Angiotensin II AT₁ receptors preserve vasodilator reactivity in skeletal muscle resistance arteries

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ANGIOTENSIN II AT₁ receptors preserve vasodilator reactivity in skeletal muscle resistance arteries. Am J Physiol Heart Circ Physiol 280: H2196–H2202, 2001.—Resistance arteries (100–150 μm) were isolated from the gracilis muscle of normotensive Sprague-Dawley rats placed on a high-salt (HS) diet (4.0% NaCl) for 3–7 days. Exposure to the HS diet eliminated vascular relaxation in response to hypoxia (P₂O₂ reduction to 35–40 Torr) and iloprost, a stable analog of prostacyclin. Vasodilator responses were restored in arteries isolated from chronically instrumented HS rats receiving a continuous intravenous infusion of either angiotensin II (ANG II; 5–6 ng·kg⁻¹·min⁻¹) or ANG II plus the AT₂ receptor blocker PD-123319 (5 μg·kg⁻¹·min⁻¹) for 3 days before the isolated vessel studies. In contrast, coinfusion of the AT₁ receptor blocker losartan (20 μg·kg⁻¹·min⁻¹) or coinfusion of both receptor blockers with ANG II eliminated the protective effect of ANG II to restore dilator responses to hypoxia and iloprost. Neither a HS diet nor ANG II infusion affected the dilation of gracilis arteries in response to direct activation of adenylyl cyclase by forskolin, suggesting that the effect of both the HS diet and the ANG II on the vasculature is mediated upstream from second messenger systems. These findings indicate that the protective effect of ANG II to maintain vasodilator reactivity in resistance arteries of rats on a HS diet is mediated via the AT₁ receptor subtype.

A N I N C R E A S I N G B O D Y O F E V I D E N C E indicates that changes in dietary salt intake and plasma angiotensin II (ANG II) levels can affect vascular structure and function. Wang and Prewitt (28, 29) reported that inhibition of ANG II production with the angiotensin-converting enzyme inhibitor captopril leads to a reduction in the cross-sectional wall area of the abdominal aorta, a reduction in the passive diameter and cross-sectional area of cremasteric feed arterioles, and a reduction of microvessel density in both hypertensive and normotensive rats. Elevations in dietary salt intake also lead to microvessel rarefaction and to significant alterations in the structure of microvessels of the cremaster muscle (10, 12). In a subsequent study, Hernandez et al. (13) reported that the intravenous infusion of a suppressor dose of ANG II prevents the microvascular rarefaction that occurs with a chronic elevation of dietary salt intake without causing an elevation in blood pressure. The latter observation suggests that the microvessel rarefaction and structural alterations observed in animals on a high-salt diet are due to the ANG II suppression that occurs in response to elevated salt intake.

Other studies (2, 3, 6–8, 14–16, 19–22, 25, 30) suggest that a high-salt diet can also lead to altered responses of blood vessels to a variety of vasoconstrictor and vasodilator stimuli. For example, recent studies (16, 30) in our laboratory have demonstrated that exposure to chronic and short-term (3 days) high-salt diets leads to a significant impairment of the relaxation of skeletal muscle resistance arteries and middle cerebral arteries in response to a variety of vasodilator stimuli acting at the level of the cell membrane. In those studies, the loss of vasodilator responses in animals on a high-salt diet could be prevented by maintaining circulating levels of ANG II via intravenous infusion of the peptide. The latter observation suggested that ANG II was acting to maintain normal vasodilator responses in resistance arteries, presumably through its interaction with ANG II receptors in the vessel wall. As opposed to more traditional roles of these ANG II receptors, which include both the control of growth and vascular tone, those findings suggested a previously undescribed role for ANG II, i.e., to maintain the normal responses of resistance arteries to different vasodilator stimuli.

The goal of the present study was to directly evaluate the role of AT₁ and AT₂, the ANG II receptor subtypes, in mediating the protective effect of ANG II to restore the responses to reduced P₂O₂ and iloprost, a stable analog of prostacyclin, in skeletal muscle resistance arteries of animals on a high-salt diet. The results of the study suggest that the protective effect of ANG II to preserve vasodilator responses in skeletal muscle resistance arteries of animals on a high-salt diet is mediated via a direct interaction of the hormone with its AT₁ receptor subtype.

