Vascular Smooth Muscle Cells From the Milan Hypertensive Rat Exhibit Decreased Functional Angiotensin II Receptors

Lilian Socorro, Gino Vallega, Amelia Nunn, Thomas J. Moore, and Mitzy Canessa

The present study was designed to study the functional properties of Angiotensin II (Ang II) binding sites in vascular smooth muscle cells in the Milan hypertensive rat (MHS), a model of low renin hypertension. Smooth muscle cells from MHS rats exhibited increased growth in culture in comparison with the Milan normotensive strain (MNS) as determined by population doubling times (24.5±2 and 34.8±2 hours, n=4, respectively). Hormone receptor number, evaluated by binding assays using \([125I]Ang II\), showed no difference in either receptor number or affinity for both cell types. The functional responsiveness of Ang II receptors was evaluated by measuring the activation of phospholipase C, Na\(^{+}\)-H\(^{+}\) exchange, and cytosolic Ca\(^{2+}\) levels. Phospholipase C activity was determined as tritium-labeled inositol trisphosphate and bisphosphate release before and after 15-second exposure to 10\(^{-7}\) M Ang II. Ang II-stimulated phospholipase C activity in MNS (p<0.02) but not in MHS cells. Na\(^{+}\)-H\(^{+}\) exchange was measured as the dimethylamiloride-sensitive \(^{22}Na\) influx into acid-loaded vascular smooth muscle cells with and without 10\(^{-7}\) M Ang II. In MNS cells, Ang II significantly stimulated (p<0.001) antiporter activity but not in MHS cells, which showed a uniformly blunted response. MHS cells exhibited higher basal cytosolic Ca\(^{2+}\) levels than MNS cells, but Ca\(^{2+}\) rapidly increased in the presence of Ang II in MNS but not in MHS cells. Direct activation of phospholipase C by GTP-\(\gamma\)-S in permeabilized cells indicated that both strains exhibited similar coupling levels by guanine-nucleotide binding proteins. In summary, cultured smooth muscle cells from MHS rats exhibit blunted phospholipase C, Na\(^{+}\)-H\(^{+}\) exchange, and cytosolic Ca\(^{2+}\) responses to Ang II despite having the same number of Ang II receptors as MNS cells. This suggests that, in this model of low renin hypertension, Ang II receptor response is blunted during increased growth rates. (Hypertension 1990;15:591-599)

It has been determined that the hypertensive state is caused, in most cases, by an increased peripheral resistance that can be the consequence of either greater reactivity to vasoactive agents or hyperplasia of the modified vessel. Genetically selected hypertension-prone rat strains have been used to study these two alternatives. For example, the spontaneously hypertensive rats (SHR) have greater vasoreactivity than their normotensive Wistar-Kyoto (WKY) counterpart in isolated vessels studies. SHR also have a greater number of angiotensin II (Ang II) binding sites in mesenteric arteries and kidney and brush border membranes in the prehypertensive stage (4 weeks old) when compared with the age-matched normotensive WKY rats. That these differences are expressed before the rat becomes hypertensive suggests that the increased vasoreactivity might have a role in the actual development of the disorder.

Phospholipase C (PLC) activation has been shown to be an imperative step in the cascade pathway induced by occupied Ang II and other vasoconstrictor receptors. This enzyme catalyzes the hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP\(_2\)) into diacylglycerol (DAG) and the calcium-mobilizing agent inositol 1,4,5 trisphosphate (IP\(_3\)). The correlation between the calcium-mobilizing activity and vasoconstrictor potency is widely accepted for a number of vasoactive agents.

The Ang II–induced PLC activation has been well studied in Sprague-Dawley (SD) rat cultured vascu-
lar smooth muscle (VSM) cells.9,10 A biphasic DAG formation and a transient IP₃ peak at 15 seconds exposure were observed. A transient increase in cytosolic Ca²⁺ follows the IP₃ generation.11 Addition of the nonhydrolyzable guanosine-5'-O-(3-thiotriphosphate) (GTP-γ-S) analogue to saponin-permeabilized VSM monolayers also resulted in inositol polyphosphate release.10 These and synergism studies between GTP-γ-S and Ang II demonstrated that the Ang II–induced PLC activation is coupled to a GTP-binding protein.10 The occupied Ang II receptor also elicits activation of Na⁺/H⁺ exchange in cultured VSM cells from SD rats.12-14

Cultured VSM cells from SHR, when compared with WKY cells, exhibited hyperresponsiveness to Ang II receptors as determined by stimulation of Na⁺/H⁺ exchange and increased growth.13 Both findings are agreement with the in vivo increased vasoactivity of the SHR genetic strain.

