Selective Increase in Renal Arcuate Innervation Density and Neurogenic Constriction in Chronic Angiotensin II-Infused Rats

Helena C. Parkington, Jonathan Dodd, Susan E. Luff, Katrina Worthy, Harold A. Coleman, Marianne Tare, Warwick P. Anderson, Amanda J. Edgley

Abstract—This study investigated the effects of angiotensin II “slow pressor” hypertension on structure and function of nerves supplying the renal vasculature. Low-dose angiotensin II (10 ng/kg per minute, initially sub-pressor) or saline vehicle was infused intravenously for 21 days in rats, and the effects were compared in renal and mesenteric arteries. Mean arterial pressure averaged 12±2 mm Hg higher than in vehicle-infused rats at 21 days. Using electron microscopy, the innervation density of renal arcuate, but not mesenteric arteries of equivalent size, was significantly higher in angiotensin II-infused than in vehicle-infused rats. Functional testing on a pressure myograph revealed that constrictions evoked by nerve stimulation in arcuate arteries were 2.3±0.7-fold greater in vessels from angiotensin II-infused compared with vehicle-infused rats (P<0.0001), whereas there was no significant difference in nerve-induced constrictions in mesenteric arteries. Sensitivity to and maximum amplitude of constrictions evoked by phenylephrine were not different in renal or mesenteric arteries between groups, suggesting that the increased neurally evoked constriction in renal arcuate arteries was not caused by postsynaptic changes. Endothelium-dependent vasorelaxation and the vessel wall physical properties were not different between the two groups in either artery. Thus, angiotensin II infusion appeared to evoke renal-specific increases in vessel innervation and increased vasoconstriction to nerve stimulation. These changes appear early and occur before changes in renal endothelial function are apparent. Thus, “slow pressor” angiotensin II hypertension is associated with increased renal innervation, compatible with a pathogenetic role. (Hypertension. 2004;43:643-648.)

Key Words: sympathetic nervous system • endothelium

Infusion of low doses of angiotensin II (Ang II) that do not cause an immediate increase in blood pressure (sub-pressor) may induce the development of a slow and progressive (“slow pressor”) hypertension. The mechanisms involved in the pathogenesis of this hypertension have not been established unequivocally but are presumed to involve the progressive potentiation or amplification of one or more actions of Ang II. These may involve increased vasoconstriction via mechanisms including enhanced sensitivity to spasmodens, increased activity of the sympathetic nervous system, or the production of reactive oxygen species leading to endothelial vasodilator dysfunction. Other possible mechanisms include progressive structural changes (via alterations in the physical properties of the vessel wall e.g., stiffness, or vessel wall smooth muscle hypertrophy) and progressive renal Na+ retention (via direct effects on tubular reabsorption or stimulation of aldosterone secretion).

Here we investigated the effects of Ang II on renal blood vessel innervation. As Hall et al have pointed out, long-term changes in arterial pressure require that the relationship between arterial pressure and renal Na+ excretion be shifted to higher pressure. There is increasing evidence that Ang II may cause changes in the structure of renal blood vessels and in their innervation. We found that 6 weeks of inhibition of Ang II production using enalapril in rabbits led to a marked increase in the density of innervation, as well as phenotypic transformation of smooth muscle cells in the wall of 20- to 35-μm diameter afferent arterioles into renin-containing secretory cells. Casellas et al subsequently found that infusion of doses of Ang II that have immediate pressor effects reduced the density of the plexus innervating renal vessels in rats across a wider range of vessel diameters (30 to 300 μm). The innervation of rabbit and rat kidney is mainly sympathetic. Thus, the aim of this study was to clarify whether chronic low doses of Ang II, documented as barely pressor initially, can affect the sympathetic innervation of arteries within the kidney. We used morphological and functional approaches and also studied a similar size artery from a non-renal systemic vascular bed, namely the well-characterized mesenteric artery.

Methods

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Animal Preparation

Under anesthesia, the tip of an osmotic minipump (model 2ML4; Alzet) was inserted into the vena cava of male Sprague-Dawley rats.

Hypertension is available at http://www.hypertensionaha.org

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The pump delivered either vehicle (0.9% NaCl containing heparin 10 IU/mL, n=21) or Ang II (10 ng/kg per minute, n=21), a dose that had negligible pressor effects (<3 mm Hg in preliminary experiments) when administered acutely. All experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

On day 21 of infusion, arterial pressure was measured in freely moving rats via a tail artery catheter.15 At the end of the recording period, blood was taken via the catheter for determination of plasma renin activity,16 creatinine concentration (Beckman Synchron CX5 Clinical System Analyste), and hematocrit.