MATERIALS AND METHODS

Experimental Animals

Male Sprague-Dawley rats (Harlan; Madison, WI) weighing 250–350 g at the time of the experiment were used for...
these studies. The Animal Care Committee of the Medical College of Wisconsin approved all of the protocols used in this study. Five experimental groups were utilized: one control group of rats maintained on a short-term, high-salt diet (4% NaCl) for 3 days, and four experimental groups of animals fed a high-salt diet and receiving a variety of intravenous infusions, including ANG II alone or ANG II with various ANG II receptor blockers (see Chronic Animal Preparation). All of the groups had tap water to drink ad libitum.

**Chronic Animal Preparation**

For the infusion studies, surgical procedures were completed as previously described (30). Briefly, the rats were anesthetized with an intraperitoneal injection containing ketamine HCl (78.0 mg/kg) and acepromazine maleate (2.2 mg/kg). Under sterile conditions, chronic indwelling catheters were placed in the femoral artery and vein and advanced into either the abdominal aorta (for blood pressure measurement) or into the inferior vena cava (to allow infusion of ANG II and ANG II with various ANG II receptor antagonists). The catheters were secured in the vessel with a 3-0 suture (Ethicon; Somerville, NJ) and anchored to the groin muscle with a surgical nylon suture (Braunamid). The catheters were filled with heparin sodium (1,000 U/ml), tunneled subcutaneously, and exteriorized at the back of the neck. The catheters were protected by being passed through a flexible spring, which was held in place by a leather jacket wrapping the upper torso of the rat. Springs were connected to a swivel outside of the cage, which allowed for free movement within the cage. All of the incisions were thoroughly cleaned with a surgical nylon suture (Braunamid). The catheters were allowed to recover for a minimum of 5 days before the beginning of the experiment.

**Infusion Protocols**

When the animals recovered from the surgery, the animals were placed on a high-salt diet for 1 wk, and the appropriate substance (ANG II, losartan, or PD-123319) was infused intravenously for 3 days before the experiment. The drug doses were as follows: ANG II alone (5–6 ng·kg⁻¹·min⁻¹), ANG II coinfused with losartan (20 μg·kg⁻¹·min⁻¹) and PD-123319 (5 μg·kg⁻¹·min⁻¹), ANG II coinfused with losartan, and ANG II coinfused with PD-123319. Blood pressure was monitored daily by the arterial catheters that were attached via a hydraulic swivel to pressure transducers (Argon; Athens, TX) for direct measurement of blood pressure. The transducer output was fed through a signal-conditioning amplifier (Stentech; Houston, TX), digitized at 100 samples/s, and analyzed with the use of software to compute systolic, diastolic, mean arterial pressure, and heart rate (Apollo Computer; Chelmsford, MA). Each daily pressure measurement represents the average of data obtained for 60 s during a period of continuous recording (minimum of 1 h) for which a pulse pressure >10 mmHg was maintained.

**Isolated Vessel Studies**

**General procedures.** On the day of the experiment, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg). The small muscular branch of the femoral artery supplying the gracilis muscle was carefully removed as described previously (4, 14, 30). Particular care was taken to handle the vessel by the connective tissue at the ends and to minimize stretching of the vessel during removal. The isolated artery was then placed in warmed physiological salt solution (PSS) bubbled with 21% O₂-5% CO₂-74% N₂. The PSS used in these experiments was composed of (in mM) 119 NaCl, 4.7 KCl, 1.17 MgSO₄, 1.6 CaCl₂, 1.18 NaH₂PO₄, 24 NaHCO₃, 0.026 EDTA, and 5.5 glucose.

