Alterations in Prostaglandin Production in Spontaneously Hypertensive Rat Smooth Muscle Cells

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We have characterized angiotensin binding sites in cultured smooth muscle cells obtained from the aorta of spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) rats. In both strains of rats the binding of $^{125}$I-angiotensin II ($^{125}$I-Ang II) in smooth muscle cells was time dependent and reached a maximum at 60 minutes. Scatchard analysis revealed a single binding site in both strains with equilibrium constants ($K_d$) of 5.35 nmol/L in SHR and 3.47 nmol/L in WKY rats. Binding capacities ($B_{max}$) in smooth muscle cells averaged 270 and 150 fmol/mg protein in SHR and WKY rats, respectively. Angiotensin peptides competed for $^{125}$I-Ang II binding with an order of potency of Ang II>angiotensin-(1–7)=angiotensin I. In smooth muscle cells of the SHR, basal prostaglandin E$_2$ (PGE$_2$) and prostacyclin (prostaglandin I$_2$ [PGI$_2$]) release were threefold and 15-fold lower than that found in WKY rat smooth muscle cells. Ang II as well as angiotensin-(1–7) stimulated PGE$_2$ and PGI$_2$ release in WKY rat smooth muscle cells. In smooth muscle cells from SHR, Ang II increased the production of both PGE$_2$ and PGI$_2$, whereas angiotensin-(1–7) enhanced only PGE$_2$ but not PGI$_2$ release. There was no significant difference between Ang II-stimulated PGE$_2$ and PGI$_2$ release or angiotensin-(1–7)-stimulated PGE$_2$ production in SHR and WKY rat smooth muscle cells. However, angiotensin-(1–7)-stimulated PGI$_2$ release was significantly lower ($p<0.0005$) in SHR compared with WKY smooth muscle cells. Collectively, the data suggest that smooth muscle cells of SHR contain a higher number of angiotensin binding sites. Basal levels of PGE$_2$ and PGI$_2$ were significantly reduced in smooth muscle cells obtained from hypertensive animals. Furthermore, the release of the potent vasodilator PGI$_2$ in response to angiotensin-(1–7) was markedly attenuated in the SHR. (Hypertension 1993;21:900–905)

KEY WORDS • receptors, angiotensin • muscle, smooth, vascular • prostaglandins • hormones

Angiotensin II (Ang II) plays an important role in the regulation of blood pressure and fluid balance. In several experimental models of hypertension, there is increased vascular responsiveness to Ang II.$^1,2$ Since Ang II binds to specific receptors on the surface of vascular smooth muscle cells (VSMC) to initiate diverse physiological responses,$^3$ differences in the actions of Ang II in blood vessels may result from the presence of different types of receptors (Ang II subtype 1 [AT$_1$] versus subtype 2 [AT$_2$]). In addition, vascular receptors may recognize specific angiotensin peptides. In keeping with this interpretation, we showed that the N-terminal heptapeptide angiotensin-(1–7) [Ang-(1–7)] mimics some of the actions of Ang II.$^4$ Production of prostaglandins is augmented by Ang-(1–7) in C6 glioma,$^5$ astrocytes,$^6$ endothelial cells,$^7$ and vas deferens.$^8$ More recently, we showed that Ang-(1–7) causes a vasorelaxant response in the areflexic rat that is prevented by cyclooxygenase inhibitors.$^9$ Vascular tissue has a large capacity to generate vasodilator prostaglandins. Prostacyclin (prostaglandin I$_2$ [PGI$_2$]) is a potent vasodilator, stimulates natriuresis, and is a potent inhibitor of platelet aggregation.$^{10,11}$ Moreover, PGI$_2$ attenuates vasoconstrictor responses to vasoactive stimuli such as Ang II.$^{10}$ In turn, Ang II augments the release of prostaglandin from a variety of organs.$^{12,13}$

In these studies, we determined whether differences exist in the binding properties of angiotensin receptors in VSMC of spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. Because data suggest that the evolution of hypertension is associated with abnormal arachidonic acid metabolism,$^{14}$ we also compared prostaglandin production in SHR and WKY VSMC in both the absence and presence of Ang II and Ang-(1–7).