Bianchi and Barlassina15 have selected another hypertensive rat, the Milan hypertensive strain (MHS), that shows renal functional abnormalities and low plasma renin levels when compared with its normotensive counterpart (MNS), suggesting a volume-expansion rather than a vasoconstriction form of hypertension. Kidney transplant from MHS into MNS gives rise to hypertension. These observations have suggested that a renal tubular defect plays an important role in the development of hypertension in this genetic model.16 Cultured VSM cells prepared from MHS exhibited higher growth rates than MNS cells associated with higher sodium-potassium-chloride cotransport activity.17 The present study was designed to study the functional properties of Ang II binding sites in VSM cells in the MHS, a model of low renin hypertension. Here, for the first time, the characterization of the Ang II receptors and their response by MNS and MHS cultured aortic VSM cells is reported. Our results indicate that MHS vascular smooth muscle cells exhibit increased growth and a similar number of Ang II binding sites compared with MNS, SHR, and WKY cells. However, in MHS cells, Ang II did not stimulate significantly PLC, Na⁺/H⁺ exchange activity, or cytosolic Ca²⁺ mobilization as observed in the normotensive strain. These data indicate that the Ang II receptor response is blunted in this model of low renin hypertension during increased growth rate.

Methods

Cell Culture

VSM cells were prepared from thoracic aortas of eight MNS (120.6±2.5 mm Hg) and eight MHS (196.6±1.3 mm Hg) rats (12 weeks old) and as described earlier.18 Cell monolayers (75 cm² flasks) were grown in Dulbecco’s minimum Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM glutamine, 100 µg/ml streptomycin, and 100 units/ml penicillin. Cells were harvested using trypsin-ethylenediaminetetraacetic acid (EDTA) (0.02% trypsin, 0.02% EDTA) and seeded at 3×10⁵ cells/dish twice a week. The dishes were observed microscopically to determine confluency. All experiments were performed at confluency between passages 3 and 12.

Angiotensin II Receptor Assay

Iodine-125–labeled Ang II binding to VSM cells was assayed after a modification of an earlier reported protocol.19 Briefly, VSM monolayers (96-well plates) prepared from both strains were incubated with tracer amounts of [¹²⁵I]Ang II (20 pM) and varying concentrations (2–4 wells at each concentration) of unlabeled ligand (10⁻¹⁰ to 10⁻⁶ M) for 90 minutes at 22°C (250 µl final volume). Unbound ligand was aspirated, the VSM monolayer was washed three times with cold saline, and the bound radioactivity was determined by separating the individual wells and placing them in a gamma counter. Binding data were analyzed and receptor affinity and number were calculated by LIGAND, a curve-fitting program designed for ligand binding analysis.20 In all cases, binding data were best characterized by a one-site model, indicating a single affinity state for the receptor.

In addition to assessing receptor binding after 90 minutes of equilibration, we also measured binding after 30 minutes in an experiment designed to estimate surface-bound versus internalized Ang II. Milan normotensive and hypertensive VSM cells in miniwells were incubated (as described above) with 4×10⁻¹¹ M [¹²⁵I]Ang II for 30 minutes. Half the wells were then washed three times with ice-cold saline and then counted to define “total binding.” An equal number of wells were incubated in an acid buffer (50 mM glycine, 150 mM NaCl, pH 3.5) for 5 minutes at 4°C. The buffer was aspirated, the cells were washed with cold saline solution, and the wells were counted to define acid-resistant or “internalized” ligand as previously described.21,22 “Cell surface binding” was calculated as “total binding” minus “internalized”; the data were expressed as percent of the [¹²⁵I]Ang II originally added and were normalized per 10⁵ cells for both strains. Nonspecific binding was determined for both control and acid-washed cells (defined as binding in the presence of 1×10⁻⁶ M Ang II) and was always less than 5% of total binding. Data from these experiments have been corrected for nonspecific binding.

