Pressure Mediates Angiotensin II–Induced Arterial Hypertrophy and PDGF-A Expression

Sheri B. Parker, Suzanne S. Wade, Russell L. Prewitt

Abstract—Angiotensin II (Ang II) may induce arterial hypertrophy either directly or through an increase in arterial pressure. To separate these 2 mechanisms, rats were implanted with osmopumps delivering either Ang II (100 ng · kg⁻¹ · min⁻¹) or saline. 5-Bromo-2′-deoxyuridine (BrdU) was delivered to both groups by osmopump (2.5 μg · kg⁻¹ · min⁻¹). Half of the rats in each group were given minoxidil (9 mg · kg⁻¹ · d⁻¹) in their drinking water. After 14 days, systolic blood pressure was 117±2, 124±3, and 115±2 mm Hg in the control, Ang II–minoxidil, and minoxidil groups, respectively, and 181±6 mm Hg in the Ang II group (P<0.05). After perfusion-fixation, the thoracic aorta, carotid artery, small mesenteric artery, external spermatic artery, and kidneys were harvested, paraffin-embedded, and used for morphological measurements, immunohistochemistry for BrdU, and in situ hybridization with a 35S-labeled riboprobe for platelet-derived growth factor–A chain (PDGF-A) mRNA. The walls of the aorta and carotid arteries hypertrophied in the Ang II group only. There were no significant morphological differences in the small arteries. BrdU was negative in all arteries but positive in the renal tubules. Expression of PDGF-A was elevated 8-fold in the thoracic aorta of the Ang II group (P<0.05). These results show that (1) arterial hypertrophy from Ang II infusion occurs in response to elevated arterial pressure, (2) hypertrophy was not associated with hyperplasia or polyploidy of vascular smooth muscle cells, and (3) PDGF-A expression correlated with elevated pressure and arterial wall hypertrophy. (Hypertension. 1998;32:452-458.)

Key Words: pressure ■ angiotensin II ■ hypertrophy ■ growth substances

Hypertension is characterized by structural alterations of the vasculature, depending on the size and function of the particular vessel. During the course of hypertension, the larger arteries hypertrophy, while the lumen diameter of smaller arterioles is reduced without a change in cross-sectional wall area. The lumen reduction in absence of hypertension is termed “inward eutrophic remodeling” and was first reported on the submucosal arterioles of the intestine in hypertensive individuals. The small arteries (150 to 250 μm ID) are found at the overlap where hypertrophy decreases and eutrophic remodeling begins, wherein they experience either an inward eutrophic or hypertrophic remodeling depending on the type of hypertension. Many factors have been proposed to explain the structural alterations that occur during hypertension. One is the hormone angiotensin II (Ang II), which is a hypertrophic and hyperplastic stimulus of vascular smooth muscle cells, as well as an inducer of platelet-derived growth factor–A chain (PDGF-A) expression. A second factor is elevated intravascular pressure itself. Like Ang II, elevated pressure is also considered a hypertrophic stimulus and is linked to PDGF-A expression. As a consequence of the effect of Ang II on blood pressure, it is often difficult to distinguish between its direct and indirect effects on vascular hypertrophy associated with hypertension.

Previous studies that provide evidence that Ang II acts as a growth factor through a nonpressor mechanism have included the use of angiotensin-converting enzyme (ACE) inhibitors, which reduced cross-sectional wall area in the aorta in the presence of elevated pressure. Results from these experiments are confounded by the fact that ACE is also responsible for the degradation of bradykinin, which is known to stimulate the production of nitric oxide as well as prostaglandins. Nitric oxide and prostaglandins have antiproliferative properties on vascular smooth muscle cells. For example, attenuation of growth in myocardial mass through converting enzyme inhibitor (CEI) therapy is blocked by the use of the bradykinin type 2 antagonist icatibant, suggesting that the CEI antiproliferative effect is due to bradykinin accumulation. The use of icatibant in conjunction with CEI prevented the reduction of neointima formation after balloon injury when compared with CEI treatment alone. These studies demonstrate that the use of CEI therapy alone is not an effective means of evaluating the role of Ang II on vascular growth.

