Receptor-Mediated Effects of Angiotensin II on Growth of Vascular Smooth Muscle Cells From Spontaneously Hypertensive Rats

Bettina Bunkenburg, Therese van Amelsvoort, Harald Rogg, and Jeanette M. Wood

This study examines the effects of angiotensin II on hypertrophy and proliferation of aortic smooth muscle cells from spontaneously hypertensive and Wistar-Kyoto rats and the receptor subtypes mediating these effects. In quiescent confluent cells, angiotensin II induced a dose-dependent increase in thymidine and leucine incorporation without stimulating cell proliferation. In nonconfluent cells, angiotensin II stimulated cell proliferation only in combination with a submaximal concentration of fetal calf serum. These effects were enhanced in cells from spontaneously hypertensive rats compared with Wistar-Kyoto rats. The effects of angiotensin II could be blocked by the AT₁ receptor antagonist DuP 753 but not by the AT₂ receptor ligand PD 123177. These effects were enhanced in cells from spontaneously hypertensive rats compared with Wistar-Kyoto rats. These data show that the angiotensin II receptors present in vascular smooth muscle cells in culture from both rat strains are of the AT₁ receptor subtype. This receptor subtype appears to mediate vascular smooth muscle cell hypertrophy and proliferation as well as vasoconstriction. Although no difference in the receptor profile was detectable between the two rat strains, the affinity for the ligands to the receptor and the receptor density tended to be greater in cells from spontaneously hypertensive rats than in cells from Wistar-Kyoto rats. These results may partly explain the greater hypotensive response to angiotensin II receptor blockade in spontaneously hypertensive rats than in Wistar-Kyoto rats, although both rat strains have the same plasma concentrations of angiotensin II.

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KEY WORDS • angiotensin II • receptors, angiotensin • hypertrophy • muscle, smooth, vascular • rats, inbred SHR

The spontaneously hypertensive rat (SHR) has a normal or low plasma renin activity and is not considered to be a renin-dependent model of hypertension. However, different blockers of the renin-angiotensin system, such as renin inhibitors, converting enzyme inhibitors, and nonpeptidic angiotensin II antagonists, lower blood pressure in SHRs after acute and chronic administration. Although the renin-angiotensin system is not activated in the circulation in SHRs, it may be at the receptor level or locally in a tissue such as the blood vessel wall.

As well as being a potent vasoconstrictor, angiotensin II (Ang II) may also influence the growth of vascular smooth muscle cells (VSMCs). Ang II has been shown to stimulate protein and DNA synthesis in cultured VSMCs and, under certain conditions, to induce proliferation of VSMCs and increase extracellular matrix formation. Aortic medial hypertrophy in SHRs is characterized by hypertrophy and polyplody of VSMCs rather than by hyperplasia, and it was shown that this can be normalized by treatment with angiotensin converting enzyme inhibitors. Thus, Ang II may contribute to the development of hypertension in SHRs not only by its activity as a vasoconstrictor but also by an effect on VSMC growth and vascular hypertrophy. In addition, Ang II may contribute to the proliferative response of VSMCs induced after vascular injury.

The aim of the present study was to determine the effects of Ang II on growth of VSMCs derived from SHRs and whether cells derived from Wistar-Kyoto (WKY) rats respond in the same way. In addition, the receptors mediating the effects were characterized, and the receptor profiles of VSMCs from SHRs and WKY rats were compared. The effects of Ang II on thymidine incorporation, leucine incorporation, and cell number of quiescent confluent VSMCs were studied in the absence of serum growth factors. The effects of Ang II on cell proliferation were determined in nonconfluent cells cultured in a medium containing a submaximal concentration of serum. The receptor types by which these effects of Ang II are mediated were studied with the use of ligands selective for the different subtypes of the Ang II receptor. In addition, the receptor subtypes present in isolated aortic VSMCs derived from both rat strains were determined by radioligand binding studies.

