Angiotensin II Facilitates Sympathetic Transmission in Rat Hind Limb Circulation

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We developed a novel method to stimulate the sympathetic innervation of the isolated, perfused rat hind limb to investigate whether a subpressor concentration of angiotensin II (Ang II) facilitates noradrenergic transmission in the vascular bed to skeletal muscle. We electrically stimulated the lumbar sympathetic trunk while perfusing the preparation with artificial medium. Seventy-five percent of the resulting frequency-dependent increases in perfusion pressure were mediated by \(\alpha_2\)-adrenergic receptors. Ang II (10 nM) significantly enhanced the effects of nerve stimulation at 1 and 10 Hz (by 42% and 35%, respectively). At a supramaximal stimulation frequency (20 Hz), Ang II prolonged the duration of the response without changing the peak increase in pressure. The reuptake inhibitor cocaine did not influence the effects of Ang II at 1 and 10 Hz but blocked the effect at 20 Hz. To control for nonspecific synergism with norepinephrine, we compared Ang II with vasopressin. Both peptides potentiated the pressor response to exogenous norepinephrine; however, vasopressin did not change the pressor response to nerve stimulation at any frequency. We conclude that Ang II, but not vasopressin, facilitates noradrenergic transmission in skeletal muscle resistance vessels, independent of its direct vasoconstrictor activity. The neurovascular preparation we describe may be useful in addressing other hypotheses concerning sympathetic transmission in skeletal muscle resistance vessels. (Hypertension 1993;21:322-328)

Key Words: autonomic nervous system • vascular resistance • norepinephrine • arginine vasopressin • hind limb • angiotensin II

Present evidence indicates that angiotensin II (Ang II) influences the vasoconstrictor activity of the peripheral sympathetic nervous system by a number of mechanisms. These actions of Ang II include facilitation of norepinephrine release, post-synaptic synergism between Ang II and norepinephrine, and perhaps also the inhibition of neuronal norepinephrine reuptake. Evidence indicating that Ang II enhances noradrenergic transmission was derived from experiments in cardiac tissue, large-caliber vessels, mesenteric artery or tail artery preparations, and various vascular beds. However, no conclusive data are available for the skeletal muscle vasculature, which is a major determinant of total peripheral resistance. In a recent report, Schwieler et al were unable to find any influence of Ang II on noradrenergic transmission in a blood-perfused canine skeletal muscle.

The available, circumstantial evidence for an interaction between Ang II and sympathetic transmission in skeletal muscle resistance vessels was derived from studies on pithed rat preparations. However, recent data suggest that in the pithed rat, the observed actions of Ang II on the pressor response evoked by electrical stimulation of the spinal cord are largely, if not entirely, due to the elevation of basal blood pressure induced by Ang II. Such an effect is nonspecific, as opposed to specific effects mediated by interactions with the sympathetic transmission.

We tested the hypothesis that Ang II enhances the pressor response induced by stimulation of the sympathetic nerves supplying a skeletal muscle resistance vessel bed. We developed a method to stimulate the sympathetic innervation of the isolated rat hindquarter, which we perfused with an artificial medium. To exclude nonspecific synergism between pressor agents, we used a subpressor concentration of Ang II and compared Ang II with arginine vasopressin (AVP). AVP had no specific effect on peripheral sympathetic transmission; instead, it showed a synergism with exogenous norepinephrine.

Methods

Animals

Male Sprague-Dawley rats (Ivanovas, Kislegg, FRG) weighing 250–300 g were kept in rooms at 24±2°C. They were fed a standard rat diet (No. C-1000, Altromin, Lage, FRG) containing 0.2% sodium by weight and were allowed free access to tap water. All procedures performed in animals were done in accordance with the guidelines of the American Physiological Society and were approved by the local government (Regierung von Mittelfranken).
**Nerve Stimulation**

Rats underwent median laparotomy under thiobarbital anesthesia (60 mg/kg i.p.) (Inactin, Byk-Gulden, Konstanz, FRG). After excision, the inferior epigastic, umbilical, internal spermatic, caudal mesenteric, and iliolumbar vessels were carefully ligated. The left iliolumbar vessels were sectioned after ligation, and the trunk of these vessels was used to tie the abdominal aorta and vena cava to the right side of the animal. In that way, we were able to avoid any gross impairment of the blood supply to the hindquarter. The preparation was then placed on a specifically designed table under a microscope (Wild-Leitz, Munich, FRG) so that the left flank was elevated by 35°. With the rat in that position and the large vessels tied to the right, the lumbar sympathetic nerve trunk was exposed at the level of the sympathetic ganglia L3-L5, where the right and left sympathetic chains are fused. In that position, the nerve could be dissected free without mechanical stress. Both anatomic and functional evidence indicate that the fibers of the lumbar sympathetic trunk project to resistance vessels in the skeletal muscle of the hindquarter. In whole animals, stimulation of these fibers increases hind limb vascular resistance.