Other studies that provide evidence that Ang II is a growth factor that acts directly on smooth muscle cells include those of Griffin et al., in which Ang II infusion at 200 ng · kg⁻¹ · min⁻¹, in the presence of hydralazine to maintain a normal blood pressure, significantly increased medial cross-sectional...
However, infusion of Ang II at 250 ng · kg⁻¹ · min⁻¹ results in a 6-fold increase in plasma levels. Thus, an infusion rate of 200 ng · kg⁻¹ · min⁻¹ may give rise to higher plasma levels than those ever reached with endogenous concentration of Ang II. A reduction in infusion dose, creating increases in plasma Ang II levels similar to those seen in renal hypertension, may more accurately describe the direct effects of Ang II on smooth muscle cell adaptations.

The purpose of this study was to investigate the role of Ang II on the vascular changes associated with hypertension. To distinguish between direct and indirect roles of Ang II during hypertension, animals were infused with Ang II at 100 ng · kg⁻¹ · min⁻¹ and treated with minoxidil to prevent a rise in blood pressure. We then evaluated modifications that often accompany hypertension. These changes included hypertrophy or hyperplastic responses of vascular smooth muscle cells as well as alterations in PDGF-A expression.

Methods

Treatment Groups

The institutional animal care and use committee approved procedures involving animals. Male Wistar rats (200 to 224 g body weight) were anesthetized with a single intraperitoneal injection of ketamine hydrochloride (80 mg/kg) and xylazine (12 mg/kg). Alzet osmotic mini-pumps (model 2002) with a 14-day capacity, an average volume of 250 µL, 1201 µL/h were filled with either 0.9% saline or Ang II in 0.9% saline solution at a concentration of 10ⁿ⁻¹ mol/L sodium nitroprusside, 10⁻² mol/L papaverine, and 10⁻⁴ mol/L verapamil in 0.9% saline until cleared of blood. The animal was perfused at 60 mm Hg with 10% buffered formalin for 5 to 10 minutes. The thoracic aorta, the carotid artery, a first-order branch of the mesenteric artery, and the distal portion of the external spermatic artery (the feeding artery to the cremaster muscle) were dissected out from the surrounding tissues and further fixed by immersion in 10% buffered formalin, processed through graded alcohol solutions, and embedded in paraffin.

Morphological Analysis

Sections (4 µm) of the paraffin-embedded tissues were stained with toluidine blue to visualize the intima-media of the blood vessel. A video-based image system with edge-tracking software (JAVA, Jandel Scientific) was used to measure internal and external circumferences of each vessel 3 times. The mean of these was used to calculate internal diameter and intimal-medial area.

Immunohistochemistry for BrdU

Incorporation of BrdU in the nuclei was used as a marker for DNA replication during the infusion period. Sections from paraffin-embedded tissues were deparaffinized, rehydrated, and blocked for endogenous peroxidases; the DNA was denatured by treatment with 2 N HCl for 30 minutes at 37°C, enzymatically pretreated with trypsin, and then incubated with 5% normal goat serum. The positive tissues were incubated with mouse monoclonal anti-BrdU (diluted 1:500) while negative controls were incubated with 5% normal goat serum. All slides were incubated with biotinylated secondary antibody IgG (H+L) (diluted 1:600), stained with Vectastain Elite avidin-biotin complex kit, and incubated with 0.1% diaminobenzidine in 0.3 mol/L NaCl, 50% formamide, 0.5 mg/mL yeast tRNA, and 4 mol/L sodium nitroprusside, 10⁻³ mol/L papaverine, and 10⁻⁴ mol/L verapamil in 0.9% saline until cleared of blood. The concentration of BrdU in the pump (30 mg/mL) was chosen to provide a dosage of 2.5 µg/kg/min. The rats were housed 3 to a cage, fed Teklad rat chow and tap water or minoxidil water ad libitum, and maintained on a 12-hour light/dark cycle.

