stimulated phospholipase C activity; however, the decrease was significant (81.2±6.4%, P<0.01, n=5) only at 1 μmol/L Ang-(1-7). These results demonstrate that pharmacological concentrations of Ang-(1-7) in the micromolar range cause a modest downregulation of the AT1 receptor on vascular cells and a reduction in Ang II–stimulated phospholipase C activity. Because the antiproliferative and vasodilatory effects of Ang-(1-7) are observed at nanomolar concentrations of the heptapeptide, these responses to Ang-(1-7) cannot be explained by competition of Ang-(1-7) at the AT1 receptor or Ang-(1-7)–mediated downregulation of the vascular AT1 receptor. (Hypertension. 2001;37:1141-1146.)

Key Words: angiotensin II muscle, smooth, vascular receptors, angiotensin-(1-7)
and a concentration-dependent decrease in the maximal response, with a pA₂ of 5.5, and suggested that Ang-(1-7) was an antagonist at the AT₁ receptor at micromolar concentrations of the heptapeptide. Roks et al. also demonstrated that Ang-(1-7) at a concentration of 10 μmol/L reduced Ang I- and Ang II-induced contraction of human internal mammary arteries, suggesting that micromolar Ang-(1-7) was an antagonist at the AT₁ receptor. Because Ang-(1-7) competes for binding to the vascular AT₁ receptor at micromolar concentrations, pharmacological concentrations of Ang-(1-7) may oppose or counteract the actions of Ang II through competitive antagonism. Alternatively, Ang-(1-7) could oppose the vasoconstrictive and proliferative effects of Ang II through downregulation or desensitization of the AT₁ receptor. This study was designed to determine whether Ang-(1-7) causes a downregulation of the AT₁ receptor or desensitizes responses to Ang II by direct actions at the AT₁ receptor.

**Methods**

**Materials**

[^3H]Sodium iodide was obtained from Amersham Pharmacia, and myo-[^3H]inositol was obtained from American Radiolabeled Chemicals, Inc. DMEM/Ham’s F-12 (DMEM/F12), inositol-free DMEM, FBS, penicillin, streptomycin, and trypsin/EDTA were obtained from Gibco. Ang II and Ang-(1-7) were purchased from Bachem, Inc. The AT₁ receptor antagonists losartan, EXP 3174, and L-158,809 were kindly provided by Merck and Co, Inc. and the AT₂ receptor antagonist PD 123319 was a gift from Parke-Davis. All other chemicals were purchased from Sigma Chemical Co.

**Cell Culture Procedures**

Aortic VSMCs were isolated from 10- to 12-week-old male Hanover Sprague-Dawley rats bred and raised at the ALACC-accredited animal facility of Wake Forest University School of Medicine. Primary cultures of VSMCs were obtained from aortic explants as previously described. The cells were cultured in DMEM/F12 supplemented with 10% FBS, 100 μg/mL penicillin, and 100 μg/mL streptomycin and maintained at 37°C in a humidified CO₂ incubator (5% CO₂ and 95% air). Confluent monolayers of VSMCs from passages 4 to 9 were used in all experiments.

**Measurement of[^125I]Ang II Binding**

Ang II was iodinated according to a modified chloramine T procedure, and the monoiodinated form was purified through HPLC according to previously established procedures.[^14][^125I]Ang II binding was measured by incubating confluent monolayers of VSMCs in binding buffer (PBS: 50 mmol/L NaHPO₄, 0.15 mol/L NaCl, pH 7.4, with 0.2% fatty acid-free BSA, 2.5 mmol/L EGTA, and 5 mmol/L MgCl₂) containing[^125I]Ang II. Nonspecific binding was determined in the presence of 10 μmol/L unlabeled Ang II. After a 1-hour incubation at room temperature, binding reactions were terminated by aspirating the binding buffer from individual wells and washing each well with ice-cold PBS (2 mL/wash). Cellular proteins were solubilized in 2N NaOH, and cell-associated radioactivity was quantified with γ-spectroscopy. Protein was measured according to the Lowry method[^15] with BSA used as the standard.

Various types of binding studies were conducted to determine competition by peptides and antagonists, the effect of pretreatment with angiotensin peptides on subsequent Ang II binding, and saturation isotherms to calculate binding constants, as described here.

First, competition studies were performed by incubating VSMCs with increasing concentrations (from 10⁻¹⁵ to 10⁻⁷ mol/L) of each peptide or with 10 μmol/L losartan. EXP 3174, or PD 123319 in the presence of 0.3 nmol/L[^125I]Ang II.