Methods

Isolation of Vascular Smooth Muscle Cells

Male SHRs and WKY rats were obtained from Iffa Credo (Lyon, France) at an age of 11 weeks (weighing approximately 270–290 g). The rats were killed by

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decapitation, and the thoracic aorta was removed. Fat and connective tissue were discarded, and the aortas were carefully washed in culture medium composed of minimal essential medium with Earle’s salts, 4 mM l-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 mM TES-HEPES, pH 7.3, and, if not otherwise stated, 10% heat-inactivated fetal calf serum. The adventitia was stripped off, and cells were isolated by enzymatic digestion of the media as described previously but with some modifications. The vessels were exposed to 0.05% elastase (type II-A) in medium for 20 minutes at room temperature. Thereafter, the vessels were opened longitudinally, cut into fine pieces, and incubated in 0.1% 0.14 mM trypsin-EDTA at 37°C. After 30 minutes this was replaced by 0.3% collagenase (type IV) in medium for 2 hours at 37°C. Thereafter, the suspension was carefully centrifuged (80–100g) for 4 minutes and washed once with medium, and the cells were plated in a 25-cm² culture flask and cultured at 37°C in a 5% CO₂ atmosphere with 95% air. One 25-cm² flask was used for three to four aortas isolated from SHRs or five aortas isolated from WKY rats. Medium was routinely changed every 2–3 days, and cells were passaged once a week at a 1:3 ratio with trypsin-EDTA. After 30 minutes this was replaced by 0.3% collagenase (type IV) in medium for 2 hours at 37°C. The medium was routinely changed for SHR or 5 days for WKY VSMCs, and the cells were plated in a 25-cm² culture flask and cultured at 37°C in a 5% CO₂ atmosphere with 95% air. One 25-cm² flask was used for three to four aortas isolated from SHRs or five aortas isolated from WKY rats. Medium was routinely changed every 2–3 days, and cells were passaged once a week at a 1:3 ratio with trypsin-EDTA. When cells reached confluence, they exhibited a hill-valley pattern typical for VSMCs in culture. Phenotypic characterization was performed with the peroxidase-antiperoxidase procedure for α-actin, with reagents obtained from Dakopatts (Instrumenten Gesellschaft, Zurich, Switzerland). The isolated cells stained positively to the α-actin antibody.

Determination of DNA Synthesis, Protein Synthesis, and Cell Number in Quiescent Confluent Smooth Muscle Cells

Cells between passages 2 and 5 were used. They were plated in a six-well multiwell plate (Costar Corp., Cambridge, Mass.) at approximately 2×10⁶ cells per well. After 3 days for SHR or 5 days for WKY VSMCs, the cells were rendered quiescent by a 48-hour serum deprivation period. Instead of 10% fetal calf serum, the medium contained 0.1% bovine serum albumin. Thereafter, the serum-free medium was replaced by fresh serum-free medium, and the different agents to be tested were added. After 24 hours, the agents were readded, and either [³H]thymidine or [³H]leucine was also added to a final activity of 1 μCi/ml. The incorporation of tritiated thymidine or leucine was stopped after a 24-hour incubation time. The medium was aspirated, washed three times with cold phosphate buffered saline pH 7.0, washed once with 10% trichloroacetic acid, and incubated at 4°C for 30 minutes in 10% trichloroacetic acid. Thereafter, the trichloroacetic acid-insoluble material was washed twice with cold 94% ethanol, and the cellular material was dissolved in 0.1N NaOH at room temperature for 4 hours. The radioactivity was measured by liquid scintillation counting. For cell number determination, no radiosilids were added. Cells were gently trypsinized and counted in a Coulter counter (Industrial D, Coulter Electronic Ltd., Instrumenten Gesellschaft, Zurich, Switzerland). All determinations were performed in triplicates or sextets with up to three isolates. For the calculation of values for thymidine or leucine incorporation, the incorporated radioactivity counted per well was divided by the mean value of cell number for each different concentration or compound and was expressed as disintegrations per minute per 10⁶ cells. Values for saline-treated cells were taken as 100%, and the percentage change over saline-treated cells was calculated.

To study the effects of Ang II on VSMC hypertrophy in culture, we measured alterations in cell number and DNA and protein synthesis in quiescent confluent VSMCs derived from SHRs. The effects of Ang II at the final concentrations of 10⁻⁸, 10⁻⁷, and 10⁻⁶ M on cell number, thymidine incorporation, and leucine incorporation were tested. The experiments were repeated with cells derived from WKY rats to determine whether they respond in the same way.