Angiotensin II–Induced Phospholipase C Activity

The time course of Ang II action on inositol phosphate release was first studied by measuring the mass production of IP₃ and inositol bisphosphate (IP₂) as previously described.23 In these experiments, we confirmed previous observations that Ang II effect peaked at 15 seconds. In subsequent experiments, VSM monolayers were grown in 35 mm dishes and labeled by adding 30 µCi/ml [³H]myoinositol for 24–48 hours as previously described.9,10 Excess isotope was washed away with buffer containing 130 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 1.0 mM MgCl₂, 20
mM HEPES-Tris pH 7.4 (Tris-buffered saline [TBS]). After 10 minutes at 37°C, the buffer was aspirated and replaced with 1 ml fresh TBS containing Ang II (10⁻⁷ M). The reactions were terminated after 15 seconds with 400 μl 50% trichloroacetic acid (TCA). To isolate inositol phosphates, the TCA-soluble material was then extracted three times with 8 ml diethylether under nitrogen three times. The samples were resolved into inositol monophosphate (IP), IP₂, and IP₃ by the method of Downes and Michel. Briefly, the samples were neutralized to pH 7-7.5, made up 5 mM Na₂B₄O₇, 180 mM NH₄COOH (IP); 0.4 M NH₄COOH, 0.1 M HCOOH (IP₂), and 1 M HCOOH (IP₃). The inositol phosphate levels in each fraction were determined by liquid scintillation spectroscopy.

To study the coupling of guanine nucleotide (GTP)-binding proteins to PLC, tritium-labeled VSM cells were permeabilized with saponin, and phosphoinositol release determined in the absence and presence of a GTP analogue. In brief, [³H]myoinositol-labeled VSM monolayers (35 mm) were washed with TBS. The cells were then permeabilized by incubating them with saponin (30 μg/ml) in a cytosolic-like buffer (25 mM NaCl, 120 mM KCl, 1 mM MgCl₂), containing 5 μM adenosine triphosphate (ATP), 15 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.2, and 13 mM LiCl to inhibit the breakdown of IP. After 10 minutes at 37°C, the buffer was replaced with 1 ml fresh saponin buffer with or without 300 μM GTPγS. The reactions were terminated after 5 minutes with TCA, and the samples were treated as outlined above to isolate IP, IP₂, and IP₃.

Na⁺-H⁺ Exchange Activity
The dimethylamiloride (DMA)-sensitive ²²Na⁺ influx was measured into acid-loaded cells (pH 6.8) as previously described. VSM monolayers (35 mm) were washed three times with Na⁺-free (sodium replaced with choline) TBS, pH 7.0, at 37°C. The monolayers were then incubated for 25 minutes with the same buffer but also containing 10 mM glucose and 1 mg/ml bovine serum albumin (BSA). For an additional 5 minutes, ouabain (1 mM) and bumetanide (0.1 mM) were also added. The buffer was aspirated and Na⁺ influx was initiated by a 2-minute incubation in a solution containing (mM): NaCl 100, KCl 5, MgCl₂ 1, CaCl₂ 2.0, ouabain 1, bumetanide 0.1, 1 μCi/ml ²²Na, and HEPES-Tris 20, pH 7.4, with or without 20 μM DMA. Cell ²²Na counts per minute were determined in a gamma counter after six washes with chilled 0.1 M MgCl₂ solution. The monolayers were extracted with 0.1% sodium dodecyl sulfate (SDS), and aliquots were used for either protein determination or for radioactive counting in a gamma counter.

Cytosolic Ca²⁺ Levels
Ionized cytosolic Ca²⁺ was measured in VSM cells in suspension with the Ca²⁺-sensitive fluorescent dye fura-2 as described by Nabel et al. Fluorescence measurements were carried out in a Perkin-Elmer spectrophotometer (Perkin-Elmer Corp., Norwalk, Connecticut) equipped with a stirrer and temperature controlled at 37°C. The fluorescence intensity ratio was calculated from recordings at 340 and 380 nm (excitation) and 505 nm (emission) after subtracting the background fluorescence observed in the absence of fura-2 in the cells. Cytosolic Ca²⁺ was calculated as previously described.

Data Analysis
The calculated values from both strains were examined for statistical significance either with non-parametric χ² test (binding assays) or Student's t test using a Statgraphic PC computer program.

