Angiotensin II Induces Vascular Smooth Muscle Cell Replication Independent of Blood Pressure

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Abstract—The purpose of this investigation was to evaluate the role of blood pressure in the proliferative response of vascular smooth muscle cells to systemic infusion of angiotensin II (Ang II). Our laboratory has previously shown that infusion of Ang II induces smooth muscle cell proliferation in rat mesenteric vessels and carotid arteries. Ang II, a strong vasopressor, raised systolic blood pressure in rats from 120 to 200 mm Hg at a dose of 435 ng kg\(^{-1}\) min\(^{-1}\) after 1 week of treatment. The question arises as to whether this development of hypertension is a primary contributor to the replicative activities observed in the arterial wall of the mesenteric arteries or the carotid arteries or whether Ang II alone, without an increase in blood pressure, is sufficient to stimulate proliferation in these vessels. In the previous studies, we found that Ang II stimulated smooth muscle cell replication in the carotid artery and in type III and type I mesenteric microvessels. This study demonstrates that although administration of hydralazine normalizes the animals' blood pressures, it does not suppress the mitogenic effect of Ang II. Thus, it appears that Ang II has a direct effect on cell proliferation. (Hypertension. 1998;31:1331-1337.)

Key Words: angiotensin II • hyperplasia • hypertrophy • muscle, smooth • mesenteric arteries • proliferation

Structural changes of resistance vessels play a major role in regulating blood pressure.\(^1\)-\(^3\) Because thickened walls of resistance vessels may act as a vascular "amplifier" to raise blood pressure,\(^4\) factors that regulate the growth of SMCs may contribute to the development of hypertension by inducing thickening of the resistance vessels. Ang II, a potent vasoconstrictor, is able to stimulate SMC growth in the vascular wall when it is administered in vivo.\(^5\)-\(^7\) When Ang II is given in vitro, it increases either the size\(^8\)-\(^9\) or the number\(^10\)-\(^11\) of SMCs. When Ang II was given in vivo, it caused vascular hypertrophy in resistance vessels of the mesentery\(^12\) and induced multiple forms of hyperproliferative responses in the mesenteric vessels.\(^3\) These results were complicated by the fact that Ang II also raised SBP. The key question is whether these hyperproliferative responses are due to the hemodynamic effect or to a direct action of Ang II.

The purpose of this study was to investigate the relationship between the mitogenic and hypertensive effects of Ang II. With cotreatment by hydralazine, the hypertensive effect of Ang II can be neutralized, thus allowing us to study the proliferative effect of Ang II without the interference of blood pressure. We found that Ang II stimulated SMC replication in the mesenteric arteries within 1 week of treatment and that this proliferative effect was not abolished by hydralazine treatment. This result demonstrates that the mitogenic activities of Ang II are not dependent on its pressor effect. Our results provide direct evidence for the first time that Ang II stimulates SMC replication in the mesenteric microvessels even without the elevation of blood pressure.

Methods

Experimental Protocol

Three-month-old male Sprague-Dawley rats (\(\sim\)450 g; Zivic Miller, Allison, Pa) were used for these studies. All animals were allowed standard rat chow and water ad libitum. On day 0, animals were anesthetized with ketamine HCl (50 mg/kg body wt), xylazine (5 mg/kg body wt), and acepromazine (1 mg/kg body wt) administered intramuscularly. Osmotic pumps (Alza Corp) were surgically implanted subcutaneously in the backs of the rats. The animals were randomly assigned to one of four groups. (1) Animals in the Ang II–treated group received a pump (model 2001) filled with Ang II (Sigma Chemical Co) and delivered at a dosage of 435 ng kg\(^{-1}\) min\(^{-1}\) in Ringer's and a second pump (model 2001) filled with BrdU diluted with Ringer's at 30 mg/mL. (2) Animals in the Ang II and hydralazine treatment group received three pumps. In addition to the Ang II and BrdU pumps, they also received a hydralazine pump (model 2ML1, 26 mg kg\(^{-1}\) d\(^{-1}\)). (3) In animals treated with Ringer's, a pump filled with Ringer's was implanted along with a BrdU pump. (4) In animals treated with Ringer's plus hydralazine, a hydralazine pump (model 2ML1, 26 mg kg\(^{-1}\) d\(^{-1}\)) and a BrdU pump were implanted. Animals recovered from anesthesia in \(\sim\)2 hours. Systolic blood pressure was measured on days 0, 3, 4, 5, and 6 to demonstrate the elevation of blood pressure due to Ang II infusion. The Animal Care Committee of the University of Washington approved all procedures.