**Morphological Studies of the Innervation**

In anesthetized rats, a bolus vasodilator solution was delivered, and parrafomaldehyde (4%) and glutaraldehyde (1%) solution perfused at the conscious arterial pressure.17 Kidneys and mesenteric blood vessels were immersed in fixative overnight at 4°C. Three second-order branches of mesenteric artery and three arcuate arteries were studied. Vessels were post-fixed and embedded in epoxy resin as described.17 Ultrathin (100 nm) longitudinal sections were cut, stained with uranyl acetate, and viewed in a transmission electron microscope (Jeol 100S) at a magnification of ×10 000.

**Functional Properties**

Segments of renal arcuate and third-order mesenteric artery were isolated and all branches tied off. The proximal end of each segment was secured in a pressure myograph (Living Systems) and was continuously superfused with bicarbonate-buffered physiological saline solution (PSS) at 15 mL/min1 and 35°C. The distal end of the segment was secured to a closed cannula, ensuring no flow through the segment. The pressure in mesenteric segments was set at 55 mm Hg (as recorded in vivo, Parkington, unpublished observations) and arcuate segments were set 10 mm Hg higher for comparison, because the pressure decrease in the renal vessels to the capillary bed (glomeruli) is less than for other systemic beds. The myograph was transferred to the stage of an inverted microscope fitted with a video camera. Vessel diameter was monitored using Diamtrak.18,19

Drugs were added externally via the superfusing PSS. Perivascular nerves in the segment were stimulated, via 2 platinum electrodes, at frequencies of 0.5 to 10 Hz at 0.1 ms duration using a Grass S88 stimulator. That the constrictions recorded reflected nerve stimulation was confirmed in all tissues using tetrodotoxin (100 nmol/L).20 Cumulative concentration–response curves to phenylephrine and acetylcholine were obtained to assess α-adrenoceptor and endothelial function, respectively. Endothelium-independent smooth muscle relaxation was tested using sodium nitroprusside, 10 μmol/L, in the presence of phenylephrine. Passive lumen diameter and wall thickness were measured in PSS containing 0 mmol/L Ca²⁺ and EGTA (5 mmol/L). All drugs were purchased from Sigma Chemical.

**Data Analysis**

**Anatomical Properties**

Varicosities, intervaricosities, and neuromuscular junctions have been defined previously.17 All densities were expressed as number per mm² of vessel surface.17 Data were analyzed for significant differences between n animals using unpaired Student t tests.

**Functional Properties**

Sigmoid curves were fitted to phenylephrine and acetylcholine responses, and the concentration required to evoke a half maximal response, EC₅₀, was calculated. Instat software (GraphPad) tested for statistical significance of treatments using two-way ANOVA followed by Student t test. Mean±SE for n animals are given, and P<0.05 was deemed statistically significant.

**Results**

After 21 days of infusion, mean arterial pressure in rats infused with Ang II was 12±2 mm Hg (n=9) higher than in vehicle-infused animals (Table). Creatinine concentration was signifi-

<table>
<thead>
<tr>
<th></th>
<th>Vehicle n</th>
<th>Ang II n</th>
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<tbody>
<tr>
<td>MAP (mm Hg)</td>
<td>109±1</td>
<td>121±2*</td>
</tr>
<tr>
<td>Creatinine (μmol)</td>
<td>37±1</td>
<td>43±2*</td>
</tr>
<tr>
<td>PRA (Ang I ng/mL per h)</td>
<td>6.3±2.6</td>
<td>0.9±0.3*</td>
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MAP indicates mean arterial pressure; PRA, plasma renin activity. *Significantly different from vehicle-infused controls (P<0.05).
of vessels from Ang II-infused rats, 57% ± 2% (n = 5) of profiles were varicosities, whereas the remaining 43% ± 2% (n = 5) were intervaricosities. In arteries from vehicle controls, 59% ± 2% of profiles were varicosities and 41% ± 2% were intervaricosities (n = 5 each). There were numerous clear synaptic vesicles within all varicosities. Microtubules were present in most intervaricosities whereas mitochondria were rarely identified (Figure 1).