Reagents
DMEM culture media, FBS, EDTA, HEPES, Tris-base, and SDS were obtained from Sigma Chemical Co., St. Louis, Missouri. Glutamine, penicillin, and streptomycin were obtained from Gibco Laboratories, Chagrins Falls, Ohio. [³H]Myoinositol (17.1 Ci/mmol), ²²Na, and [³H]Ang II were obtained from New England Nuclear, Boston, Massachusetts. GTPγS was obtained from Boehringer Mannheim, Indianapolis, Indiana. Fura 2-AM was from Molecular Probes, Eugene, Oregon. Bumetanide was from Leo Laboratories, Vernouillet, France, and Ang II was obtained from Calbiochem, La Jolla, California. DMA was kindly provided by Dr. E. Cragoe from Merck Sharp & Dohme, New York.

Results
Milan Hypertensive Vascular Smooth Muscle Cells Exhibit Increased Growth Rate
To determine growth rates, VSM cells from MHS and MNS rats were first made quiescent by culturing them for 72 hours in DMEM with 0.3% FBS; the medium was then changed to DMEM with 10% FBS for 4 additional days, and the cell number determined after trypsinization. As previously reported, VSM of the hypertensive strain grew faster than the normotensive strain. The population doubling time of serum-grown VSM cells was 24.5±2.1 hours for MHS and 34.8±2.1 hours for MNS (n=4). VSM cells from MNS rats tended toward slow growth, enlarged size, and became polypliod. Total growth arrest was avoided by splitting the culture before it reached 50–60% confluency. Because of these differences in cell growth, it was difficult to obtain simultaneous data in exactly comparable passages, but the differences in passage number between strain at a given experiment were never more than two. Furthermore, to circumvent this problem, cells from both strains were characterized between consecutive passages 3 to 12. Because the interstrain differences persisted
through all passages, the average values of a given parameter are presented below.

**Vascular Smooth Muscle Cells From Milan Rats Have Similar Number of Angiotensin II Receptors**

To assess whether differences in Ang II binding capacity exist between cultured smooth muscle cells from MNS versus MHS, their surface density of Ang II receptors was measured. Binding data from five experiments are displayed in Table 1 and from a single experiment in Figure 1. Although MHS rats showed a slightly greater receptor number than MNS rats, there were no significant interstrain differences in either receptor number (expressed in fmol/mg protein) or affinity in five experiments (Table 1). In addition to relating Ang II binding capacity to cell protein, cell number was used to normalize the binding data to obtain the receptor density per cell that may reflect better the ratio of surface membrane between both cell types. Because of the different growth rates already reported, we determined the relation between protein content (r=mg protein/10^6 cells) and cell number (y=cell number/well x 10^3) in four experiments to estimate the number of Ang II binding sites per 10^6 cells. The linear regressions were y=-13.1+2.1x (r=0.9933) for MHS and y=-3.3+1.42x (r=0.9992) for MNS. The number of Ang II receptors in femtomoles per 10^6 cells still showed a trend, although not statistically significant, to higher values in MHS than in MNS cells (Table 1).

In our studies of VSM cell response to Ang II (described below) all parameters are measured after brief (seconds or minutes) exposure to Ang II. Therefore, in addition to assessing receptor binding after 90 minutes of equilibration, we also measured binding after 30 minutes in an experiment designed to estimate surface-bound versus internalized [125I]Ang II. After 30 minutes incubation with 4x10^-11 M Ang II, MHS cells again displayed more total binding than MNS cells (2.7 vs. 1.7% of added ligand per 10^6 cells), but the proportion of that total binding that was not displaced by acid-washing ("internalized") was similar in the two strains (MHS 53%, MNS 62%).

**Angiotensin II Does Not Stimulate Phospholipase C Activity in Smooth Muscle Cells From Milan Hypertensive Rats**

To determine the functional integrity of the Ang II receptors, the stimulation of the phosphatidylinositol (PI)-specific PLC activity was studied by measuring the release of IP_3, IP_2, and IP after incubation with Ang II in VSM cells of both strains as a function of time. In MNS cells, a rapid rise in IP_3 which reached a peak at 15 seconds, was observed (Table 2) as previously reported by Alexander et al; this was not the case in MHS cells, which did not show a significant increase in IP_3 at any time intervals. On the basis of these experiments, we further studied PLC activity after 15 seconds of incubation with Ang II. As shown in Figure 2, Ang II-activated IP release was not significantly different from basal values in both cell types. The release of both IP_3 and IP_2 in the presence of Ang II was significantly lower in MHS than MNS cells. Table 3 shows the average of six experiments showing that angiotensin-stimulated PLC activity (45% increase in IP_3 and 300% in IP_2 formation) is significant in MNS cells but not in MHS cells.
Angiotensin II Does Not Stimulate Na⁺-H⁺ Exchange Activity in Milan Hypertensive Rat Cells