One week after pump implantation, animals were anesthetized as described above and euthanized with an intravenous injection of pentobarbital. Two rings of the carotid artery were taken from each animal and immersion fixed in either 4% paraformaldehyde or methyl Carnoy's fixative. Rings were routinely processed and paraffin embedded. The mesenteric bed including the gut was cut
Selected Abbreviations and Acronyms
- Ang II = angiotensin II
- bFGF = basic fibroblast growth factor
- BrdU = bromodeoxyuridine
- Ringer’s = Ringer’s lactate
- SBP = systolic blood pressure
- SMC = smooth muscle cell
- TGF = transforming growth factor

Mesenteric Artery Dissection and Preparation
A random loop of small intestine was cut out and cleaned while being viewed under a dissection microscope to retrieve microvessel types I, II, and III as described elsewhere and in Figure 2. A minimum of 3 type III and 8 to 10 type I vessels from each animal were routinely processed, embedded in paraffin, and examined.

Histochemistry and Morphometry
Five-micron-thick paraffin sections of carotid arteries were cut. Three sections at least 50 μm apart were stained with anti-BrdU by utilizing a specific monoclonal antibody (a kind gift of M. Daemen) and standard ABC detection as described elsewhere. Positively stained cells were visualized with 3,3’-diaminobenzidine (Sigma), and slides were counterstained with hematoxylin. The number of BrdU-stained cells and total cells (estimated by counting nuclei) was counted per vessel cross section (3 sections per rat). A replication index was calculated by using the following equation: percent positive cells = number of BrdU-positive nuclei per 3 cross sections/total number of nuclei per 3 cross sections × 100.

Systolic Blood Pressures
Systolic blood pressures were taken as described previously. In brief, individual conscious rats were put into restrainers and conditioned to handling on two consecutive days. Then SBPs were measured by tail-cuff plethysmography (Narco Biosystems). Three measurements per animal were taken to obtain a mean value for the day. For the baseline value, blood pressures were measured 2 days before treatment and on days 0, 3, 4, 5, and 6 after Ang II pumps had been implanted.

Measurement of Vascular Cross-sectional Area
For type III mesenteric vessels, two nonconsecutive cross sections (5 μm) were stained with the elastin fiber–specific stain orcein, and cross-sectional areas were assessed by a computer program (Optimus, BioScan, Edmonds, Wash). Cross-sectional-area was determined by taking measurements between the external and internal elastic laminas.

Statistics
Values are given as mean±SEM. ANOVA was performed. Comparisons of two group means were made with subsequent Fisher’s protected least-square difference test. P<0.05 was considered significant.

Results
Effect of Ang II on SBP
To study the mitogenic effect of Ang II on the blood vessels in vivo, we used osmotic pumps to deliver Ang II subcutaneously at a rate of 435 ng·kg⁻¹·min⁻¹. Compared with Ringer’s-treated animals, SBPs from Ang II–treated animals were significantly higher on days 3, 4, 5, and 6. Blood pressure reached a peak of 200 mm Hg on day 6 in the Ang II–treated group. Ringer’s-infused animals did not show any significant changes in blood pressure during the treatment period (Figure 1). Hydralazine treatment lowered SBP significantly. By day 6, hydralazine-treated animals showed a mean SBP of 75 mm Hg in contrast to a mean SBP of 120 mm Hg in the Ringer’s-treated animals.

Figure 1. Blood pressure measurement (mean±SEM) in rats receiving continuous subcutaneous infusion of Ang II, Ringer’s, Ang II plus hydralazine (AngII+Hyd), or Ringer’s plus hydralazine (Hyd+Ringer’s) for 1 week. Ang II was given at 435 ng·kg⁻¹·min⁻¹, and hydralazine was infused at 26 mg·kg⁻¹·d⁻¹. Ang II raised blood pressure significantly, whereas hydralazine treatment abolished the hypertensive effect of Ang II.
(Figure 1). Cotreatment of Ang II–treated animals with hydralazine inhibited the development of hypertension. SBP was \( \approx 85 \) mm Hg in animals infused with both Ang II and hydralazine on day 6 (Figure 1).

**Effect of Ang II on Cross-sectional Area of the Arterial Wall**

We examined two major branches in the mesenteric vascular tree. The radial vessels, usually two branch levels from the superior mesenteric artery, are classified as type III microvessels. Type I vessels are the smallest, penetrating the gut (Figure 2).

Ang II increased cross-sectional area in the tunica media of the type III microvessels (50% increase compared with Ringer’s animals; Figure 3). Antihypertensive treatment with hydralazine did not block this growth induced by Ang II (Figure 3). Additionally, hydralazine treatment alone had no effect on cross-sectional area when compared with that in Ringer’s control animals (Figure 3).