After isolation, the vessel was placed in a heated (37°C) chamber that allowed the lumen of the artery to be perfused with PSS and the outside of the vessel to be superfused with PSS from separate reservoirs. The artery was cannulated at both ends with tapered glass micropipettes (diameter 100–150 μm) and secured onto the inflow and outflow pipettes with the use of a 10-0 nylon suture (22-μm diameter, Look; Norwell, MA). Any side branches were tied off with a single strand teased from a 6-0 silk suture (Ethicon). The inflow pipette was connected to a reservoir perfusion system that allowed the intraluminal pressure and luminal gas concentrations to be controlled (4, 5, 17). Vessel diameters were measured utilizing television microscopy and an on-screen video microscaler (model IV-550, FOR.A; Tokyo, Japan).

After the artery was mounted on the micropipettes, it was stretched to its in situ length in warmed, oxygenated (100 mmHg) to approximate the pressure encountered in vivo. The viability of the artery was assessed by measuring vessel diameter after a brief interruption of superfusion with administration of 1 μM norepinephrine to the vessel chamber. After the viability of the vessel was determined, the norepinephrine was washed out for 10 min, and the artery was equilibrated for 30 min with continuous superfusion and perfusion with PSS. Any vessel that did not constrict in response to norepinephrine or that did not show active tone at rest was not used in the study.

**Response to reduced PO₂.** After the initial control period (PSS equilibrated with 21% O₂), we determined the response to reduced oxygen availability in gracilis arteries from each group of animals. PO₂ reduction was achieved by a simultaneous perfusion and superfusion of the artery for 20 min with PSS equilibrated with 0% O₂-5% CO₂-95% N₂. The vessel chamber was covered with glass microscope slides throughout the experiment, except when diameters were measured. The PO₂ in the perfusate was reduced by bubbling the PSS in the perfusion reservoir with the same gas mixtures and by using gas-impermeable delivery lines. Under these conditions, control values for PO₂ during 21% O₂ perfusion/superfusion are ~140 Torr, whereas equilibration of the PSS reservoirs with 0% O₂ reduces both the luminal and extraluminal PO₂ to 35–40 Torr (4, 5). After the period of PO₂ reduction, the perfusate and superfusate were reequilibrated with 21% O₂ for a minimum of 20 min, and the recovery of the vessel from hypoxia was verified by measuring vessel diameter at the end of the recovery period in 21% O₂.

**Response to vasodilator agents.** The responses of skeletal muscle resistance arteries to the stable prostacyclin analog iloprost (10 pg/ml) and forskolin (10 μM) were assessed in each group of rats. The doses selected were based on previous studies (14) of vasodilator responses completed in our laboratory. To administer the drugs, the superfusion was stopped in the bath, and an appropriate amount of drug was added to the PSS to achieve the final desired concentration in the vessel chamber. Vessel diameters were monitored constantly and was measured at the point of its maximum value after addition of the drug. All of the measurements were determined with the vessel fully pressurized by clamping the outflow pipette.

After the response of the vessels to the different vasodilator stimuli was determined, active tone in the arteries was determined by measuring the diameter increase that occurred during maximal dilation with a Ca²⁺-free relaxing
solution that contained (in mM) 92.0 NaCl, 4.7 KCl, 1.17 MgSO4·7H2O, 20.0 MgCl-6H2O, 1.18 NaH2PO4, 24.0 NaHCO3, 0.026 EDTA, 2.0 EGTA, and 5.5 dextrose.

Compounds used for infusion and isolated vessel studies. For infusion protocols, the human analog of ANG II was obtained from Sigma (St. Louis, MO). Losartan was a gift from DuPont (Wilmington, DE), and PD-123319 was a gift from Parke-Davis (Ann Arbor, MI). For isolated vessel studies, forskolin and norepinephrine were purchased from Sigma. Iloprost was a gift from Berlex Laboratories (Wayne, NJ).

Statistical Analysis

All data were expressed as means ± SE. One-way analysis of variance (ANOVA) was used to compare separate treatment groups receiving a single dose of drug. Conscious blood pressures during drug infusion days were also compared with a mean value for the control days by use of one-way repeated measures ANOVA. Differences in means after one-way ANOVA were determined with a Student-Newman-Keuls test a posteriori.