Systolic Blood Pressures

To assess the development of hypertension, indirect systolic tail-cuff blood pressures were routinely obtained with a Narco Bio-Systems electromyographmanometer after the rats were warmed at 35°C for 5 minutes. The mean of 3 measurements was recorded from each rat every other day, in addition to trial periods before surgery, until the end of the experiment. Ang II-infused rats whose blood pressure was not elevated to >150 mm Hg by day 14 (n=1) and Ang II-MIN rats whose systolic blood pressure was elevated to >140 mm Hg by day 14 (n=3) were eliminated from the study.

Tissue Preparation

After 14 days of treatment, the animals were anesthetized with sodium pentobarbital (60 mg/kg). The tail artery was cannulated (PE-10) for measurement of mean blood pressure, and 0.1 mL/100 g (1000 U/mL) heparin was injected intra-arterially. The chest was opened, and the inferior vena cava was cut to allow free flow of venous return. The animal was then perfused through the aorta from a catheter inserted into the apex of the left ventricle with a vasodilator solution of 10⁻¹ mol/L sodium nitroprusside, 10⁻² mol/L papaverine, and 10⁻⁴ mol/L verapamil in 0.9% saline until cleared of blood. The animal was perfused at 60 mm Hg with 10% buffered formalin for 5 to 10 minutes. The thoracic aorta, the carotid artery, a first-order branch of the mesenteric artery, and the distal portion of the external spermatic artery (the feeding artery to the cremaster muscle) were dissected out from the surrounding tissues and further fixed by immersion in 10% buffered formalin, processed through graded alcohol solutions, and embedded in paraffin.

In Situ Hybridization and Quantification

To determine the expression of PDGF-A mRNA, an in situ technique based on the method of Wilcox was used. Paraffin sections of arteries were mounted on Superfrost/Plus slides. 3⁻² labeled sense and antisense riboprobes for PDGF-A were transcribed from a 368-bp restriction fragment of the cDNA inserted into the polycloning site of pBluescript SK (+). Briefly, slides were deparaffinized by 2 washes in xylene. The vessels were then rehydrated in graded alcohol and washed with 0.5× SSC. Vessels were then treated with proteinase K at room temperature, washed 3 times with PBS, and fixed in 4% paraformaldehyde for 15 minutes at 4°C. Slides were then washed 3 times with PBS. The vessel sections were then covered with 200 µL of prehybridization solution (10% dextran sulfate, 1× Denhardt’s solution, 1 mmol/L EDTA, 10 mmol/L Tris, 0.3 mol/L NaCl, 50% formamide, 0.5 mg/mL yeast RNA, and 10 mmol/L DTT) and incubated for 3 hours at 38°C to 42°C in a humidified chamber. Slides were then covered with 100 µL of hybridization solution (prehybridization solution containing 10⁸ cpm of probe per mL) and incubated overnight at 55°C in a humidified chamber. Slides were washed with 2× SSC containing 1 mmol/L...
EDTA and 10 mmol/L β-mercaptoethanol and then treated with ribonuclease A (30 μg/mL) for 30 minutes at room temperature. Slides were then washed with 2× SSC containing 1 mmol/L EDTA and 10 mmol/L β-mercaptoethanol 4 times for 30 minutes, followed by 0.5× SSC. Finally, the vessel sections were dehydrated in graded alcohol solutions and air-dried.

Results were quantified by densitometric analysis of the slides with a Molecular Dynamics PhosphorImager SF. Slides were exposed on the phosphorimager cassette for 5 days. Sense probe binding of all groups was not detectable or was very low. By use of a volume integration function in the software, the total density minus the background was determined for each vessel cross section. The density counts for binding of sense probe were then subtracted from the counts for binding for antisense probes and divided by the cross-sectional area of the vessel to determine the mRNA levels for PDGF-A.

Materials

The cDNA for human PDGF-A was obtained from Tucker Collins at Brigham & Women’s Hospital (Boston, Mass). Radiolabeled cytidine triphosphate was obtained from Du Pont. Minoxidil was a gift from Brenda Ling at Upjohn Laboratories (Kalamazoo, Mich). All other chemicals or biochemicals were obtained from Sigma Chemical Co, Fisher Scientific, Gibco BRL, or Promega.