There was a greater density of intervaricosities in the adventitia of renal arcuate arteries from Ang II-infused compared with vehicle-infused rats (P = 0.022) (Figure 2). There was also a higher density of varicosities in the adventitia of arteries of Ang II-infused rats (P = 0.01) (Figure 2). Varicosities were identified as forming neuromuscular junctions (contacting) or as noncontacting (Methods17). There was no difference in the density of neuromuscular junctions in renal arcuate arteries of Ang II-infused versus vehicle-infused rats (P = 0.4) (Figure 2) and no difference in the percentage of varicosities that formed a neuromuscular junction with a smooth muscle cell in the two groups (3.2% ± 0.3% and 3.3% ± 0.3%, respectively).

Structure of Nerves in Mesenteric Arteries
The density of axon bundles in mesenteric arteries did not differ between Ang II-infused and vehicle-infused groups (P = 0.8) (Figure 2). The average number of axons per bundle was also not different (10.7 ± 0.4 versus 10.9 ± 0.6 axons per bundle, P = 0.8) and neither the density of intervaricosities (P = 0.6) in vessels from the two groups nor the density of varicosities (P = 0.8) differed between the two groups (Figure 2). Neuromuscular junctions occurred in 0.6% ± 0.3% of varicosities of mesenteric vessels of Ang II-infused rats and in 1.0% ± 0.3% of varicosities in vessels from vehicle-infused rats (P = 0.4) (Figure 2).

Functional Properties of Isolated Renal Arcuate and Mesenteric Vessels
Measured at 65 mm Hg, the outside diameters of segments of renal arcuate artery averaged 250 ± 17 μm (n = 9) and 278 ± 18 μm (n = 9) from Ang II-infused and vehicle-infused animals (P = 0.3). In segments of mesenteric artery at 55 mm Hg, the outside diameters averaged 225 ± 9 μm (n = 9) and 232 ± 7 μm (n = 9), respectively (P = 0.5).

Nerve Stimulation
Transmural stimulation of the nerves evoked brief constrictions whose amplitudes increased as the frequency or duration of stimulation was increased. In arcuate arteries obtained from Ang II-infused rats, responses were larger than those from the vehicle-infused group (P < 0.0001) (Figure 3). For mesenteric arteries, these was no difference in the responses to nerve stimulation in the Ang II-infused versus vehicle-infused group (P = 0.3).

Responses to nerve stimulation were reduced by ∼two-thirds in the presence of the α-adrenoceptor antagonist phentolamine (0.2 μmol/L) in both arteries (data not shown).

Sensitivity of the Smooth Muscle to Phenylephrine
The α-adrenoceptor agonist phenylephrine evoked concentration-dependent constriction of renal arcuate and mesenteric segments (Figure 4A). Sensitivity to and maximum constriction evoked by phenylephrine were similar in arcuate artery segments from Ang II-infused (to 126 ± 9 μm, EC50 0.33 ± 0.05 μmol/L, n = 8) and vehicle-infused rats (to 130 ± 11 μm, EC50 0.27 ± 0.02 μmol/L, n = 9). The maximum

Figure 2. Density of axon bundles, intervaricosity density, overall varicosity density, and neuromuscular junction density (×10⁶/mm² vessel surface) in renal arcuate and mesenteric arteries from rats infused for 21 days with either Ang II or vehicle control (Veh).

Figure 3. Constriction (diameter relative to unstimulated diameter) evoked by perivascular nerve stimulation in renal and mesenteric arteries of control rats (solid symbols) and after 21 days of infusion with Ang II (open symbols). Nerves were stimulated at a range of frequencies (1 to 10 Hz), each for a duration of 2 and 3 seconds. * Significant difference between responses in Ang II-infused versus vehicle-infused tissues for both stimulus durations.
Constriction and sensitivity to phenylephrine were also not different in mesenteric segments from Ang II-treated (constriction to $145\pm13$ µm, $EC_{50} 0.96\pm0.10$ µmol/L, $n=8$) versus vehicle-infused rats (constriction to $131\pm12$ µm, $EC_{50} 0.79\pm0.09$ µmol/L, $n=8$) (Figure 4A).

Constrictions evoked by PSS containing 100 mmol/L KCl for 1 minute were similar in arcuate segments from Ang II-infused (constriction to $169\pm17$ µm, $n=9$) and vehicle-infused rats (maximum to $186\pm17$ µm, $n=9$). Likewise, in mesenteric arteries, the KCl-induced constrictions were to $134\pm8$ µm ($n=9$) and to $143\pm10$ µm ($n=9$), respectively.