**Methods**

**Cell Culture**

Smooth muscle cells were obtained from the aorta of 14-week-old male SHR and WKY rats using published procedures.$^{15}$ Briefly, 2-cm² segments of the aorta were cut and minced into 1-mm² pieces after the removal of endothelium, adventitia, and outer third of the medial layer. The pieces were incubated in Dulbecco's modified Eagle medium (DMEM)/F12 (1:1) (GIBCO, Grand Island, N.Y.) with 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37°C in 5% CO$_2$ and 95% air. VSMC began migrating from explants as early as 5 to 10 days. The pieces of tissue were removed after appearance of a halo (approximately 10 cells deep) around the explant. For subcultures, cells were dissociated by treating with 0.05% trypsin-EDTA and, after washing, were plated on collagen-coated culture dishes.
ated with trypsin (0.01% trypsin, 0.02% EDTA in saline), seeded in 24-well tissue culture plates, and grown to confluency. Cells were used between passage 2 to 5. VSMC were distinguished from endothelial cells by routinely examining the cultures by phase contrast microscopy. On initiation of cultures, cells were tested for their characteristic morphology as well as their positive reactivities with antibodies to VSMC specific α-actin.

Measurement of 125I-Angiotensin II Binding
Specific binding of 125I-Ang II to VSMC angiotensin receptors was measured in phosphate-buffered saline at 4°C using published procedures.16 For Scatchard analysis, cells were incubated with increasing concentrations of 125I-Ang II (0.05 nmol/L–2.0 nmol/L) for 60 minutes in the absence and presence of 10 μmol/L Ang II. For competition studies, cells were incubated with 0.2 nmol/L 125I-Ang II in the presence of increasing concentrations of angiotensin peptides (from 10^{-10} to 10^{-5} mol/L). The equilibrium constant (K_D) and maximal number of binding sites (B_max) were calculated using the computer program EBDA/LIGAND (Equilibrium Binding Data Analysis; Elsevier-BIOSOFT).

Prostaglandin Measurements
Confluent cell cultures were washed two times with Hanks balanced salt solution (HBSS) and incubated with Ang II or Ang-(1-7) (range, 10^{-10} to 10^{-5} mol/L) in HBSS at 37°C for 15 minutes. At the end of incubation, the medium was removed to measure prostaglandin E2 (PGE2) and 6-ketoprostaglandin F1α (6-keto-PGF1α) (a stable metabolite of PGI2) by radioimmunoassay as described previously.17

Analysis of Data
Results are expressed as mean±SEM. The increase in prostaglandin synthesis induced by various agents is expressed as percentage increase above basal value to control for variations in unstimulated prostaglandin levels in cells from different passages. Actual release data (basal versus stimulated) were analyzed by analysis of variance followed by Student's t test corrected for multiple comparisons by the Bonferroni method.18 Comparisons of percentage increase between the two peptides were by unpaired nonparametric analyses. STATVIEW+GRAPHICS was used for these analyses.

Materials
Prostaglandins were purchased from Sigma Chemical Co., St. Louis, Mo. [3H]PGE2 and [3H]6-keto-PGF1α were purchased from DuPont-New England Nuclear, Boston, Mass. DMEM, FBS, F12, penicillin, and streptomycin were obtained from GIBCO. Angiotensin peptides were provided by Dr. Mahesh C. Khosla of The Cleveland Clinic Foundation, Cleveland, Ohio.

Results
Angiotensin II Binding
Binding of 125I-Ang II to angiotensin receptors in SHR and WKY VSMC was time dependent and reached a plateau at 60 minutes. Saturation binding assays over the 125I-Ang II concentration range of 0.05–2.0 nmol/L showed that SHR VSMC had a significantly greater capacity for binding Ang II when compared with

![Figure 1](http://hyper.ahajournals.org/)

**FIGURE 1.** Plots show saturation binding of 125I-angiotensin II (Ang II) in spontaneously hypertensive rat (SHR) (upper panel) and Wistar-Kyoto (WKY) rat vascular smooth muscle cells (VSMC) (lower panel). Scatchard analysis of 125I-Ang II binding is given in the inset. Cells were incubated with increasing concentrations of 125I-Ang II for 60 minutes at 4°C. The equilibrium constant (K_D) and maximal number of binding sites (B_max) were calculated as described in "Methods." Each point is the mean of at least three experiments obtained with cells from passages 2–5.
The ability of SHR and WKY VSMC to generate prostaglandins is shown in Table 1. We consistently found that basal PGE\textsubscript{2} and PGI\textsubscript{2} release were lower in SHR VSMC compared with WKY VSMC. PGI\textsubscript{2} production was about 15-fold lower in SHR VSMC compared with their normal control WKY VSMC ($p<0.0005$). Similarly, basal PGE\textsubscript{2} was also significantly lower (about threefold) in the SHR VSMC ($p<0.0005$).