Another parameter used to estimate the functional activity of occupied Ang II receptors is the capacity of this agonist to stimulate Na⁺-H⁺ exchange activity. We have previously shown that Ang II activates this transport system by modulating the Kₐ for external Na⁺ and increasing its turnover rate. The basal transporter activity was estimated as DMA-sensitive ²²Na⁺ influx into acid-loaded cells in the absence (basal) and in the presence of Ang II. Ang II-stimulated (Δ Ang II–basal) Na⁺ exchange was determined after a 2-minute incubation with 10⁻⁷ M Ang II and analyzed with paired t test (Table 4). MNS cells exhibited significant Ang II–stimulated Na⁺-H⁺ exchange activity (p<0.001), whereas the basal and stimulated levels in MHS cells were not different (5.55±1.43 vs. 1.78±1.91 nmol Na⁺/mg protein x min, MNS vs. MHS cells, mean±SEM). Furthermore, the differences between both strains become more striking when the ²²Na⁺ influx was evaluated per cell number in the dish (8.03±2.29 vs. 0.81±0.85 nmol Na⁺/10⁶ cells x min, MNS vs. MHS cells, Mean±SEM). Figure 3 depicts the frequency distribution of Ang II–stimulated Na⁺-H⁺ exchange. The MHS cells were uniformly less Ang II–responsive than MNS cells, which exhibit a more heterogeneous response.

Bypassing Angiotensin II Receptors Shows Integrity of Coupling Apparatus

In a previous study,¹⁰ we showed that activation of PLC by Ang II in vascular smooth muscle appeared to be mediated by a pertussis toxin–insensitive G protein. GTP-γ-S stimulated inositol phosphate formation and Ang II–induced inositol phosphate formation was potentiated by a submaximal concentration of GTP-γ-S. We therefore hypothesized that the reduced responses to Ang II in the hypertensive strain could be alternatively explained either by a defect distal to the receptor or by a more specific, Ang II receptor–G protein coupling defect. To discern between these alternatives, the Ang II receptor was bypassed studying the effect of GTP-γ-S on inositol phosphate formation. A direct and irreversible G protein activation was obtained by incubating the saponin-permeabilized, [³H]myoinositol-labeled cells with GTP-γ-S (300 μM). Compared with intact cells, the basal labeling of IP₃ was twofold higher in the permeabilized cells and 10-fold higher for IP₂. As shown in Table 5, measurements of GTP-γ-S–induced PLC activity in both MNS and MHS VSM cells gave comparable levels of this activity. This finding argues for a similar coupling system in both cell types.

Angiotensin II Does Not Mobilize Cytosolic Ca²⁺ in Milan Hypertensive Rat Cells

We also examined the effect of Ang II on cytosolic Ca²⁺ in VSM cells of both strains. Figure 4 summarizes measurements of the basal and the Ang II–

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**TABLE 2. Time Course of Inositol Trisphosphate and Inositol Bisphosphate Release By Normotensive and Hypertensive Milan Rat Vascular Smooth Muscle Cells on Incubation With Angiotensin II**

<table>
<thead>
<tr>
<th>Time (sec)</th>
<th>MNS IP₃</th>
<th>MNS IP₂</th>
<th>MHS IP₃</th>
<th>MHS IP₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>28</td>
<td>24</td>
<td>16.5</td>
<td>16.5</td>
</tr>
<tr>
<td>5</td>
<td>28</td>
<td>31</td>
<td>17.0</td>
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<tr>
<td>10</td>
<td>25</td>
<td>26</td>
<td>20.6</td>
<td>23.0</td>
</tr>
<tr>
<td>15</td>
<td>45</td>
<td>55</td>
<td>21.6</td>
<td>22.0</td>
</tr>
<tr>
<td>20</td>
<td>35</td>
<td>36</td>
<td>15.0</td>
<td>25.0</td>
</tr>
</tbody>
</table>

Values are in pmol/10⁶ cells·Ang II 10⁻⁷ M. IP₃, inositol trisphosphate; IP₂, inositol bisphosphate.