Statistics

Results are expressed as mean±SE. Statistical analyses were performed using ANOVA followed by Student-Newman-Keuls multi-comparison test, with P<0.05 being viewed as significant.

Results

Tail-cuff systolic blood pressures were significantly elevated in Ang II animals (Figure 1) compared with the other 3 groups, beginning 4 days after implantation and continuing for the duration of the study. Consumption of minoxidil before surgery initially lowered the blood pressure of treated animals. Ang II-MIN animals ingested 9.4±0.2 mg·kg⁻¹·d⁻¹ minoxidil; the CON-MIN animals ingested 8.7±0.3 mg·kg⁻¹·d⁻¹. Mean arterial blood pressure measured through an indwelling catheter in the tail artery was significantly elevated in the Ang II animals (117.4±11.2 mm Hg) when compared with that in the saline (88.7±6.7 mm Hg, CON; 73.9±4.2 mm Hg, CON-MIN) and Ang II-MIN animals (90.3±9.3 mm Hg). The body weight of CON animals (329.5±8.3 g) was significantly lower than that of the CON-MIN animals (366.5±8.6 g) but not different from those of the Ang II (352.1±4.9 g) or Ang II-MIN (343.4±6.8 g) groups.

Cross-sectional wall area was significantly elevated in the thoracic aorta and carotid artery of the Ang II group when compared with both control and Ang II-MIN animals (Figure 2). There were no significant differences among the groups in the cross-sectional wall area of the small mesenteric artery and external spermatic artery. The ID of the thoracic aorta was significantly increased when compared with that of the CON-MIN group. The small mesenteric artery ID was significantly reduced in the Ang II-MIN arteries. The ID of the carotid artery in the Ang II-MIN group was significantly decreased when compared with that of the CON group. The small mesenteric artery ID was significantly reduced in the Ang II animals compared with CON-MIN and Ang II-MIN arteries. The ID of the external spermatic artery was significantly decreased in the Ang II group when compared with that of the CON-MIN arteries.

Wall-to-lumen ratio of the Ang II thoracic aortas was 0.059±0.004, unchanged from a control value of 0.060±0.003 because both the lumen and wall thickness increased with angiotensin infusion. However, the CON-MIN aortas had a smaller wall-to-lumen ratio than controls at 0.049±0.002 (P<0.05), and the Ang II-MIN wall-to-lumen ratios were smaller than both control and Ang II values at 0.045±0.002 (P<0.05). There were no significant differences in wall-to-lumen ratios among any of the other vessels. Immunohistochemistry for BrdU showed positive-staining nuclei in tubular cells of all kidneys except 1, confirming the delivery of BrdU by the osmopump and the ability of the method to detect BrdU incorporation (Figure 4). However, no...
positive-staining nuclei were detected in the thoracic aortas or carotid or small mesenteric arteries among all 4 animal groups, regardless of the date of pump implantation. A sample aortic section from each group is shown in Figure 5.

In situ hybridization with the 35S-labeled riboprobe for PDGF-A was quantified through the use of a phosphorimeter, and sample phosphorimages of aortic cross sections from the Ang II and control groups are shown in Figure 6. Expression of PDGF-A mRNA was significantly elevated in the thoracic aorta of the Ang II animals compared with all other groups (Figure 7). PDGF-A mRNA followed a similar pattern of expression in the carotid artery, but the 3.8-fold increase in the Ang II group did not achieve statistical significance.

Discussion
The present study was an attempt to differentiate between the direct and indirect effects of Ang II on structural changes in the vasculature. Ang II infusion at 100 ng·kg⁻¹·min⁻¹ was sufficient to elicit a significant rise in systolic blood pressure 4 days after pump implantation, which continued for the duration of the study (Figure 1). Treatment with minoxidil, a K<sub>ATP</sub> channel activator, maintained blood pressure in a normal range in Ang II–infused animals. This allowed us to evaluate the direct effects of Ang II in the absence of a confounding increase in arterial pressure.