The lumbar sympathetic trunk was placed on a bipolar stimulation electrode and embedded using a rapidly hardening, two-component cement (Silgel S4i, Bisco, Bielefeld, FRG). An additional grounding electrode was implanted subcutaneously in the chest wall. Rats were then transferred to the perfusion apparatus.

The electrode was connected to a stimulator (model S48, Grass Instruments, Quincy, Mass.). Nerve stimulations were performed after hindquarter perfusion was begun (see below). Only the frequency of stimulation was varied (0.5-25 Hz); all other parameters were maintained constant. Stimulations were done with supramaximal voltage (15 V). Stimulation trains of 10-second duration were applied. In preliminary experiments, we found that such short stimulations were most appropriate to achieve reproducible and frequency-dependent pressor effects. The single pulse duration was 0.5 msec, the stimulation delay, 0.01 msec.

**Hindquarter Perfusion**

Perfusion was performed as previously described. Briefly, rats received an intravenous injection of 500 units Na-heparin. Five minutes thereafter, the abdominal aorta and the inferior vena cava were cannulated, and the perfusion was begun immediately. The hindquarters were perfused in a nonrecirculating system with modified Tyrode’s solution containing 2 g/L glucose and 40 g/L of the artificial colloid Ficol 70 (Pharmacia LKB Biotechnology, Uppsala, Sweden). The perfusate was gassed with O₂/CO₂, adjusted to pH 7.4, and maintained at 38°C. The hindquarter perfusion was performed at a constant flow rate (10 mL/min) using a two-channel peristaltic pump (Harvard Apparatus, South Natick, Mass.). The perfusion pressure was monitored via a side arm of the aortic perfusion cannula by transducers (Gould Inc., Cleveland, Ohio) connected to a polygraph (Hellige, Freiburg, FRG). All experimental substances were infused into the perfusion system at a rate of 100 μL/min by means of a syringe pump (model 22, Harvard Apparatus). All experimental protocols were begun after an initial perfusion period of 20 minutes (washout period).

**Experimental Protocols**

Because stimulation of sympathetic nerves has not previously been combined with the isolated, perfused rat hindquarter, we first determined the basal characteristics of the stimulation-induced pressor response. The reproducibility of nerve stimulation was tested by applying seven repetitive stimulations of 10 Hz each at 5-minute intervals (n=6 preparations). The frequency dependence of stimulation-induced increases of perfusion pressure was investigated using stimulations of increasing frequency (0.5, 1, 5, 10, 15, 20, and 25 Hz) in random order at 5-minute intervals (n=7).

To test the effects of experimental substances on pressor responses to nerve stimulation, we performed the following protocol: An initial period of three stimulations of increasing frequencies (1, 10, and 20 Hz) at 5-minute intervals was followed by a second identical period. During the second period, experimental substances, which had been dissolved in 0.9% NaCl, were infused. The drugs tested included saline as a vehicle control (n=8), the α₁-adrenergic receptor antagonist prazosin (5 μM, n=7), Ang II (10 nM, n=7), AVP (0.3 nM, n=7), the reuptake blocker cocaine (15 μM, n=7), and cocaine combined with Ang II (n=7). The concentration of AVP was just below the pressor threshold.

Infusion of all substances was begun 5 minutes before the first stimulation of the second period. The increases in hindquarter perfusion pressure obtained during the second stimulation period were compared with those in the saline infusion (vehicle control) group with respect to both magnitude and duration.

We also tested for the effects of substances on the pressor response to exogenous norepinephrine. In each preparation, four repetitive bolus doses of 1 μg norepinephrine, dissolved in 30 μL 0.9% NaCl, were injected at 5-minute intervals. Experimental substances were infused during the last two bolus injections. The following substances were tested: prazosin (n=6), Ang II (n=8), AVP (n=8), cocaine (n=8), and saline (n=7) as a vehicle control. All substances were infused at the same concentrations as indicated above. The mean of the increases in vascular resistance elicited by the first two injections of norepinephrine was compared with the mean of the increases after norepinephrine administration during infusion of experimental substances.