RESULTS

Conscious Blood Pressure Measurements

Figure 1 shows the daily mean values of blood pressure determined via indwelling femoral artery catheters in rats maintained on a high-salt diet for 1 wk while receiving an intravenous infusion of ANG II with and without the various angiotensin receptor antagonists. Infusion of ANG II alone (5–6 ng·kg⁻¹·min⁻¹) resulted in a slight elevation of mean arterial pressure by the third day of infusion relative to the mean of the blood pressure determined during the saline control days. Infusion of ANG II with blockade of both receptors and coinfusion of ANG II and losartan had no effect on the arterial blood pressure, whereas infusion of ANG II with PD-123319 led to a small but significant increase in the mean arterial pressure during drug infusion.

Isolated Vessel Studies

To determine the effect of selective blockade of angiotensin receptors on the protective effect of ANG II to maintain the response of the arteries to vasodilator stimuli in animals on a high-salt diet, vasodilator responses were tested in isolated skeletal muscle resistance arteries from high-salt animals receiving an intravenous infusion of ANG II with and without the various angiotensin receptor antagonists. Previous studies (30) in our laboratory have demonstrated that resistance arteries of rats on a low-salt diet exhibit a significant dilation in response to all of the vasodilator stimuli employed in this study.

Figure 2 summarizes the response of gracilis arteries to reduced PO2 in rats maintained on a high-salt diet while receiving an intravenous infusion of ANG II with the various combinations of receptor blockers. In these experiments, exposure to a high-salt diet for 3 days eliminated hypoxic relaxation of the vessels, whereas infusion of low-dose ANG II in animals on a high-salt diet restored the vasodilator response to reduced PO2. When both angiotensin receptors were blocked by coinfusion of ANG II with losartan and PD-123319, the dilator response to reduced PO2 was eliminated. Coinfusion of the AT1 receptor antagonist losartan with ANG II in animals on the high-salt diet also prevented the restoration of hypoxic dilation by ANG II infusion. However, if ANG II was infused with the AT2 receptor antagonist PD-123319, the response to reduced PO2 was restored.

Similar results were obtained in studies assessing the response of isolated skeletal muscle resistance arteries to the stable prostacyclin analog iloprost (Fig. 3). In those studies, ANG II infusion, either alone or in conjunction with the AT2 receptor blocker PD-123319,
restored the relaxation of the vessels in response to iloprost. However, coinfusion of losartan alone or losartan plus PD-123319 eliminated the protective effect of ANG II to restore the vasodilator response to iloprost.

In addition to studies of vessels from high-salt animals receiving a continuous intravenous infusion of ANG II, we completed two series of control experiments. In one of these (Fig. 4A), acute incubation of vessels from animals on a high-salt diet with ANG II (10^{-10} M) in the chamber failed to restore vessel relaxation in response to hypoxia and iloprost, demonstrating that the protective effect of ANG II to restore dilator responses in animals on a high-salt diet was not a permissive effect due to the acute presence of ANG II. In a second series of control experiments (Fig. 4B), vessel responses to hypoxia and iloprost were determined in arteries isolated from rats on a low-salt (0.4% NaCl) diet for 3 days and continuously incubated with the angiotensin receptor antagonists losartan (10^{-7} M) and PD-123319 (10^{-7} M) during the experiment. The effectiveness of ANG II receptor blockade was verified by the elimination of ANG II-induced constriction of the vessel. In those experiments, arteries from animals on a low-salt diet exhibited normal dilator responses to reduced PO2 and iloprost in the presence of the blockers, demonstrating that the ANG II antagonists themselves did not directly affect vessel responses to these dilator stimuli.

As noted above, our earlier studies (14, 30) have demonstrated that skeletal muscle resistance arteries of animals on a low-salt diet exhibit a significant dilation in response to direct activation of adenyl cyclase with forskolin. In the present study, the response of the vessels to forskolin was not compromised after short-term elevations in dietary salt intake (Fig. 5), and the dilator response to forskolin was unaffected by infusion of ANG II with the various angiotensin receptor antagonists.