Infusion of Ang II alone caused a significant increase in cross-sectional wall area of the thoracic aorta and carotid arteries (Figure 2). This effect was prevented when blood pressure was maintained in the normal range by minoxidil, suggesting that pressure was the mediator of the arterial changes at this dose of Ang II infusion. There is evidence from the literature that pressure plays a considerable role in vascular alterations associated with hypertension. In models of aortic coarctation hypertension, there were significant increases in cross-sectional wall area in the arteries subjected to high pressure, whereas the normotensive regions showed no significant alterations.²,¹² In addition, interventions that protect the vascular bed from elevated pressure in the SHR by use of a ligature prevented the increase in medial thickness normally associated with this genetically hypertensive rat.²⁴,²⁵ Nonetheless, other studies have suggested a nonpressor role for Ang II in these hypertrophic changes. CEI treatment in renal hypertensive animals resulted in a significant decrease in aortic wall area in the presence of elevated arterial pressure.¹ However, these results may have been confounded by CEI potentiation of bradykinin. In addition, Ang II infusion caused a significant increase in wall area of the thoracic aorta.²⁶ However, these changes were also associated with an increase in systolic blood pressure, leaving elevated pressure and Ang II indistinguishable from one another. Our data provide strong evidence that regulation of wall structure in hypertensive animals during Ang II infusion is mediated by elevated pressure.

The ID of the larger arteries in both minoxidil-treated groups was significantly increased (Figure 3). Typically, in many models of hypertension, the lumen of the larger arteries does not change despite an increase in cross-sectional wall area.¹¹ Minoxidil treatment induces a significant increase in cardiac index as well as skeletal muscle blood flow.²⁷ Changes in blood flow can regulate arterial lumen size

Figure 3. Internal diameter of the thoracic aorta, carotid artery, small mesenteric artery, and external spermatic arteriole for CON, Ang II, CON-MIN, and Ang II-MIN groups. Internal diameter was calculated from the internal circumference determined by the use of a video-based image system with edge-tracking software on toluidine blue-stained tissues. Values are mean±SEM. Significance levels at P<0.05 vs: *control rats, +control minoxidil rats, and #Ang II–minoxidil rats by ANOVA and Student-Newman-Keuls posttest.

Figure 4. Representative photomicrographs of renal tubules from kidneys treated with primary antibody for bromodeoxyuridine (positive) and negative controls that received no primary antibody.

NEGATIVE CONTROL POSITIVE CONTROL
through changes in shear stress.\textsuperscript{28,29} Consequently, it is quite possible that the diameter changes seen in the minoxidil-treated animals are due to the increase in flow initiated by the vasodilator treatment.

In the present study, there were no significant changes in the cross-sectional wall area of either the small mesenteric artery or the external spermatic artery. In the Ang II group, these vessels exhibited a trend, though not statistically significant, toward inward eutrophic remodeling. It should be noted, however, that the perfusion pressure was 60 mm Hg during fixation, and any differences in passive distensibility could produce different diameter results if the vessels were fixed at their in vivo pressures. Characteristic structural alterations during renal hypertension include hypertrophy of smaller mesenteric arteries\textsuperscript{6} and lumen reductions in the first- and second-order arterioles.\textsuperscript{3,4} The lack of structural changes in cremaster arterioles of rats subjected to aortic coarctation hypertension\textsuperscript{12} indicates a role for pressure as a stimulus for remodeling. Pressure has been implicated as a stimulus for structural alterations of the mesenteric artery when sodium loading of SHR was used to maintain pressure in the presence of CEI therapy, resulting in wall-to-lumen ratios that corre-

Figure 5. Representative photomicrographs of thoracic aortas of CON, Ang II, CON-MIN, and Ang II-MIN groups treated with primary antibody for bromodeoxyuridine. No positive-staining nuclei were detected.

Figure 6. Representative phosphorimages of thoracic aortas from control (CON) and Ang II–infused rats hybridized to an antisense \textsuperscript{35}S-labeled riboprobe for PDGF-A mRNA and exposed for 5 days.