**Endothelium-Dependent Relaxation**

Segments of renal arcuate and mesenteric arteries were constricted to $70\%$ of control diameter with phenylephrine. Stimulation of the endothelium with acetylcholine evoked relaxation to $0.86\pm0.06$ of maximum, with an $EC_{50}$ of $0.87\pm0.28$ µmol/L ($n=6$) in renal arcuate arteries of Ang II-infused rats. This was not different from responses in segments of vehicle-infused animals (maximum relaxation to $0.87\pm0.09$, $EC_{50} 0.80\pm0.52$ µmol/L, $n=7$) (Figure 4B).

Acetylcholine elicited almost complete relaxation in mesenteric segments from Ang II-infused and vehicle-infused rats (maximum: $0.96\pm0.04$, $n=8$ and $0.98\pm0.02$, $n=8$) (Figure 4B), and sensitivity to acetylcholine was not different with Ang II versus vehicle infusion ($EC_{50} 0.26\pm0.07$ µmol/L, $n=8$ and $0.21\pm0.04$ µmol/L, $n=8$).

Endothelium-independent smooth muscle relaxation evoked by sodium nitroprusside was not different in arcuate arteries obtained from Ang II-infused (0.78±0.04, $n=7$) compared with vehicle-infused rats (0.75±0.05, $n=7$). Similar, for mesenteric arteries, nitroprusside-induced relaxation was not different in Ang II-treated (0.93±0.02, $n=8$) versus vehicle-treated animals (0.93±0.02, $n=8$).
Passive Wall Properties

In Ca\(^{2+}\)-free, EGTA-containing PSS, there was no difference in passive wall properties in either renal arcuate or mesenteric arteries obtained from Ang II-infused versus vehicle-infused rats. Parameters tested included external diameter (Figure 4D), media (wall) cross-sectional area (arcuate artery Ang II 17.395±1.457 μm\(^2\) versus control 17.676±1.664 μm\(^2\), n=6; mesenteric artery Ang II 10.751±1.855 μm\(^2\) versus control 10.385±1.514 μm\(^2\), n=6), and media/lumen ratio (Figure 4C).

Discussion

This study has demonstrated that chronic low-dose intravenous Ang II infusion results in a significant increase in the density of the nerve network supplying the renal arcuate artery. This low dose of Ang II induced a barely detectable effect on arterial pressure in acute experiments but produced a modest elevation of arterial pressure of approximately 12 mm Hg by 21 days of infusion, at which time there was no evidence of endothelium-dependent vasodilator dysfunction. The changes in innervation structure were associated with an increase in the functional response to nerve stimulation, with a 2.5-fold greater constriction of arcuate arteries of Ang II-infused rats compared with vehicle controls. In contrast, this increase in nerve-evoked constriction was not observed in the mesenteric arteries. Thus, in mesenteric arteries from the same animals, chronic Ang II infusion was without significant effect on either the innervation density or the amplitude of nerve-induced constriction.

Chronic Ang II treatment induced a significant ∼150% greater density in axon bundles in the renal arcuate artery without a change in the number of axons per bundle and an increase in the density of varicosities and intervaricosities. This suggests that there may have been an increase in the number of varicosities along individual axons as well as an increase in the length and/or branching of axons. Whereas overall varicosity density was greater in arcuate arteries exposed to Ang II, there was no observed difference in the density of neuromuscular junctions, ie, varicosities that make specific close contacts. This suggests that most of the new varicosities that appeared during Ang II treatment did not form specific neuromuscular junctions. We hypothesized that the functional consequence might be an increased effector response to neurotransmitter release via diffusion after release from the varicosity, and we tested for this. The renal vasculature in the rat is innervated by sympathetic nerves. The marked augmentation of the response of the renal arcuate artery to nerve stimulation is unlikely to reflect an action of Ang II on the density of α-adrenoceptors on the smooth muscle cells, or on postreceptor mechanisms accessed by stimulation of these receptors because neither the maximum response nor the sensitivity to applied phenylephrine were different between the Ang II-infused and vehicle-infused groups, similar to previous observations. Therefore, we propose that the most likely explanation for the augmented response to nerve stimulation is the increase in the density of nerves evident in the renal vessels of rats infused with Ang II for 21 days. This confirms previous observations in which the slow pressor response to Ang II was reduced after denervation of the kidney. Although the greater number of non-contacting sympathetic nerve varicosities found in the electron microscopy studies is the most economical explanation for the increase in the nerve-evoked constrictions, the present study cannot exclude the possibility that a reduction in re-uptake of released norepinephrine by varicosities contributes to the enhanced responses.