Figure 3 shows the Ang II– and Ang-(1-7)–induced prostaglandin production in SHR and WKY VSMC. Ang II and Ang-(1-7) produced a dose-dependent stimulation of PGE\textsubscript{2} release in WKY and SHR VSMC (Figure 3, top panel). Ang II (10$^{-7}$ mol/L) produced a maximal response of 96±13% and 61±10% above baseline for PGE\textsubscript{2} release in WKY and SHR VSMC, respectively. Incubation with higher Ang II concentrations (10$^{-6}$ to 10$^{-5}$ mol/L) did not further stimulate PGE\textsubscript{2} release. In fact, higher concentrations appeared to be less effective in both groups. The difference between the SHR and WKY groups did not reach statistical significance (Figure 3, top left panel). Ang-(1-7) maximally stimulated PGE\textsubscript{2} formation at 10$^{-7}$ mol/L in both cell lines. At higher doses, prostaglandin stimulation tended to fall in both groups. No significant difference in PGE\textsubscript{2} production was observed in response to Ang-(1-7) in the two groups except at a concentration of 10$^{-5}$ mol/L (68±11% above basal in WKY versus 4±10% above basal in SHR; $n=6$; $p<0.0005$) (Figure 3, top right panel). In both SHR and WKY VSMC, Ang II–stimulated PGI\textsubscript{2} generation was similar with maximal stimulation at 10$^{-4}$ mol/L (Figure 3, lower left panel). In contrast, Ang-(1-7)–stimulated PGI\textsubscript{2} generation in SHR VSMC was significantly lower when compared with the WKY VSMC at concentrations above 10$^{-8}$ mol/L (Figure 3, lower right panel). PGI\textsubscript{2} release in response to Ang-(1-7) in SHR VSMC was not significantly different than basal levels.

**Discussion**

Several studies demonstrated an increased responsiveness to Ang II in vascular tissue in SHR.\textsuperscript{1,2,19} One mechanism whereby cell sensitivity to angiotensins may increase is by an elevation in the number of receptors. Our results demonstrate an increased number of angiotensin binding sites in VSMC obtained from aorta of SHR versus WKY rats. These results are in agreement with findings in mesenteric vasculature,\textsuperscript{20} kidney,\textsuperscript{21,22} neuronal cultures,\textsuperscript{23} cerebral microvessels,\textsuperscript{24} and brain nuclei.\textsuperscript{25} In addition, the number of Ang II binding sites in proximal tubular brush border membranes was higher in SHR versus WKY rats at 4 weeks of age.\textsuperscript{26} Moreover, an increase in a\textsubscript{2A}-adrenergic receptor binding sites has been shown in SHR\textsuperscript{26} suggesting that differences in the number of binding sites is not unique to Ang II.
et al. have also reported an increase in responsiveness to both norepinephrine and Ang II in blood vessels obtained from the kidney of SHR.

There was no difference between SHR and WKY VSMC for displacement of radiolabeled Ang II by angiotensin peptides. Similar to the present study, previous observations have also reported that Ang-(1-7) is a poor competitor for Ang II binding sites in other cell types. The decreased ability of Ang-(1-7) to displace radiolabeled Ang II may reflect a relatively low abundance of AT1 receptors in these cells. Past studies from this laboratory have shown that Ang-(1-7) has a greater affinity for AT3 receptors. However, since we have also seen that the actions of Ang-(1-7) were not totally blocked by either AT1 or AT3 antagonists in C6 glioma cells, competition binding studies with Ang II may not predict the total functional capacity of this peptide.