Second, the effect of pretreatment with Ang-(1-7) or Ang II on subsequent binding of[^125I]Ang II to VSMCs was determined through preincubation with increasing concentrations of peptide in Hanks’ balanced salt solution for 30 minutes at room temperature. After this preincubation, surface-bound Ang-(1-7) or Ang II was removed with a 5-minute treatment with ice-cold 50 mmol/L glycine/50 mmol/L HCl, pH 3.0, before the measurement of[^125I]Ang II binding.

Third, saturation isotherms were constructed after treatment (30 minutes at room temperature) of VSMCs with vehicle or 1 μmol/L Ang-(1-7). Surface-bound Ang-(1-7) was removed with acidic glycine, and acid-stripped cells were incubated with increasing concentrations of[^125I]Ang II (0.075 to 2.4 nmol/L) for 1 hour at room temperature.

**Measurement of Phosphoinositide-Specific Phospholipase C**

Phospholipase C (PLC) activity was assayed by the production of[^2]H-labeled inositol phosphates in VSMCs prelabeled with myo-[^3H]-inositol, as previously described. Confluent monolayers of VSMCs in 6-well culture plates were labeled for 72 hours with myo-[^3H]-inositol (2 μCi/well) in inositol-free DMEM that contained 0.5% FBS. The radiolabeled VSMCs were preincubated with increasing concentrations of Ang-(1-7) (0.1 to 10 μmol/L) in HEPES-buffered Krebs-Ringer solution (125 mmol/L NaCl, 5 mmol/L KCl, 1.2 mmol/L MgSO₄, 6 mmol/L glucose, 1 mmol/L CaCl₂, and 25 mmol/L LiHPO₄, pH 7.4) and 10 μmol/L LiCl, for 30 minutes at 37°C. The cells were then treated with 10 nmol/L Ang II, and the reaction was continued for 30 minutes at 37°C. Inositol phosphates were extracted with ice-cold perchloric acid, separated through ion exchange chromatography, and quantified with liquid scintillation spectroscopy as previously described.[^16]

**Statistics**

All data are expressed as the mean±SEM of ≥3 experiments. Competition binding data were analyzed by nonlinear regression with the computer program Prism (GraphPad). Saturation isotherms were analyzed with the EBDA/LIGAND computer program (Elsevier-BIOSOFT). Repeated measures 1-way ANOVA or ANOVA with Dunnnett’s post hoc test were used to compare treatment groups. The criterion for statistical significance was P<0.05.

**Results**

**Competition for the AT₁ Receptor by Ang-(1-7)**

VSMCs were incubated with increasing concentrations of Ang II and Ang-(1-7) to determine whether Ang-(1-7) competed for[^125I]Ang II binding to VSMCs. As shown in Figure 1, Ang-(1-7) competed poorly for binding to the AT₁ receptor on VSMCs isolated from the thoracic aorta of Sprague-Dawley rats with an IC₅₀ value of 2.0 μmol/L compared with an IC₅₀ value of 1.9 nmol/L for Ang II. The[^125I]Ang II binding to VSMCs was characterized as AT₁ based on ≥95% competition of specific binding by either 10 μmol/L concentration of the AT₁ receptor antagonist losartan or EXP 3174 and no competition by 10 μmol/L PD 123319, an AT₂ receptor antagonist (Figure 1).

**Regulation of the AT₁ Receptor by Ang-(1-7)**

Ang II binds to AT₁ receptors on VSMCs and induces a rapid internalization of the Ang II-AT₁ receptor complex. Because we observed that Ang-(1-7) binds to the vascular AT₁ receptor, albeit at a 1000-fold higher concentration than Ang II, Ang-(1-7) binding to the AT₁ receptor could reduce subsequent responses to Ang II by causing internalization and...
Effect of pretreatment with Ang-(1-7) on the Binding Affinity and Density of the AT$_1$ Receptors on VSMCs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$K_d$, nmol/L</th>
<th>$B_{max}$, fmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.5±0.35</td>
<td>607.5±301.2</td>
</tr>
<tr>
<td>Ang-(1-7)</td>
<td>1.18±0.29</td>
<td>437.7±261.5*</td>
</tr>
</tbody>
</table>

VSMCs were pretreated with vehicle or 1 μmol/L Ang-(1-7) for 30 minutes at room temperature. Surface-bound peptide was removed through treatment with acidic glycine. Acid-stripped cells were incubated with increasing concentrations of [125I]Ang II (0.075 to 2.4 nmol/L) to saturate the receptor. Nonspecific binding was measured in the presence of 10 μmol/L Ang II. Scatchard analysis was used to calculate the $K_d$ and $B_{max}$ values of the AT$_1$ receptor in the presence and absence of Ang-(1-7). Each value is the mean±SEM of 4 or 5 experiments. *$P<0.05$ of Ang-(1-7)–treated cells compared with untreated cells.