ATP, another cotransmitter, when released from sympathetic nerves, activates purinergic P2X receptors on vascular smooth muscle to give rise to a brisk depolarization, an excitatory junction potential that requires that the neuromuscular junction be “contacting.” The density of neuromuscular junctions was not increased by Ang II, suggesting that increases in ATP release are unlikely to be the cause of the increased sensitivity of the renal arcuate arteries to nerve stimulation after treatment. However, the low incidence of neuromuscular junctions and the possibility of a diffuse distribution of purinoceptors mean that this conclusion is made cautiously.

In contrast to the arcuate artery, the constrictor response to stimulating the nerves in mesenteric arteries of rats was not influenced by Ang II pretreatment, and this concurred with the morphological evidence of no change in the density of innervation in this bed.

This study provides further evidence that the renal innervation may be plastic and that chronic Ang II levels may be an important factor in establishing renal innervation density. We have previously reported that prolonged (6 weeks) treatment of rabbits with enalapril resulted in changes in the innervation of the renal vasculature, which were, paradoxically, similar to those seen here from low-dose Ang II infusion. In another recent case, Casellas et al reported that high doses of Ang II (400 ng/Kg per minute subcutaneous) infused for 10 days, which produced a marked (50 mm Hg) increase in systolic pressure, resulted in a decrease in innervation density in renal arcuate arteries. The reason for the differences in these results and those of the present study is not clear but may be explained by the higher dose of Ang II used, resulting in considerably higher arterial pressures achieved, in the study by Casellas. Alternatively, in contrast with the current study in which vessels were fixed rapidly in situ for examination with electron microscopy, Casellas et al excised, isolated, and permeabilized the vessels before fixation and light microscopic visualization with a marker for synaptic vesicles (synaptophysin). This approach may have lead to synaptic vesicle release before fixation and thus may, in combination with the low resolution of the light microscope, have led to underestimation of nerve densities in their study.

Ang II infusion at the low dose used in the present experiments led to a small increase in arterial pressure after 21 days but had no detectable effects on cardiac mass or renal arcuate and mesenteric vessel dimensions. Although it is not possible to rule out that the small increase in blood pressure contributed to the greater innervation density and enhanced constriction to nerve stimulation evoked by Ang II, this effect was apparently specific to the renal vasculature and was not detectable in the mesenteric artery. Regardless of the mechanism by which the Ang II acted, the increased innervation density seen in the renal vasculature could be prohypertensive via a number of mechanisms. For example, increased neurotransmitter release during sympathetic nerve activity in the kidney could increase renal vasoconstriction.
and/or tubular Na⁺ reabsorption, resulting in a rightward shift of the renal arterial pressure/body fluid balance (‘’pressure natriuresis’’) relationship⁹ and thus higher arterial pressure.⁹,¹⁰,¹⁰ Interestingly, human studies have shown that sympathetic nerve activity is increased predominantly in the kidney, but not the mesenteric circulation, early in some forms of hypertension,¹¹–¹⁴ including in borderline forms of the disease.¹⁵ The exact contribution of these changes in renal sympathetic structure and function to the increase in blood pressure in this model of experimental hypertension remains to be determined. Furthermore, the fate of the innervation in the smaller arterioles in the present study is unknown. Although Ang II had no effect on the density or function of the innervation in mesenteric vessels, further studies are required to examine whether Ang II affects the innervation density of other components of the cardiovascular system.

**Perspectives**

This study has demonstrated that Ang II induces significant increases in the sympathetic vasoconstrictor innervation in the kidney, but not in the mesenteric bed, within 21 days. This is achieved at a dose that has negligible pressor effects acutely and that is without effects on vascular endothelial vasodilator function or passive wall properties in either bed. These results provide support of strengthened cooperative interaction between the renin–angiotensin and sympathetic nervous systems. Although this would be beneficial in blunting pressure changes and restoring homeostasis after hypovolemic perturbation in young healthy individuals, subtle increases in Ang II in the absence of such stimuli are likely to contribute to an increase in blood pressure. The “feed-forward” interaction between the two systems requires more extensive study to determine whether the effects of Ang II on the innervation properties of the kidney may be an important factor in long-term arterial pressure regulation.

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**References**

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