It has been suggested that abnormal metabolism of the arachidonic acid-cyclooxygenase system is involved in the development of genetic hypertension, either directly (by altering the balance between vasodilator and vasoconstrictor prostanoids) or through an interaction with the renin-angiotensin system. This hypothesis gained further support from the observation that cyclooxygenase inhibitors increase blood pressure in hypertensive patients, especially those receiving some form of antihypertensive treatment. Prostaglandins participate in the regulation of blood pressure by exerting local actions within the kidney and in blood vessels. In the current experiments, we observed that an increased number of angiotensin receptors in VSMC of SHR are associated with reduced levels of PGI2 production. In patients with mild essential hypertension, biosynthesis of PGI2 is selectively impaired. This decrease in PGI2 may be due to a reduction in PGI2 synthetase or an enhancement in thromboxane synthetase activity. An enhancement in thromboxane A2 (TXA2) production in SHR (in prehypertensive as well as in an established hypertensive stage) and in Dahl salt-sensitive rats has been reported. In addition, thromboxane synthetase inhibitors attenuate the development of hypertension and stimulate vasodilator prostaglandin production. Moreover, studies in SHR showed an exaggerated renal vascular response induced by Ang II before but not after indomethacin administration, suggesting a deficiency in endogenous vasodilator prostanoids, an exaggerated Ang II-induced production of TXA2, or both. Exogenous vasodepressor prostaglandins slow VSMC growth, whereas vasoconstrictor thromboxanes participate to some extent in rapid VSMC growth in SHR.

Therefore, an increase in vasoconstrictor thromboxane synthesis and a decrease in vasodilator PGI2 production may stimulate a proliferative response in VSMC and increase arterial wall thickness.

Conflicting data have been reported concerning basal prostaglandin levels in genetic rat models of spontaneous hypertension. Release of prostaglandins in SHR has been reported to be similar, increased, or decreased when compared with WKY controls. The increase in vasodepressor prostaglandins in SHR has been interpreted as an adaptive phenomenon that counterbalances the elevation of blood pressure. In addition, increased arterial pressure is reported to be a potential activator of PGI2 synthetase. It has been demonstrated that the biosynthesis of PGI2 was increased only in rats with the established phase of hypertension, whereas in the prehypertensive stage, vascular PGI2 generation was reduced when compared with control rats. Thus, different basal prostaglandin levels may occur during different stages of hypertension.

In the present study, we showed that Ang-(1-7) stimulated PGE2 and PGI2 production in WKY VSMC and PGE2 production in SHR VSMC. It has been reported previously that Ang-(1-7) has no biological activity. However, we have shown that Ang-(1-7) is one of the principal products of angiotensin metabolism in the central nervous system and in the periphery.
Recent studies in cultured human astrocytoma cells,\textsuperscript{6} C6 glioma cells,\textsuperscript{6} and cultures of porcine aortic endothelial cells and vascular smooth muscle cells\textsuperscript{2,23} further demonstrated that Ang-(1-7) is equipotent or more potent than Ang I or Ang II in stimulating PGF\textsubscript{2}\alpha and PGI\textsubscript{2} release. These studies in cell cultures as well as in the pithed rat clearly indicate that Ang-(1-7) is an active hormone of the renin-angiotensin system possessing myotonic actions that may in part be related to the release of vasodilator prostaglandins. In the present study we found that basal as well as Ang-(1-7)-stimulated release of vasodepressor PGI\textsubscript{2} were significantly impaired in SHR VSMC. The explanation for impairment in Ang-(1-7)-stimulated PGI\textsubscript{2} but not PGE\textsubscript{2} is not known. However, previous reports have indicated a difference in Ang II-stimulated PGE\textsubscript{2} versus PGI\textsubscript{2} production. For example, prolonged Ang II infusion significantly enhanced PGI\textsubscript{2} but not PGE\textsubscript{2} generation as measured in plasma, urine, or renal cortical slices.\textsuperscript{33,44} The mechanism for the decreased effect of Ang-(1-7) but not of Ang II on PGI\textsubscript{2} production in SHR VSMC remains to be elucidated. Independent stimulation of PGE\textsubscript{2} versus PGI\textsubscript{2} by angiotensin peptides is potentially physiologically significant; for example, PGI\textsubscript{2} but not PGE\textsubscript{2} inhibited DNA synthesis in VSMC.\textsuperscript{45}

In conclusion, we found that angiotensin receptors in vascular VSMC from SHR aorta are increased in number as compared with WKY controls. Furthermore, SHR VSMC exhibited decreased basal production of vasodepressor prostanoids. Finally, we found attenuated production of PGI\textsubscript{2} in response to Ang-(1-7) in SHR VSMC. We suggest that the effects of increased numbers of angiotensin receptors and reduced basal and Ang-(1-7)-stimulated production of PGI\textsubscript{2} are part of a mechanism that may promote increased vascular contractility and reduced vasodilator capacity in SHR.

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References


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