**TABLE 3. Angiotensin II–Induced Phospholipase C Activity in Normotensive and Hypertensive Milan Rat Vascular Smooth Muscle Cells**

<table>
<thead>
<tr>
<th>Rat strain</th>
<th>IP₃</th>
<th>IP₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNS</td>
<td>1.38±0.11</td>
<td>3.06±0.80</td>
</tr>
<tr>
<td>MHS</td>
<td>1.05±0.09*</td>
<td>1.45±0.35*</td>
</tr>
</tbody>
</table>

* p<0.02, significantly different from MNS.
TABLE 4. Angiotensin II–Induced Na\(^+\)-H\(^+\) Exchange in Normotensive and Hypertensive Aortic Vascular Smooth Muscle Cells

<table>
<thead>
<tr>
<th>Rat strain</th>
<th>Basal (nmol Na(^+)/mg protein/min)</th>
<th>Ang II (nmol Na(^+)/mg protein/min)</th>
<th>∆ Ang II-basal (nmol Na(^+)/10(^6) cell/min)</th>
<th>∆ Ang II-basal (nmol Na(^+)/10(^6) cell/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNS (n=21)</td>
<td>16.2 ± 2.1</td>
<td>25.8 ± 2.38</td>
<td>5.55 ± 1.43(^*)</td>
<td>8.03 ± 2.29(^*)</td>
</tr>
<tr>
<td>MHS (n=18)</td>
<td>23.8 ± 2.2</td>
<td>26.6 ± 2.52</td>
<td>1.78 ± 1.9(^*)</td>
<td>0.81 ± 0.85(^*)</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM. Angiotensin II (Ang II)–stimulated, dimethylamiloride-sensitive \(^{22}\)Na influx was measured in acid-loaded cells, as described in Figure 2 and Methods, by using 10\(^{-7}\) M Ang II and 100 mM extracellular Na\(^+\). MNS, Milan normotensive strain; MHS, Milan hypertensive strain.

\(^*\)p<0.01.
\(^\dagger\)p<0.01. $p$ values are for paired \(t\) test.

stimulated cytosolic Ca\(^{2+}\) levels of VSM cells from MHS and MNS cells. The resting cytosolic Ca\(^{2+}\) was higher in VSM cells from MHS than from MNS cells (\(p<0.02\)). As previously reported\(^{1,18,28}\), 10\(^{-8}\) M Ang II caused a transient increase in Ca\(^{2+}\) in VSM cells from MNS cells that reached a maximum value (about fourfold) within 1 minute and subsequently returned to basal values within 6–8 minutes. In contrast, in MHS cells Ang II produced only a sluggish increase in cytosolic Ca\(^{2+}\) that was not statistically significant.

Discussion

The present study was designed to characterize functional Ang II receptors in cultured VSM cells from the normotensive and hypertensive Milan rat strains, a genetic model of low renin hypertension. To assess whether differential characteristics between MHS and MNS rats were maintained in vascular cells, stimulation of PLC activity by the vasoconstrictor agonist Ang II was studied.

The data reported here show that there were no significant differences in the number and affinity of Ang II binding sites between both strains (Figure 1 and Table 1). As in a previous report\(^{17}\), we found that VSM cells from MHS rats grow faster (24.5 hours population doubling time) than those from MSN rats (34.8 hours population doubling time). The ratio of protein content to cell number was also different for both strains because cells from the hypertensive strain appear to decrease their cell size. For this reason, the number of Ang II binding sites were expressed two ways: as femtomoles Ang II bound per milligrams protein and per 10\(^6\) cells. By both criteria, there was a trend to greater number of Ang II

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**Figure 3.** Graph showing frequency distribution of angiotensin II (Ang II)–stimulated Na\(^+\)-H\(^+\) exchange in vascular smooth muscle (VSM) cells from Milan hypertensive (MHS) and normotensive (MNS) rats. VSM cells were acid loaded in Na\(^+\)-free Tris-buffered saline (TBS) and total Na\(^+\) influx (100 mM NaCl 1 μCi/ml \(^{22}\)Na) was measured for 2 minutes in presence and absence of 20 μM dimethylamiloride (DMA) and 10\(^{-7}\) M Ang II. DMA-sensitive Na\(^+\) influx was calculated from the difference between total Ang II–stimulated Na\(^+\) influx and DMA-insensitive influx.