DISCUSSION

In addition to its classic actions in regulating vascular smooth muscle tone and fluid balance, ANG II has been demonstrated to have important effects on microvessel structure and reactivity (13, 16, 18, 21, 30). For example, recent studies have suggested an additional role of ANG II, namely to maintain normal

Fig. 3. Mean ± SE diameter change in response to iloprost (10 pg/ml) in skeletal muscle resistance arteries isolated from rats maintained on a short-term (3 days) HS diet (n = 9) or after 1 wk on a HS diet and receiving intravenous low-dose ANG II (n = 9), ANG II + losartan + PD-123319 (n = 6), ANG II + losartan (n = 7), or ANG II + PD-123319 (n = 6) for 3 days before the isolated vessel experiment. *P < 0.05, significant difference from salt-fed control without ANG II infusion.

Fig. 4. A: effect of low-dose ANG II (10^{-10} M) in the tissue bath on the response to reduced PO2 and iloprost in resistance arteries of animals on a HS diet. B: effect of losartan (10^{-7} M) and PD-123319 (10^{-7} M) in the tissue bath on the response to reduced PO2 and iloprost in resistance arteries from animals on a low-salt diet.

Fig. 5. Mean ± SE diameter increase in response to forskolin (10 μm) in skeletal muscle resistance arteries isolated from rats maintained on a short-term (3 days) HS diet (n = 9) or after 1 wk on a HS diet and receiving intravenous low-dose ANG II (n = 9), ANG II + losartan + PD-123319 (n = 6), ANG II + losartan (n = 7), or ANG II + PD-123319 (n = 6) for 3 days before the isolated vessel experiment. There were no significant differences in the response to forskolin in any of the groups.
vasodilator reactivity in arterioles and resistance arteries (9, 16, 30). Both chronic and short-term elevations in dietary salt intake lead to an impaired relaxation of skeletal muscle resistance arteries in response to acetylcholine, hypoxia, and iloprost (30). The impaired vasodilator responses after short-term exposure to a high-salt diet can be prevented by maintaining circulating levels of ANG II (30), suggesting that this peptide hormone has a protective effect to restore the mechanisms responsible for vascular relaxation in these vessels. On the basis of these findings, the goal of the present study was to elucidate the role of the specific ANG II receptor subtypes (AT_1 and AT_2) in mediating the protective effect of ANG II to restore normal vasodilator responses to hypoxia and iloprost in skeletal muscle of resistance arteries of animals on a high-salt diet.

To investigate this question, we used protocols similar to those of Hernandez et al. (13), in which the fall in ANG II levels in animals on a high-salt diet was opposed by intravenous infusion of a low dose of ANG II. To mimic the conditions of a short-term elevation in dietary salt intake, rats were placed on a high-salt diet for 1 wk before using the animal for an isolated vessel study. This allowed us to suppress the renin-angiotensin system initially and to then restore plasma ANG II levels by 3 days of low-dose ANG II infusion.

The dose of ANG II used in these experiments was a nearpressor dose in animals on a high-salt diet because the final day of drug treatment lead to a slight elevation in mean arterial pressure relative to an average of the two control days (Fig. 1). Despite the small increase in blood pressure, the infusion of ANG II in rats on a high-salt diet led to a restoration of the vasodilator responses to reduced PO_2 (Fig. 2) and iloprost (Fig. 3). These findings are in agreement with our earlier reports (16, 30), which demonstrated that ANG II restored vasodilator responses to reduced PO_2 and iloprost in resistance arteries of animals on a high-salt diet. Further support for a role of the renin-angiotensin system in maintaining normal vasodilator responses in resistance vessels is provided by a recent study (9) demonstrating that chronic administration of the angiotensin-converting enzyme inhibitor captopril (100 mg·kg^{-1}·day^{-1}) for 4–8 wk not only leads to alterations in vessel structure but also causes a significant blunting of arteriolar relaxation in response to a variety of vasodilator stimuli. Taken together, these observations imply that ANG II modulates both vascular growth and vasodilator responses in the microcirculation.