To determine the receptor subtypes mediating VSMC hypertrophy induced by Ang II, we studied the effects of the Ang II receptor ligands DuP 753 and PD 123177 on thymidine and leucine incorporation in cells derived from SHRs. First, the effective dose range of the Ang II receptor ligands for blocking the Ang II-induced VSMC hypertrophy was evaluated. The effects of different concentrations of DuP 753 or PD 123177 in combination with Ang II (10⁻⁷ M) on thymidine incorporation were measured in cells derived from SHRs. Thereafter, the effects of the selected dose of DuP 753 and the highest dose of PD 123177 in combination with Ang II (10⁻⁷ M) on both thymidine and leucine incorporation were determined. In addition, the effects on these parameters of DuP 753 or PD 123177 when given alone were tested. DuP 753 and PD 123177 were always added up to 30 minutes before the addition of Ang II.

To test the stability of Ang II under these experimental conditions, we plated SHR VSMCs into six-well multiwell plates at 2×10⁶ cells per well and allowed them to grow to confluence. After cells were rendered quiescent in serum-free medium for 48 hours, medium was changed for fresh serum-free medium. Unlabeled Ang II (final concentration, 10⁻⁶ M) and [¹²⁵]Ang II (approximately 0.1 nM final concentration, 800,000 cpm) were added, and the samples were incubated at 37°C in a 5% CO₂ atmosphere. Aliquots of the medium were taken out at 0 (just after the addition of Ang II), 1, 3, 7, and 24 hours. Degradation was measured by thin-layer chromatography (TLC) with precoated TLC plates (Sil RP 15W/UV₂₅₄, Hans Mohler + Co., Basel, Switzerland) using the solvent system NaCl 3.6% /acetoniitrile (70:30, vol/vol). The plates were read with an automatic TLC linear analyzer (Tracemaster 20, Berthold, Regensburg, Switzerland).

Proliferation Studies

Cells were plated in six-well multiwell plates in culture medium at 2×10⁶ cells per well. After approximately 12 hours, cell attachment was complete, and the medium was exchanged for medium containing 1–10% fetal calf serum. This time was taken as the zero point for these experiments. The compounds to be tested first were added at the zero point and then every 24 hours. Cell number was determined with a Coulter counter at the times indicated in the figures, with six separate wells for each agent or agent concentration and with up to two different isolates. Medium was routinely changed every 2 days.
In the first series of experiments, the culture conditions necessary for Ang II to increase cell proliferation were determined. Experiments were performed in medium containing 1%, 5%, or 10% fetal calf serum with VSMCs derived from SHRs. Cells were supplemented once daily with either Ang II (10^{-7} M final concentration) or saline.

To investigate whether there is a difference in the proliferatory response to Ang II between cells derived from SHRs or WKY rats, we tested the effects of Ang II at 10^{-9}, 10^{-8}, and 10^{-7} M (final concentration) or saline on cell number in cells derived from both rat strains in medium containing 1% fetal calf serum.

To determine the receptor subtype mediating the Ang II-stimulated VSMC hyperplasia, we studied the effects of DuP 753 on the growth curve of VSMCs from SHRs. DuP 753 was given at the final concentration of 10^{-3} M, which was shown to block the Ang II–induced hypertrophy (described above). DuP 753 was either given alone or in combination with Ang II at 10^{-7} M. The culture medium contained 1% fetal calf serum, and DuP 753 was added always 30 minutes before the addition of Ang II.

**Radioligand Receptor Binding Studies**

**Membrane preparation.** Cells were plated at 2×10^4 cells/cm² for SHR cells and 3–4×10^4 cells/cm² for WKY cells in a 500-cm² culture dish (Nunc, Roskilde, Denmark) and were grown to confluence for 7 days. Only the second to the fourth subcultures were used. The cells were washed with phosphate buffered saline, pH 7.0, removed from the plate with a rubber policeman, and centrifuged for 5 minutes at 5,000g. These and all following steps were carried out at 0–4°C. Cells were homogenized in 2 mM sodium bicarbonate (Polytron, Kinematica GmbH, Lucerne, Switzerland; 8 seconds at setting 8) and were centrifuged at 47,800g for 30 minutes. The pellet was resuspended in 25 mM Tris-HCl, pH 7.5, containing 10 mM MgCl₂ with the use of a tight-fitting pestle homogenizer. The suspension was frozen in liquid nitrogen at a protein concentration of approximately 2 mg/ml and was stored at −80°C. Protein concentration was assayed with the method of Bradford using bovine serum albumin as standard.