We previously reported that Ang II treatment increased the size of the type I microvessel approximately 2-fold to 5-fold. As with type III microvessels, this increase was not abolished by cotreatment with Ang II and hydralazine (Figure 4).

**Effect of Ang II and Antihypertensive Treatment on DNA Replication in the Carotid Artery and Microvessels**

Rats were given Ang II and hydralazine to determine their effects on DNA replication in the media of the carotid artery. The drugs were infused via osmotic pumps for 1 week. At the end of the week, the left carotid artery was fixed, histologically processed, and stained for BrdU. Ang II treatment stimulated SMC DNA replication in the media of the carotid artery (16-fold, Figure 5A; also see BrdU labeling in Figure 6). In rats infused with Ang II and hydralazine, measurement of DNA replication showed no significant difference from that in Ang II–infused animals \( (P > 0.05, \text{Figure } 5A) \). Despite the pressure-normalizing effects of hydralazine, the increase of DNA replication in the media of the carotid artery was not prevented. Thus, rats treated with Ang II and hydralazine showed levels of DNA replication similar to those found in animals receiving Ang II alone. Hydralazine alone produced a replication index similar to that seen in Ringer’s animals \( (P > 0.05) \).

In type III microvessels, Ang II stimulated medial SMC replication. The increase in replication rate was 26-fold compared with the control, Ringer’s infused animals. Cotreatment with Ang II and hydralazine, although blunting the increase in blood pressure, did not diminish the increase in DNA replication. Ang II plus hydralazine treatment produced an \( \approx 37 \)-fold increase of DNA replication versus that observed in the Ringer’s animals (Figure 5B; also see BrdU labeling in Figure 7).

Similarly, Ang II treatment induced an extraordinarily high replication rate in the type I vessels of the mesentery. Using the BrdU immunostaining technique, we were able to stain the proliferating SMCs, and these results are summarized in Figure 8. The labeling index was 49.1±3.8% in the Ang II–treated group. Again, hydralazine treatment did not abolish this proliferative effect (64.0±6.6%; see Figure 8). The Ringer’s group of rats showed minimal cell replication.

**Discussion**

Ang II, a potent vasoconstrictor, has been studied extensively for its role in stimulating proliferation of SMCs. This study was undertaken to determine whether Ang II could stimulate SMC DNA replication in vivo in the absence of elevated pressure. We found that Ang II stimulated SMC replication in the carotid arteries and type I and III mesenteric microvessels, without elevated blood pressure.

The existing evidence for mitogenic effects of Ang II, primarily based on in vitro studies, is confusing. Geisterfer et al. used rat aortic SMCs to evaluate the role of Ang II in regulating SMC growth. They found that Ang II failed to stimulate SMCs to replicate either in serum-free condi-
tions or with 10% fetal bovine serum. However, Ang II was able to increase protein synthesis in these cells. On the other hand, Campbell and Robertson\(^\text{14}\) reported elevated DNA synthesis in human SMCs due to Ang II treatment. The differences between these two studies may originate from the sources of SMCs that they used in these experiments. We and others have shown that rat SMCs are diverse, including markedly different growth factor requirements that depend on the donor’s original age and the manner in which the cells were placed in culture.

Gibbons et al\(^\text{15}\) offered a different hypothesis to explain the effects of Ang II in vitro.\(^\text{15}\) They demonstrated that Ang II induced both hypertrophy and hyperplasia in the same rat SMC line through a mechanism suggested to be modulated by TGF-\(\beta\)\(^\text{1}\) because cotreatment with neutralizing anti–TGF-\(\beta\)\(^\text{1}\) and Ang II increased DNA synthesis in SMCs. On the contrary, Stouffer and Owens\(^\text{16}\) reported the opposite findings.\(^\text{16}\) When using anti–TGF-\(\beta\)\(^\text{1}\) antibody, they detected decreased DNA synthesis in rat aortic SMCs treated with Ang II. The differences may be ascribed to differences in the strains of rats used in the two experiments because Gibbons et al used SMCs derived from Sprague-Dawley rats, whereas Stouffer and Owens obtained SMCs from spontaneously hypertensive rats. It is conceivable that SMCs from different rat strains may exhibit differential responses to growth stimuli, such as Ang II or TGF-\(\beta\)\(^\text{1}\).

The proliferative effect of Ang II may also depend on bFGF. We have shown that in vivo at least part of the proliferative effect of Ang II is due to bFGF.\(^\text{17}\) Using antisense oligomers complimentary to bFGF, Itoh et al\(^\text{18}\) were able to inhibit Ang II–induced DNA synthesis in SMCs in vitro.\(^\text{18}\) Therefore, the growth-stimulating effects of Ang II may involve TGF-\(\beta\)\(^\text{1}\) and bFGF and also depend on the source of the SMCs.