Figure 7. Mean values for PDGF-A expression in thoracic aortas of CON, Ang II, CON-MIN, and Ang II-MIN groups. mRNA content was determined by in situ hybridization with a \textsuperscript{35}S-labeled riboprobe and quantified on a phosphorimager. Values are mean±SEM of density in arbitrary units divided by wall cross-sectional area. \(p<0.0001\) compared with all other groups by ANOVA and Student-Newman-Keuls posttest.
lated with raised blood pressure. Nevertheless, Ang II is also implicated as a direct hypertrophic factor in the small mesenteric resistance vessels. In a study by Griffin et al., Ang II infusion induced a pressure-independent hypertrophy of the small mesenteric artery. Our infusion dose was half of that used by Griffin et al. At this reduced dose, the concentration of Ang II may have been too low to have a direct effect, and pressure was not high enough to induce remodeling. In addition, we used a first-order branch from the mesenteric artery, whereas Griffin et al. used a third-order branch.

The lack of hypertrophy in the large arteries with minoxidil treatment is best explained by a decrease in mechanical stress caused by lowering blood pressure. At 3 and 9 days after aortic coarctation, products of inositol lipid hydrolysis and increased levels of proto-oncogene expression were found in the high-pressure, but not normotensive, region of the aorta. This proto-oncogene expression may regulate smooth muscle cell growth. Studies in our laboratory indicate that pressure induces proto-oncogene expression in isolated small artery. This proto-oncogene expression significantly correlates with alterations of wall stress. Thus, in the presence of elevated pressure, the inositol triphosphate pathway and proto-oncogene expression are viable explanations for pressure-induced vascular alterations.

Hypertension is associated with hypertrophy of the vascular smooth muscle cell and accumulation of the extracellular matrix. This hypertrophic, not hyperplastic, smooth muscle cell adaptation is found in many models of hypertension. The accompanied increase in cell mass may be associated with polyplody, in which smooth muscle cells undergo DNA synthesis, without division. The lack of any BrdU staining in our vessels suggests that polyplody did not occur during hypertrophy. Therefore, pressure appears to be the cause of this response in the present study. Ang II also can be a hypertrophic stimulus. Ang II induced an increase in protein synthesis, not DNA synthesis in intact rat aortic segments. Outside of cellular adaptations, Ang II also induces an increase in fibronectin expression, independent of pressure. In the present study, however, Ang II infusion did not elicit hypertrophy of the arterial wall unless pressure was also elevated.

Another outcome of this study is that the hypertrophic response of the aortic wall, mediated by elevated pressure, was accompanied by an increase in PDGF-A expression. Pressure has been linked previously to PDGF-A expression, wherein the reduction of blood pressure of SHR significantly decreased growth factor expression. Furthermore, cyclic stretch of vascular smooth muscle cells cultured on fibronectin has been shown to increase PDGF-A expression. Alternatively, application of Ang II in cell culture also induced PDGF-A expression. Ang II infusion, accompanied by an elevated systolic blood pressure, caused a significant increase in PDGF-A expression in the thoracic aorta. In addition to pressure and Ang II, adrenergic stimulation is implicated in PDGF-A expression, in which phenylephrine-induced PDGF-A mRNA was significantly greater than Ang II-induced expression at a similar pressure rise. It has been shown that Ang II infusion increased plasma norepinephrine levels. This suggests that Ang II–induced PDGF-A expression may be due to an increase in circulating norepinephrine levels. However, our study demonstrated no increase in PDGF-A expression when the pressure was returned to normal values in Ang II–infused animals. These results suggest that at this dosage of Ang II, the increase in PDGF-A expression is mediated through elevated pressure, and PDGF-A may be part of the signaling pathway to transduce an elevation of pressure into a hypertrophic response.

In summary, the results of these studies indicate that the hypertrophic response of arterial vascular smooth muscle cells to Ang II infusion is mediated through an increase in arterial blood pressure. Second, PDGF-A expression, which may be a mediator of the hypertrophic response, is also associated with the elevation in pressure rather than a direct effect of Ang II.

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References


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