**Chemicals**

Ang II and AVP were purchased from Bachem, Heidelberg, FRG. Stock solutions (1 mg/mL) were dissolved in 0.1% bovine serum albumin (fraction V, Sigma Chemical Co., St. Louis, Mo.) in 0.9% NaCl and kept at -70°C. Solutions were diluted with 0.9% NaCl. Cocaine hydrochloride, dissolved in water, was obtained from the Pharmacy Department of the University of Erlangen-Nürnberg. All other compounds were purchased from Sigma and dissolved in 0.9% NaCl. The concentrations indicated above resemble the final concentrations achieved in the perfusate after infusion into the perfusion system.
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FIGURE 1. Original tracings of hind limb perfusion pressure recordings from three representative nerve stimulation experiments. Perfusion pressure is indicated on the left; time scale is indicated by a bar ("5 min") in lower left corner. Stimulation frequencies marked with an arrow are shown below the tracings. On the right side, substances infused during the second period of three stimulations ("experimental period") are indicated. The \( \alpha_1 \)-adrenergic receptor antagonist prazosin (5 \( \mu \)M, middle tracing) suppressed the effects of nerve stimulation. Note that cocaine (15 \( \mu \)M, bottom tracing) did not affect peak response to nerve stimulation but rather prolonged the duration of the increase in perfusion pressure.

Analysis of Data

The magnitude of the increase in perfusion pressure elicited by nerve stimulation and norepinephrine injection was measured in millimeters of mercury. Because the perfusion flow was maintained constant, the pressure increases reflect changes in vascular resistance. Pressure changes measured during infusion of experimental substances were compared with the changes during infusion of saline.

Significance of differences between groups was assessed by analysis of covariance and subsequently the Newman-Keuls test. To correct for the differences in susceptibility to nerve stimulation between different preparations, we used as a covariate the pressor response to the respective stimulation frequency during the first period of three stimulations. The data from nerve stimulations during infusion of substances are presented as the percent of control response. If there was no difference between groups with regard to the magnitude of pressor response, we also analyzed the duration of the response. We measured the time in seconds that elapsed until 25%, 50%, and 75% recovery from maximum increase in vascular resistance had occurred. Statistical significance of these data was assessed by analysis of variance and subsequent Newman-Keuls test.

Statistical analysis was performed with a CSS statistical software package (StatSoft Inc., Tulsa, Okla.). A value of \( p < 0.05 \) was considered significant. All data are given as the mean\( \pm \)SEM.

Results

The baseline perfusion pressure ranged between 28 and 36 mm Hg. There were no significant differences in baseline perfusion pressure between any of the groups. None of the substances changed the baseline perfusion pressure.

Original tracings of nerve stimulation experiments are shown in Figure 1. Electrical stimulation of the lumbar sympathetic chain increased the perfusion pressure immediately. Pressor responses to seven repeated applications of a constant stimulation frequency were reproducible (Figure 2, top panel). Furthermore, the increase in vascular resistance evoked by nerve stimulation was clearly dependent on the stimulation frequency (Figure 2, middle panel). The lowest frequency that induced a significant increase in hindquarter resistance (threshold) was 1 Hz. The maximum pressor response was reached between 15 and 20 Hz (Figure 2, middle panel). Two subsequent frequency–response curves of three stimulations each (1, 10, and 20 Hz) could be performed in the same preparation without any difference between pressor responses to the same frequency (Figure 2, bottom panel). The \( \alpha_1 \)-adrenergic receptor antagonist prazosin inhibited the pressor response to 1 Hz by 70\( \pm \)11%, the pressor response to 10 Hz by 78\( \pm \)6%, and to 20 Hz by 75\( \pm \)5%, compared with saline.

Ang II significantly enhanced the pressor response at 1 Hz (by 42%) and 10 Hz (by 35%) but not at 20 Hz stimulation (Figure 3, top panel). In contrast, AVP had
no effect at any frequency (Figure 3, top panel). At 20 Hz stimulation, Ang II did not affect the peak increase in perfusion pressure but instead significantly prolonged the duration of the pressor response (Figure 4, top panel). The time that elapsed until 75% recovery from pressor response had occurred was 79±10 seconds in the presence of Ang II and 28±13 seconds in the absence of the peptide (p<0.05). In contrast, AVP had no significant effect on the duration of the pressor response at any stimulation frequency (Figure 4, top panel).