**Binding assay.** Binding studies were performed with varying concentrations of unlabeled competitors as indicated in the figures. Total reaction volume was 100 μl. Incubation was performed for 60 minutes at 25°C with 5–10 μg of membrane proteins in 25 mM Tris-HCl, pH 7.5, containing 10 mM MgCl₂, 110 mM NaCl, 1% dimethyl sulfoxide, and 0.2% bovine serum albumin, with the use of 0.5 nM [125I]–Ang II as radioligand and the peptidase inhibitors as described. All following steps were performed as described previously. Non-specific binding was determined in the presence of 10 μM unlabeled Ang II. The integrity of the radioligand before and after incubation was determined by TLC. As controls showed, no notable degradation of the radioligand occurred.

The equilibrium dissociation constant (Kᵦ) and concentration of receptor sites (B₂TOT) were calculated for the natural ligand Ang II. The binding data were analyzed as reported previously, with the use of the LIGAND iterative curve-fitting program. The concentration of the unlabeled ligands that induced 50% inhibition of the binding of the radioactive Ang II (IC₅₀) was estimated from the inhibition curves. The IC₅₀ values then were used to calculate the inhibition constants (Kᵦ) with the Cheng-Prusoff equation. The data were obtained from experiments performed at least twice with two independent isolates from each rat strain, and each experiment was carried out in duplicate.

**Materials**

The medium for cell culture was purchased at Fakola, Basel, Switzerland; bovine serum albumin, elastase, and collagenase were obtained from Sigma Chemical Co., St. Louis, Mo.; all other chemicals and ingredients for cell culture were purchased at Gibco, Basel, Switzerland. The radioisotopes [methyl-3H]thymidine (2.0 Ci/mmol, 1 mCi/ml) and [1,4-3H(N)]leucine (5.0 Ci/mmol, 1 mCi/ml) were obtained from New England Research Products, Zurich, Switzerland. [125I]–Ang II (2,200 Ci/mmol) was obtained from Anawa, Wangen, Switzerland. Ang II (Hypertensin) was obtained at CIBA-GEIGY, Basel, Switzerland, and Ang II (human sequence) for binding studies was obtained from Bachem, Bubendorf, Switzerland. The nonpeptidic Ang II antagonist selective for the AT₁ receptor subtype, DuP 753 (losartan), was synthesized in our laboratories. The peptidic ligand specific for the AT₂ receptor subtype, CGP 42112A, and PD 123177, a nonpeptidic Ang II ligand selective for the AT₁ receptor subtype, formerly known as EXP655, were synthesized in our laboratories.

**Statistics**

Differences in cell numbers and thymidine and leucine incorporation between more than two different treatment groups for cells derived from the same strain were tested by analysis of variance, and individual differences were assessed by Dunnett’s multiple comparison test. For these data, differences between the treatment groups or between the two rat strains were determined using the Student’s t test for unpaired samples. If not otherwise stated, data are expressed as the arithmetic mean±SEM; a value of p<0.05 was considered significant.

For the data obtained from the receptor ligand binding studies, differences between SHRs and WKY rats were assessed using the Wilcoxon-Mann-Whitney test. Data are expressed as median and range, and significance was taken at a value of p≤0.05.

**Results**

**Determination of DNA Synthesis, Protein Synthesis, and Cell Number in Quiescent Confluent Vascular Smooth Muscle Cells**

To study the effects of Ang II on VSMC hypertrophy in culture, we measured alterations in cell number and the incorporation of [3H]thymidine and [3H]leucine in quiescent confluent VSMCs derived from SHRs and WKY rats. Ang II caused a dose-dependent increase in both thymidine and leucine incorporation in cells derived from both rat strains. The effects on DNA synthesis were greater than on protein synthesis (Figure 1). For both responses, cells derived from SHRs were more responsive than cells derived from WKY rats at all three Ang II concentrations. No significant changes in cell...
Cell Number

Thymidine Incorporation

Leucine Incorporation

FIGURE 1. Bar graphs show dose-response effects of angiotensin II on cell number and [3H]thymidine and [3H]leucine incorporation in quiescent confluent vascular smooth muscle cells derived from spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. Experiments were performed under serum-free conditions. Values are mean±SEM of two separate experiments (in sextets) performed on different isolates for each strain. *p<0.05 compared with saline-treated cells within the same strain by Dunnett's multiple comparison test.

number were observed at the lower Ang II concentrations. A small but significant decrease in cell number was observed in VSMCs from SHRs at 10^{-7} M Ang II (Figure 1).