Effect of Ang-(1-7) on Ang II–Induced PLC Activity

Ang II significantly stimulated inositol phosphate release from VSMCs, measured as the release of total inositol phosphates from cells prelabeled with myo-[3H]-inositol. A 30-minute incubation of VSMCs with Ang II (10 nmol/L) increased phosphoinositide–specific PLC activity by 102.6±19.6% above basal levels ($P<0.05$, $n=5$). A similar incubation with 1 μmol/L Ang-(1-7) had no effect on inositol
After exposure to the peptides for 30 minutes at room temperature, surface-bound peptide and antagonist were removed by treatment with acidic glycine, and [125I]Ang II binding was measured. The data are presented as the percentage of specific binding. *P<0.05 compared with total specific binding in cells pretreated with vehicle. Each value represents the mean±SEM of 4 experiments with VSMCs isolated from different rat aortas.

The percent of Ang II–induced inositol release above basal was measured, and the amount of release is presented as the percent of Ang II–stimulated inositol phosphate release. These results clearly demonstrate that the short-term treatment of VSMCs with pharmacological concentrations of Ang-(1-7) downregulates the vascular AT1 receptor.

Although 1 μmol/L Ang-(1-7) alone did not activate PLC in VSMCs, it did reduce subsequent binding of Ang II to the AT1 receptor and attenuated activation of AT1-coupled cellular responses by Ang II. The AT1 receptor on VSMCs is rapidly internalized on stimulation by Ang II, a phenomenon accompanied by a reduction in subsequent responses. This suggests that Ang-(1-7), at micromolar concentrations, binds to the AT1 receptor to induce receptor internalization without activating subsequent cellular responses. Previous work with substance P and serotonin receptors clearly indicates that receptor internalization can occur independent of changes in functional responsiveness. 17–19 Studies by several investigators suggest that different motifs in the structure of the AT1 receptor are required for receptor internalization compared with intracellular signal generation by Ang II. 20–22 The truncation of a section of the carboxyl terminus of the AT1 receptor prevented receptor internalization without affecting coupling to PLC. 21,23 A point mutation in the AT1 receptor that eliminates Ang II coupling to PLC (Asp74→Asn) did not block receptor internalization. 22 In addition, the potent antagonist [Sar1,Ile8]Ang II, a sarcosine analog of Ang II, was internalized but did not activate PLC in Chinese hamster ovary cells transfected with the AT1 receptor, as well as in adrenal medullary cells. 22,24 This suggests that Ang-(1-7), as well as sarcosine analogs of Ang II, bind to and activate the AT1 receptor to produce receptor internalization without coupling to G proteins to activate PLC. In contrast, the nonpeptide antagonist L-158,809 did not cause internalization in our study. Thus, it is possible that high concentrations of Ang-(1-7) and the peptide antagonists produce receptor internalization through weak agonist properties. This would also be consistent with our observation of a weak pressor response to high doses of Ang-(1-7) in the pithed rat, an effect that was mediated by an AT1 receptor. 11

Mahon et al 12 suggested that Ang-(1-7) is a noncompetitive antagonist at AT1 receptors in rabbit aortic rings, causing a rightward shift in the dose-response contraction curve to Ang II administered 60 minutes after Ang-(1-7). However, the concentrations of Ang-(1-7) used in their study ranged from 1 to 30 μmol/L. Roks et al 13 also suggested that 10 μmol/L Ang-(1-7) was antagonistic at the AT1 receptor in human VSMCs. This would be consistent with our observation that Ang-(1-7) had an IC50 value of 2 μmol/L at the AT1 receptor in VSMCs. Our evidence suggests that short-term exposure of cells to micromolar levels of Ang-(1-7) downregulates the AT1 receptor over a time frame consistent with receptor
internalization. Thus, when Ang II is given after a prior exposure to Ang-(1-7) (as we did in our study) or in the presence of a large excess of Ang-(1-7) (as in studies described by Roks et al\textsuperscript{13} and Mahon et al\textsuperscript{12}), one might expect antagonist actions of Ang-(1-7). However, endogenous circulating levels of Ang-(1-7), as well as tissue concentrations of the peptide, are in the picomolar range.\textsuperscript{25–27} Although treatment with ACE inhibitors increased circulating levels of Ang-(1-7) from 9- to 25-fold,\textsuperscript{25,28} there is no evidence showing an increase in endogenous Ang-(1-7) levels to the micromolar range. In addition, the majority of the in vivo and in vitro actions of Ang-(1-7) occur at doses lower than 1 to 30 \(\mu\)mol/L. Because Ang-(1-7) downregulated Ang II binding and receptor activation only at micromolar concentrations, the vasodilatory and antiproliferative responses that occur at nanomolar concentrations of the heptapeptide cannot be explained by antagonist effects of Ang-(1-7) at the AT\(_1\) receptor. Ueda et al\textsuperscript{29} recently reported that the infusion of Ang-(1-7) antagonized the Ang II-mediated vasoconstriction of human forearm resistance vessels, suggesting that Ang-(1-7) is an endogenous antagonist of the human AT\(_1\) receptor. However, the authors reported that Ang-(1-7) significantly reduced the response to Ang II at a dose of 10 \(\mu\)mol/L, a concentration significantly lower than the IC\textsubscript{50} value for the AT\(_1\) receptor (\(> 1 \) \(\mu\)mol/L). In light of our observations, the responsiveness to Ang-(1-7) in the study by Ueda et al\textsuperscript{29} may be due to activation of a non-AT\(_1\) receptor by Ang-(1-7) and the release of a compensatory vasodilator such as NO or prostaglandins.