Pharmacological blockade of the neuronal catecholamine reuptake by cocaine had no effect on the peak increase of vascular resistance at any stimulation frequency (Figure 3, bottom panel). However, cocaine markedly prolonged the duration of the pressor responses to all stimulation frequencies. At 10 Hz stimulation in the presence of cocaine, 120±26 seconds elapsed until 75% recovery from maximum pressor response was achieved (versus 15±2 seconds in vehicle controls, p<0.05). Data for 20 Hz stimulations are shown in the bottom panel of Figure 4. When Ang II and cocaine were combined, the peak pressor responses at 1 and 10 Hz were significantly enhanced, compared with saline or cocaine alone (Figure 3, bottom panel). At 20 Hz stimulation, neither the duration of the pressor response nor the peak increase was different between cocaine alone and cocaine plus Ang II (Figure 4, bottom panel).

The pressor response to 1 µg bolus injections of norepinephrine was 9.3±0.4 mm Hg in the absence and 3.5±0.7 mm Hg in the presence of prazosin (p<0.05), corresponding to a 62% inhibition of exogenous norepinephrine by α1-adrenergic blockade. Ang II potentiated the pressor response to norepinephrine by 48%, AVP by 45%, and cocaine by 103%, compared with saline (see Figure 5).

Discussion
The aim of the present study was to test whether Ang II facilitates sympathetic transmission in an isolated,
skeletal muscle, resistance vessel bed. To investigate this issue, we developed a method to stimulate nerve traffic to resistance arteries of the artificially perfused rat hindquarter. Our main finding was that Ang II enhanced the effects of nerve stimulation at low and intermediate frequencies, whereas the peptide prolonged the duration of the pressor response at a high stimulation frequency. In contrast, AVP had no effect on nerve stimulation, whereas both Ang II and AVP enhanced the pressor effects of exogenous norepinephrine to the same extent. This observation rules out the possibility that the effect of Ang II on stimulation-evoked pressor responses might be due to "nonspecific" synergism of vasopressors.

The isolated rat hindquarter, perfused with an artificial buffer, has been a useful tool in the study of resistance vessel physiology, biochemistry, and structure under a variety of pathophysiological conditions. However, to our knowledge, stimulation of sympathetic innervation has not been combined with the isolated rat hindquarter perfused with an artificial buffer. Other investigators have stimulated the lumbar sympathetic chain in rats in vivo and demonstrated enhanced hindquarter vascular resistance. By means of intravital microscopy, Ohyanagi et al. could visualize constriction of skeletal muscle arterioles in the rat cremaster muscle during stimulation of the lumbar sympathetic chain. The method we describe provides a useful tool to study the effects of sympathetic innervation on skeletal muscle and cutaneous resistance vessels in vitro.

The pressor responses to nerve stimulation in our in vitro preparation were relatively small if compared with the effects of lumbar nerve stimulation in blood-perfused hindquarters in vivo. Hindquarter vessels are maximally dilated, if perfused in the absence of blood, and high concentrations of agonists are usually necessary to evoke vasoconstriction in such a preparation.

The bulk (75%) of the pressor response after electrical stimulation was mediated by a₁-adrenergic receptors, as evidenced by blockade with prazosin. Nonadrenergic transmitters, e.g., purines, and postsynaptic a₂-adrenergic receptors, which have been clearly demonstrated in the rat hindquarter circulation, may account for the prazosin-resistant component.

We evaluated the effects of two vasoconstrictor peptides, Ang II and AVP, on neurogenic vasoconstriction in the isolated rat hindquarter. Both peptides influence the sympathetic nervous system centrally. However, the prevailing evidence suggests that AVP does not affect the vasoconstrictor activity of the peripheral sympathetic nervous system, which is in agreement with our data.