To determine the receptor subtype mediating the Ang II-induced VSMC hypertrophy, we tested the effects on thymidine incorporation of different concentrations of the Ang II receptor ligands DuP 753 or PD 123177 in combination with 10^{-7} M Ang II. Thymidine incorporation was increased to 183±6% in the presence of 10^{-7} M Ang II. The AT_1 receptor antagonist DuP 753 reduced thymidine incorporation in a dose-dependent manner (thymidine incorporation was 105±3%, 131±4%, and 154±6% of values for saline-treated cells in the presence of DuP 753 at 10^{-5}, 10^{-6}, and 10^{-7} M, respectively, in combination with 10^{-7} M Ang II). The AT_2 ligand PD 123177 had no effects up to the highest dose tested.

The AT_1 receptor antagonist DuP 753 at a dose of 10^{-5} M decreased not only the Ang II-stimulated thymidine incorporation but also leucine incorporation (Figure 2). The nonpeptidic AT_1 ligand PD 123177 had no effects even at the highest dose tested (Figure 2). DuP 753 and PD 123177 when given alone at the highest dose had no effects on thymidine or leucine incorporation (Figure 2).

The stability test with Ang II showed that, after an incubation time of 1 hour at 37°C in a 5% CO_2 atmosphere under serum-free conditions, a 76% degradation occurred. After 24 hours, only 1% of Ang II was detected.

Proliferation Studies

The influence of Ang II on the proliferation of VSMCs from SHRs was studied in the absence or presence of different concentrations of fetal calf serum. Cell number (initial number of cells at the zero point, approximately 12 hours after plating, was 31.4±0.5x10^4) had increased sixfold, 13-fold, and 17-fold at day 9 in the presence of either 1%, 5%, or 10% fetal calf serum, respectively. Ang II in concentrations up to 10^{-5} M had no influence on cell proliferation in the absence of serum. In medium containing 1% fetal calf serum, Ang II (10^{-7} M) induced a small but significant additional increase in cell number (1.1-fold, 1.1-fold, 1.2-fold, 1.4-fold, and 1.6-fold over saline-treated cells at days 1, 2, 5, 7, and 9, respectively) but had no significant effects in the presence of 5% or 10% fetal calf serum. Thus, the Ang II-induced increase in cell number in medium containing 1% fetal calf serum was much less than the proliferatory effect of 5% or 10% fetal calf serum.

We compared the proliferatory effect of Ang II in the presence of 1% fetal calf serum on cells from SHRs and WKY rats. The addition of Ang II induced a dose-dependent increase in cell number of VSMCs derived from SHRs that persisted over the 10 days of the experiment (Figure 3). The Ang II-induced increase in cell number was significantly different compared with the saline-treated cells on days 6, 8, and 10 for 10^{-7} M Ang II and on days 8 and 10 for 10^{-8} M Ang II. At day 10, the number of cells in wells exposed to 10^{-7} M Ang II was 188±9x10^4 cells per well, compared with 152±6x10^4 cells per well for saline-treated cells. In cells derived from WKY rats, only a small increase in cell number was observed that was not dose dependent (Figure 3). The Ang II-induced increase in cell number was statistically significant from day 3, but there was no difference between the three different doses of Ang II. SHR cells grew slightly faster than WKY cells. Cell number was the same at the zero point (SHR, 21.3±1.3x10^4 cells per well; WKY, 22.0±0.6x10^4 cells per well) but was greater (23%) in VSMCs from SHRs at day 10.

Finally, we studied the effects of DuP 753 given alone or in combination with Ang II on the proliferation of
VSMCs derived from SHRs. The Ang II-induced increase in cell number was significant (compared with the saline-treated cells) from day 3 on (Figure 4). DuP 753 at $10^{-5}$ M completely inhibited the Ang II-induced cell proliferation in the presence of 1% fetal calf serum (Figure 4). DuP 753 at $10^{-5}$ M when given alone under the same conditions had no significant effect on cell number (Figure 4).

Radioligand Receptor Binding Studies

Although there was considerable variation between different isolates, the $K_i$ values for Ang II, the natural ligand, tended to be lower in cells derived from SHRs ($p<0.05$) (median, 0.9 nM; range, 0.7–2.0 nM; number of separate experiments, six) than from WKY rats (median, 2.0 nM; range, 1.6–2.1 nM; number of separate experiments, six). The $B_{max}$ values were approximately threefold higher in VSMCs derived from SHRs ($p<0.05$) (median, 2,836 fmol/mg protein; range, 1,069–5,268 fmol/mg protein; number of separate experiments, six) than in VSMCs from WKY rats (median, 1,046 fmol/mg protein; range, 880–1,760 fmol/mg protein; number of separate experiments, five).