The mitogenic effect of Ang II in vivo is even more difficult to evaluate. In vivo studies have shown that Ang II is able to stimulate SMC DNA replication in large and small arteries.\(^\text{5,6}\) The etiology of this mitogenic effect is difficult to assess, however, given the concomitant development of hypertension in the treated animals. Moreover, Ang II has been shown to augment SMC DNA replication in the neointima, even months after balloon injury.\(^\text{7}\) Evidence for a local effect of Ang II rather than an indirect effect of blood pressure comes from antagonist studies in nonhypertensive models. Studies with an angiotensin type I receptor antagonist have shown that Ang II is important in the replicative response after balloon injury.\(^\text{19}\) There is

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**Figure 4.** BrdU labeling of type I microvessels. A, Micrograph of type I microvessel stained for BrdU from an Ang II–treated animal. B, Micrograph of type I microvessel from an animal receiving hydralazine and Ang II cotreatment. C, Micrograph of type I microvessel from Ringer’s-infused animal. D, Micrograph of type I microvessel from an animal receiving Ringer’s and hydralazine cotreatment (×40 objective).
Figure 5. DNA labeling by BrdU staining in blood vessels. Control rats were infused with Ringer's (vehicle). Ang II was infused at 435 ng kg⁻¹ min⁻¹. Hydralazine (hyd) was given via osmotic pump at 26 mg kg⁻¹ d⁻¹. All rats also received continuous infusion of BrdU for 1 week (via a separate pump) to label replicating cells. Top, BrdU labeling index for SMCs of carotid arteries. Bottom, BrdU labeling index for SMCs of type III microvessels. Values are mean±SEM.

Figure 6. Medial SMC replication in carotid arteries. A, Micrograph of carotid artery stained for BrdU from Ang II–treated animal. B, Micrograph of carotid artery from animal receiving hydralazine and Ang II cotreatment. C, Micrograph of carotid artery from Ringer’s-infused animal. D, Micrograph of carotid artery from animal receiving Ringer’s and hydralazine cotreatment (×20 objective).
also evidence that other mediators may be involved in Ang II–stimulated SMC replication. Our laboratory previously reported that anti-bFGF antibody blocked the proliferative effect of Ang II in carotid arteries and type II and III mesenteric microvessels. Therefore, the mitogenic effect of Ang II may be dependent on the presence of bFGF. Ang II may also mediate its effect in part via the \( \beta_1 \) adrenergic receptor, since blocking this receptor can attenuate the proliferative response to Ang II while stimulating this receptor can promote SMC growth. These pathways may all act together to elicit the mitogenic effects of Ang II in vivo.

Other data also suggest a direct effect of Ang II on the vessel wall. For example, Griffin et al found an increase in cross-sectional area after 1 week of Ang II treatment, independent of blood pressure. However, they did not determine whether this increase was due to proliferation of SMCs. We found that Ang II was a mitogen for vascular SMCs in a variety of vessels, including the carotid artery and type I and III microvessels in this study. The dramatic increase in SMC replication observed after 1 week of Ang II infusion was blood pressure independent. We also found that Ang II caused significant structural changes in the type III microvessels, ie, an increase in cross-sectional area independent of blood pressure, which confirmed the findings of Griffin et al. This suggests that the mitogenic effect of Ang II may be responsible for the change in mass. Since changes in resistance vessel mass have profound effects on the responsiveness of blood pressure to vasoconstrictors, this proliferative event could be important to the establishment of a maintained hypertensive state if it results in hyperplasia as well as hypertrophy.
Our data combined with those of Griffin et al raise the important issue of whether the remodeling of vessel walls to increase their resistance to vasoactive agents in hypertension may depend in part on the number of cells in the walls. The idea that hyperplasia is important is of interest because DNA does not turn over in vessel walls under normal conditions. If cell replication is important, then two kinds of drug effects may be relevant to antihypertensive therapy. First, we may want to consider drugs that block cell replication. These may or may not be the same drugs that induce the other effects of antihypertensive medications. Thus, the present study suggests that hydralazine may not be as effective as angiotensin-converting enzyme inhibitors in treating blood pressure. Second, we may be interested in drugs that modulate cell number. In this regard, deBlois et al reported that calcium channel blockers were able to induce apoptosis and reduce cell numbers by 40% in the aorta of spontaneously hypertensive rats.

The results of this investigation help to separate the hypertensive effects of Ang II from its mitogenic effects. These findings strongly suggest that Ang II behaves as a mitogen in vivo, although the precise mechanism is not clear. Nonetheless, our results have important implications for understanding the mechanism for vascular hypertrophy in hypertensive patients.

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References
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