In contrast to AVP, Ang II exerted complex effects on noradrenergic transmission in our model. At 1 and 10 Hz stimulation, Ang II enhanced the pressor response. Similar observations were reported for mesenteric preparations. However, we did not measure norepinephrine release, we cannot precisely determine the mechanisms by which Ang II enhanced the pressor response to nerve stimulation. However, several arguments point to presynaptic facilitation of transmitter release by Ang II. The enhanced pressor response cannot be explained by reuptake inhibition, because the
enhancement by Ang II was still observed in the presence of cocaine. Postsynaptic potentiation of the effects of norepinephrine is also an unlikely explanation, because the enhancement of noradrenergic transmission was not observed with AVP. However, the latter potentiated the pressor effects of exogenous norepinephrine, as did Ang II. Finally, others have shown that enhancement of the effects of nerve stimulation by Ang II corresponds to increased transmitter release in the perfused mesentery.2,28 At 20 Hz stimulation, Ang II significantly prolonged the duration of the increase in hindquarter resistance. This effect was similar to the prolongation caused by cocaine, although cocaine was more potent. No additional effect of Ang II was observed in the presence of cocaine at 20 Hz stimulation. Because Ang II also potentiated exogenous norepinephrine, our data suggest that Ang II may act as a norepinephrine reuptake blocker in resistance vessels, as reported by Campbell and Jackson2 for the isolated, perfused mesentery. However, some authors have found no evidence for reuptake inhibition by Ang II.3,6,8 The pressor effect of 20 Hz stimulation in the presence of Ang II could also be due to enhanced transmitter release and slow diffusion of norepinephrine away from the synaptic site. Furthermore, it is possible that Ang II shows different effects at different stimulation frequencies. Ellis and Burnstock4 described that, in the guinea pig vas deferens, Ang II enhanced the release of ATP at 2 Hz but not at 20 Hz stimulation. The prolongation of the pressor effect of 20 Hz stimulation by Ang II in our study was specific, since AVP did not exhibit such an effect at any stimulation frequency.

In contrast to our findings, Schwieler et al10 did not detect any effect of Ang II on sympathetic transmission in their model of an isolated canine skeletal muscle, even though they used high concentrations of the peptide. Differences in experimental conditions may partly explain this discrepancy. In contrast to our in vitro preparation, Schwieler et al10 used a blood-perfused skeletal muscle in vivo. Thus, plasma constituents influencing sympathetic nerve transmission could possibly account for the discrepancy between their results and our data. In addition, Schwieler et al could not maintain constant baseline pressure and flow during Ang II infusion. Furthermore, they could not test for a cocaine-like effect of Ang II, because all experiments were done during reuptake blockade.10 In view of the finding that marked differences in the facilitating action of Ang II exist between different rat strains,3 it is conceivable that differences between species may also account for the discrepant finding of our study and that of Schwieler et al.10

Reuptake inhibition could be an explanation for the enhancement of the pressor response to exogenous norepinephrine by Ang II. Other mechanisms may contribute as well, because subpressor concentrations of Ang II potentiate the effects of a number of vasoconstrictors other than norepinephrine.2 Activation of calcium channels3 or a sodium transporter4 has been implicated in this potentiation. Similar mechanisms may contribute to the enhancement of the pressor response to exogenous norepinephrine by subpressor AVP. In view of this marked potentiation, it is puzzling that AVP failed to influence noradrenergic transmission. Some authors have suggested that AVP inhibits ganglionic transmission in rabbits,29 whereas others reported inconsistent findings.5,13 Alternatively, AVP may solely potentiate the effects of the extrasynaptic adrenergic receptors. Synaptic and extrasynaptic α-adrenergic receptor populations differ; α-receptors are more prominent among extrasynaptic receptors.30 Our data are consistent with these findings, because prazosin blocked exogenous norepinephrine less effectively than the responses to nerve stimulation.

The subpressor concentration of Ang II used in our study was higher than blood levels under normal or pathophysiological conditions.31 However, Ang II is extensively metabolized in the hindquarter vascular bed.12,20 Thus, high amounts of exogenous peptide may be necessary to evoke the same tissue concentrations provided by the endogenous formation of Ang II. A local vascular renin-angiotensin system was recently described in the perfused rat hindquarter by ourselves12,20,32 and others.33,34 Blood levels of Ang II possibly represent only a spillover of higher tissue concentrations. In support of this notion, we previously observed that induction of local Ang II formation exerted marked pressor effects, even though only low concentrations of Ang II were released.17 Some authors27,28 reported that a local renin-angiotensin system in the mesentery may influence noradrenergic transmission. However, others have argued that nonspecific effects of the converting enzyme inhibitors used may account for the blunted sympathetic transmission.35,36

In summary, our data show that exogenous Ang II facilitated noradrenergic transmission in an isolated, perfused skeletal muscle, resistance vessel bed. This effect was seen only with Ang II and not with AVP and was not due to postsynaptic synergism with norepinephrine. Further studies will be necessary to elucidate the precise mechanisms of Ang II action and to investigate whether or not local formation of endogenous Ang II within skeletal muscle resistance vessels facilitates noradrenergic transmission.

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