To characterize the receptor subtype or subtypes present in VSMCs from SHRs, we determined the binding properties of different highly selective Ang II receptor ligands. The following profile of relative affinities of the various ligands is characteristic for the AT$_1$ receptor subtype: Ang II $>$ DuP 753 $>$ CGP 42112A $>$ PD 123177. VSMCs derived from both SHRs and WKY rats showed this characteristic binding profile (Figure 5). The $K_i$ values measured for DuP 753 and CGP 42112A were consistently lower in VSMCs from SHRs than from WKY rats ($p<0.05$) (Table 1).

Discussion

Our studies were designed to compare the effects of Ang II on the growth of VSMCs derived from SHRs and WKY rats and to characterize the receptor subtypes mediating these effects. Our results show that Ang II can induce either a hypertrophic or a hyperplastic response in VSMCs derived from either rat strain, depending on the culture conditions. In confluent, quiescent cells deprived of serum, Ang II induced a dose-dependent increase in thymidine and leucine incorporation without increasing cell number. This is in agreement with other reports showing an effect of Ang II on either DNA or protein synthesis in cells derived from other normotensive rat strains. Because cell number did not increase, this indicates that Ang II induced hypertrophy and not hyperplasia. Cell number actually decreased slightly with the highest dose of...
Angiotensin II on Smooth Muscle Growth

FIGURE 3. Line graphs show comparison of the effects of angiotensin II on the proliferation of vascular smooth muscle cells derived from spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. Cells were plated \(2 \times 10^5\) cells per well into six-well multiwell plates in normal medium containing 10% fetal calf serum. When cell attachment was complete (approximately 12 hours after seeding), medium was exchanged for medium containing 1% fetal calf serum. This time was defined as the zero point. Angiotensin II or saline was added at the zero point and every 24 hours thereafter; medium was replaced routinely every 48 hours. Cell numbers were determined (in sextets) at times shown. Values represent mean±SEM calculated from separate experiments (SHR, \(n=3\); WKY, \(n=2\)) performed on different isolates.

Ang II. The reason for this is not known but may be due to loss of adhesion of some of the cells as the cell size increases and space becomes limited.\(^{20}\)

Our results have also shown that Ang II can induce a hyperplastic response in nonconfluent cells, but this is dependent on the presence of a low concentration of fetal calf serum. In medium without serum, no effects of Ang II on cell proliferation were observed. In the presence of 1% serum, Ang II stimulated cell proliferation. However, the increase in cell proliferation induced by Ang II was weak compared with the effects of 5% or 10% fetal calf serum alone, and, with these higher concentrations of fetal calf serum, no additional effects of Ang II could be observed. These findings indicate that Ang II requires factors present in serum to stimulate proliferation of VSMCs. This is in agreement with a recent observation that Ang II alone was not mitogenic but induced a proliferative response in the presence of epidermal growth factor or platelet-derived growth factor BB in VSMCs derived from SHRs.\(^{21}\) The dependence of the proliferative response on the presence of serum growth factors may explain the many conflicting reports concerning this effect of Ang II.

The maximum effects of Ang II on either VSMC hypertrophy or hyperplasia were observed with a dose of \(10^{-7}\) M, which is much higher than circulating concentrations of Ang II or the \(K_i\) of the receptor. However, the Ang II concentration required to mediate these effects was probably much lower. The Ang II degradation experiment showed a rapid and substantial degradation of Ang II in these high cell density cultures.