In addition, evidence from several studies clearly shows that the actions of Ang-(1-7) are independent of AT\(_1\) receptor activation. The vasodilation of canine or porcine coronary arteries induced by Ang-(1-7) was prevented by removal of the endothelium or by blockade of NO release,\textsuperscript{30,31} suggesting that Ang-(1-7) stimulated NO production to counter the vasoconstrictor effects of Ang II. These effects were not blocked by AT\(_1\) or AT\(_2\) receptor antagonists. Muthalif et al\textsuperscript{32} showed that Ang-(1-7) stimulated arachidonic acid release from rabbit VSMCs, an effect that was partially prevented by \([\beta-Ala\]\textsuperscript{5}Ang-(1-7), the selective Ang-(1-7) receptor antagonist, or by the AT\(_2\) antagonist PD 123319 but not by an AT\(_1\) receptor antagonist. Ang-(1-7) caused the vasodilation of renal afferent arterioles, an effect blocked by \([\beta-Ala\]\textsuperscript{5}Ang-(1-7).\textsuperscript{8} Further, Ang-(1-7) blocked mitogen-stimulated growth of rat VSMCs, responses that were not reversed by an AT\(_1\) or AT\(_2\) receptor antagonist\textsuperscript{\textsuperscript{23}} but were prevented by the selective Ang-(1-7) receptor antagonist \([\beta-Ala\]\textsuperscript{5}Ang-(1-7).\textsuperscript{32} suggesting that Ang-(1-7) activates a unique receptor on VSMCs to counteract the mitogenic response to Ang II. These in vitro studies are further supported by observations in combined ACE inhibitor/lotsartan–treated rats where a monoclonal antibody to Ang-(1-7)\textsuperscript{9} or \([\beta-Ala\]\textsuperscript{5}Ang-(1-7)\textsuperscript{32} unmasked a role for endogenous Ang-(1-7), working in part through prostaglandins, in the blood pressure–lowering effects.\textsuperscript{34} Furthermore, there is evidence for a \([\beta-Ala\]\textsuperscript{5}Ang-(1-7)–sensitive, non-AT\(_1\), non-AT\(_2\) receptor in the aorta\textsuperscript{32} and mesentery\textsuperscript{34} of the animals treated with ACE inhibitors and lotsartan. Thus, Ang-(1-7) has unique effects in the vascular system that are coupled to a non-AT\(_1\), receptor and do not result from antagonism of the AT\(_1\) receptor.

In the present study, we showed that prior treatment of VSMCs with Ang-(1-7) attenuated \([\text{[3H]}\text{Ang}\text{II}]\) binding to the AT\(_1\) receptor and reduced Ang II–stimulated PLC activation. However, significant downregulation of Ang II binding and Ang II–mediated responsiveness occurred only at micromolar concentrations of Ang-(1-7), in agreement with its IC\textsubscript{50} value for competition at the AT\(_1\) receptor. These results suggest that at micromolar concentrations, Ang-(1-7) acts as a weak agonist at the AT\(_1\) receptor to downregulate the receptor and to reduce subsequent cellular responses to Ang II.

**Acknowledgments**

This work was supported in part by grants HL-51952 and NS-31664 from the National Institutes of Health and a Grant-in-Aid from the American Heart Association, North Carolina Affiliate.

**References**


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Hypertension. 2001;37:1141-1146
doi: 10.1161/01.HYP.37.4.1141

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