**Figure 4.** Bar graph showing basal and angiotensin II (Ang II)–stimulated cytosolic Ca\(^{2+}\) levels in vascular smooth muscle (VSM) cells from Milan hypertensive and normotensive strain. Ang II–stimulated cytosolic Ca\(^{2+}\) was measured at the peak of the rising phase (±15 seconds). VSM cells were suspended in Hanks balanced salt solution and 10\(^{-8}\) M Ang II was added to the thermoregulated cuvette. Similar results were obtained with cells grown on glass slides.
Angiotensin II Receptors in Milan Vascular Cells

receptors in VSM cells from MHS rats than in those from MNS rats; however, the differences were not statistically significant because of the large dispersion of the data (Table 1). Because Ang II binding measurements were performed at equilibrium and suggested differences in receptor number, the bound ligand-receptor complex functionality was evaluated by measuring the Ang II-induced PLC and Na⁺-H⁺ exchange activities. VSM cells from MHS aorta had lower vasoconstrictor-induced PLC and Na⁺ exchange activity than those of the normotensive strain (Tables 2 and 3). The lower Ang II–stimulated activities in the MHS VSM cells were observed through all the studied passages (passages 4 through 9), and they do not seem to be due to a defect in the coupling apparatus between the Ang II receptor and the enzyme. GTP-γ-S–induced IP3 and IP2 release from saponin-permeabilized cells showed no difference between strains. Because it is not known what proportion of the different GTP-sensitive proteins activated is coupled to the Ang II receptor, it should be stated that our results do not necessarily imply integrity of the specific G proteins coupling Ang II receptors to PLC in MHS. Thus, under our experimental conditions, a genetically defective (or “uncoupled”) G protein subpopulation might not be detectable.

Binding assays using [125I]Ang II have shown a higher number of receptors on prehypertensive SHR mesenteric and renal tubular membranes than those from age-matched WKY rats (184 vs. 93 fmol/mg protein3 and 155 ± 5 vs. 108 ± 5 fmol/mg protein,4 mean±SD). This trend was also shown in basal PLC activity, which was higher in aortic wall membranes coming from prehypertensive SHR than in those from age-matched WKY rats.29 In hypertensive SHR, however, contradictory results have been reported. The difference in Ang II receptor number fades when mesenteric, renal tubular membranes,4 or cultured aortic VSM cells (139±68 vs. 217±138 SHR vs. WKY rats, mean±SD13) of both strains are compared. Basal and vasoconstrictor-induced PLC activity still showed higher values in the SHR aortic membranes,29 platelets,30 and aortic VSM cells31 when contrasted with those of WKY rats. Freshly prepared material3,4,29,30 and cultured cells10,28 from SHR exhibit high PLC activities, whereas no differences in Ang II binding sites were observed in comparison with the WKY cells.13 GTP binding proteins have not yet been studied on cells prepared from SHR and WKY strains; therefore, no comparison can be stated about the efficiency of their coupling apparatus.

In the case of VSM cells from MHS and MNS rats, the present study shows that there is a pronounced disagreement between functional assays and equilibrium measurements of the number of Ang II receptors. Equilibrium binding assays require relatively long incubations and, when done in intact cells, do not differentiate between cell receptor binding and internalization. Experiments conducted to differentiate between these two processes appear to indicate that at 30 minutes no significant differences in internalization could be observed. Thus, because binding studies do not discern between functional and non-functional receptors, testing of Ang II–induced responses allows an assessment of the functional receptors. In VSM cells from MHS compared with MNS rats, PLC, cytosolic Ca2⁺, and Na⁺-H⁺ exchange exhibited reduced responsiveness to Ang II despite their similar number of binding sites. This led us to conclude that the Ang II receptor response is desensitized in the VSM of the Milan hypertensive strain (Table 6).