In our study we characterized the receptor subtypes mediating these effects of Ang II on VSMC growth. The nonpeptidic AT\(_1\) receptor antagonist DuP 753 blocked the effects of Ang II on DNA and protein synthesis in confluent, serum-deprived VSMCs from SHRs, whereas...
The radioligand binding studies confirmed that the AT1 receptor is responsible for the effects of Ang II observed in VSMCs from SHRs in culture. The AT1-selective ligand DuP 753 fully displaced 125I-Ang II from the receptors in concentrations at which it shows selectivity and affinity for this entity as described previously.17 On the other hand, the nonpeptidic AT2 receptor ligand PD 123177 showed almost no activity. In addition, CGP 42112A displaced the radioligand only at concentrations at which it loses its selectivity and acts at the AT1 receptor subtype.17 Hence, these binding properties demonstrate the presence of a pure population of AT1 receptors in aortic VSMCs from SHRs in culture. However, it cannot be ruled out that the AT2 receptor is present in vivo, as shown recently in rat aortic tissues by autoradiography23 or in human renal artery,17 but either is lost from VSMCs under the in vitro culture conditions or is present in another vascular cell type. Although there is some evidence to suggest that the AT2 receptor may be involved in growth,24 our results indicate that the AT1 receptor subtype mediates both the hypertrophic and hyperplastic effects of Ang II on VSMCs in culture. Because this receptor subtype also mediates constriction of VSMCs, Ang II may contribute to the development of hypertension via its hypertrophic as well as its vasoconstrictor effects. Ang II may also contribute to

**TABLE 1. Inhibition Constants of Various Synthetic Ligands for the Angiotensin II Receptor of Vascular Smooth Muscle Cells**

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<th>Ligand</th>
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<td>n</td>
<td>Median</td>
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<tr>
<td>DuP 753</td>
<td>4</td>
<td>30</td>
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<tr>
<td>CGP 42112A</td>
<td>4</td>
<td>932</td>
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<td>PD 123177</td>
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Each competition binding experiment was performed in duplicate with two different isolates of vascular smooth muscle cells from spontaneously hypertensive rats (SHR) or Wistar-Kyoto (WKY) rats (where n=number of separate experiments) and was individually analyzed. Values given are median and range. The inhibition constant (Ki) values for DuP 753 and CGP 42112A were significantly lower in cells derived from SHR than from WKY rats (p<0.05, Wilcoxon-Mann-Whitney test).
the hyperplastic response after vascular injury via effects on cell proliferation.\(^{12,25,26}\)

The response to Ang II of either VSMC hypertrophy in quiescent serum-deprived confluent VSMCs or VSMC proliferation in nonconfluent cells in the presence of serum was enhanced in SHR cells compared with WKY cells. These results are consistent with observations that VSMCs from SHRs show an increased responsiveness to various levels of fetal calf serum, to Ang II, and to several growth factors.\(^{13,27,28}\) and that they are less susceptible to the growth-inhibitory effects of heparin and transforming growth factor-\(\beta\).\(^{29}\) The increased responsiveness to Ang II of VSMCs derived from SHRs compared with cells from WKY rats does not appear to be due to a difference in the expression of the Ang II receptor subtypes. As in SHRs, only the AT\(_1\) receptor subtype could be detected in cultured cells derived from WKY rats. However, \(B^*_\max\) values tended to be higher in VSMCs from SHRs. Although data need to be obtained from many more isolates to confirm this, our findings are consistent with a previous report of a greater receptor density in VSMCs derived from SHRs than from WKY rats.\(^{2}\) In addition, we observed a lower \(K_d\) value for Ang II and a lower \(K_I\) value of the two synthetic ligands DuP 753 and CGP 42112A for the AT\(_1\) receptor. This indicates a higher affinity of these ligands for the AT\(_1\) receptor in VSMCs derived from SHRs than from WKY rats. These results may explain the greater sensitivity of VSMCs from SHRs to Ang II in vitro. Additionally, it may also explain why blockers of the renin-angiotensin system are effective antihypertensive agents in SHRs, although these rats do not have elevated plasma concentrations of renin and Ang II.\(^{3}\)

In summary, in this study we have shown that in rat VSMCs in culture Ang II can stimulate both hypertrophy without increasing cell number and, under different experimental conditions, cell proliferation. These effects are mediated by the AT\(_1\) receptor subtype, because they are completely blocked by a ligand selective for this receptor subtype. The hypertrophic and proliferative effects of Ang II observed on VSMCs support the role of Ang II mediating not only vasoconstriction but also influencing vascular growth. The responsiveness of VSMCs derived from SHRs compared with WKY rats was enhanced. Although no difference in the VSMC Ang II receptor profile was detectable between the two rat strains, cells derived from SHRs tended to have a higher affinity for the AT\(_1\) receptor ligands and a greater receptor density. These results may partly explain the greater hypertensive response to Ang II receptor blockade or converting enzyme inhibition in SHRs than in WKY rats, although both rat strains have similar plasma concentrations of Ang II.

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