An important finding is that serum-grown MHS cells exhibited a higher basal cytosolic Ca2⁺ level than MNS cells but no differences in basal Na⁺-H⁺ exchange activity (Table 4) as previously reported in SHR cells.13 Thus, elevation of cytosolic Ca2⁺ is not strongly associated with activation of this antiporter in cultured VSM cells from this hypertensive strain. However, VSM cells from MHS rats have elevated activity of sodium-potassium-chloride cotransport13 as previously reported in red blood cells and kidney proximal tubular cells.13 In contrast, studies performed in VSM cells from SHR and WKY rat strains (Table 6) indicate that both PLC29 and Na⁺-H⁺ antiporter13 activities stim-

### Table 5. GTP-γ-S–Induced Phospholipase C Activity in Permeabilized Vascular Smooth Cells of Normotensive and Hypertensive Milan Rats

<table>
<thead>
<tr>
<th>Rat strain</th>
<th>Increase above control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IP3</td>
</tr>
<tr>
<td>MNS</td>
<td>1.38±0.21</td>
</tr>
<tr>
<td>MHS</td>
<td>1.33±0.23</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM, n=4. [H]Myoinositol-labeled vascular smooth muscle monolayers were washed and permeabilized with saponin (30 μg/ml) in a cytosolic-like buffer. Cells were then exposed to 300 μM GTP-γ-S for 5 minutes. Trichloroacetic acid–soluble material was then resolved in inositol monophosphate (IP), inositol bisphosphate (IP2), and inositol trisphosphate (IP3). Values are calculated as ratios of counts per minute in stimulated samples over counts per minute in controls. MNS, Milan normotensive strain; MHS, Milan hypertensive strain.

### Table 6. Cultured Vascular Smooth Muscle Cells From Genetic Strains of Hypertensive Rats

<table>
<thead>
<tr>
<th></th>
<th>SHR</th>
<th>MHS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth rate</td>
<td>faster</td>
<td>faster</td>
</tr>
<tr>
<td>Na-H exchange</td>
<td>increased</td>
<td>same</td>
</tr>
<tr>
<td>Cytosolic Ca²⁺</td>
<td>increased</td>
<td>increased</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Binding sites</td>
<td>same</td>
<td>same</td>
</tr>
<tr>
<td>Stimulation of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospholipase C</td>
<td>higher</td>
<td>lower</td>
</tr>
<tr>
<td>Na-H exchange</td>
<td>higher</td>
<td>lower</td>
</tr>
<tr>
<td>Cytosolic Ca²⁺</td>
<td>increased</td>
<td>NS</td>
</tr>
</tbody>
</table>

In comparison with normotensive strains. SHR, spontaneously hypertensive rats; MHS, Milan hypertensive strain.
ultated by Ang II exhibit enhanced responsiveness in the hypertensive strain. Because the number of Ang II receptors was not found to be different in cultured VSM cells from SHR and WKY rats, it can be suggested that, in this genetic model of hypertension, the functional responsiveness of the Ang II receptor is increased in the hypertensive strain. Thus, in the SHR model, increased growth is associated with higher cytosolic Ca\textsuperscript{2+}, Ang II-stimulated PLC activity and Na\textsuperscript{+}-H\textsuperscript{+} exchange activities compatible with the increased vasoreactivity observed in this genetic model of hypertension. In the case of the Milan rats, the faster growth of the hypertensive strain is associated with hyporesponsive Ang II receptors. In both genetic models of hypertension, functional Ang II receptors appear to be differentially regulated by cell growth. The Milan hypertensive strain develops a mild low renin type of hypertension early in life that does not progress with age and does not show increased vasoreactivity. We hypothesize that in VSM cells from MHS rats, the Ang II receptors are most likely desensitized during increased growth. In contrast, in VSM cells from the SHR strain, which exhibit increased vasoreactivity, the responsiveness of the Ang II was increased during increased growth. Basal and Ang II-stimulated levels of Na\textsuperscript{+}-H\textsuperscript{+} exchange were higher in SHR than in WKY cells.

In summary, the present study demonstrates that, in the Milan hypertensive strain, a genetic model of low renin hypertension, VSM cells exhibit increased growth but a decreased functional response to a vasoconstrictor such as Ang II.

Acknowledgment

We are grateful to Drs. Giuseppe Bianchi and Patricia Ferrari for kindly providing us with the Milan rat strains.

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**KEY WORDS** • angiotensin receptors • sodium-hydrogen exchange • vascular smooth muscle • renal hypertension • phospholipases • Milan rats
Vascular smooth muscle cells from the Milan hypertensive rat exhibit decreased functional angiotensin II receptors.

L Socorro, G Vallega, A Nunn, T J Moore and M Canessa

Hypertension. 1990;15:591-599
doi: 10.1161/01.HYP.15.6.591

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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