After demonstrating that ANG II infusion restored the relaxation of skeletal muscle resistance arteries in response to reduced PO_2 and iloprost in animals on a high-salt diet, we determined the role of the specific angiotensin receptor subtypes in mediating the protective effect of angiotensin to restore the response to vasodilator stimuli in these vessels. In those experiments, blood pressure did not increase in rats maintained on a high-salt diet and receiving a simultaneous intravenous infusion of ANG II plus losartan (AT_1) and PD-123319 (AT_2), because any pressor effect of the ANG II was prevented by pharmacologically blocking both of its receptor subtypes (Fig. 1). Coinfusion of ANG II with the AT_2 antagonist PD-123319 resulted in a small but significant elevation of blood pressure during the third day of drug infusion (Fig. 1), consistent with previous reports (18, 24), indicating that activation of the AT_2 receptor has a depressor effect.

Similar to our previous report (30), intravenous infusion of a low dose of ANG II alone restored vasodilator responses to reduced PO_2 and iloprost in isolated resistance arteries of rats maintained on a high-salt diet (Figs. 2 and 3, respectively). Coinfusion of ANG II with both angiotensin receptor antagonists or with the AT_1 receptor antagonist losartan alone eliminated the protective effect of ANG II to restore vascular relaxation, because vessels of animals receiving a confusion of ANG II with either losartan alone or with both of the receptor blockers failed to dilate in response to hypoxia (Fig. 2) and iloprost (Fig. 3). In contrast, infusion of ANG II with the AT_2 receptor antagonist PD-123319 restored the normal response of the vessels to these vasodilator stimuli in arteries of animals on a high-salt diet (Figs. 2 and 3). Taken together, these observations suggest that the protective effect of ANG II to maintain the response of the vessels to reduced PO_2 and iloprost is mediated by interaction of the peptide with the AT_1 receptor subtype.

The impairment of vascular relaxation in response to hypoxia and iloprost in resistance arteries of animals on a high-salt diet appears to be mediated via intrinsic alterations in the mechanisms of vascular relaxation in smooth muscle cells themselves, because hypoxic relaxation of rat gracilis arteries and middle cerebral arteries appears to be mediated by the release of prostacyclin from the endothelium (14, 17), and this is unaffected by high-salt diet (14). Prostacyclin causes dilation by activation of a prostacyclin receptor in the vascular smooth muscle cell membrane, resulting in a G_protein-mediated activation of adenyl cyclase. In the present study, the vessels exhibited an impaired relaxation in response to hypoxia and to direct application of iloprost. In contrast, the high-salt diet did not impair the dilation of the vessels in response to direct activation of adenyl cyclase with forskolin (Fig. 5). Taken together, these observations suggest that the high-salt diet alters vascular function at either the level of the membrane receptors or at the level of the G proteins in the signal transduction cascade of vascular smooth muscle relaxation. Previous studies have indicated that the coupling of the G protein to activation of adenyl cyclase is impaired both in reduced renal mass hypertension (27) and in spontaneously hypertensive rats (23). The present studies provide initial evidence that a high-salt diet can also affect receptor/G protein coupling to downstream steps in the cAMP-signaling cascade.

One question that remains unanswered is: What are the mechanisms by which the interaction of ANG II with the AT_1 receptor acts to preserve normal relaxation in the vascular smooth muscle cells? The interaction of ANG II with the AT_1 receptor leads to a
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variety of second messenger responses in the vascular smooth muscle cell. These include the following: 1) increases in the activity of phospholipases C, D, and A2; 2) elevations in inositol trisphosphate and diacylglycerol; 3) increases in intracellular Ca2+ levels via release from membrane stores and increases in the open-state probability of L-type Ca2+ channels; 4) elevation of arachidonic acid levels in the cell; and 5) activation of protein kinase C, mitogen-activated protein kinase, and various tyrosine kinases (1, 11, 26). ANG II also has prolonged effects on the cell, which include stimulation of gene transcription, sustained activation of phospholipase D, and increases in NADP and NADP oxidase activity (11). The current studies do not address the potential interaction of these second messengers with Gs proteins or membrane receptors, but it is conceivable that one or more of these second messengers may control intracellular enzymes or regulate the expression of genes that have a role in preserving normal receptor function or G protein coupling in the cells. In the light of these initial findings, elucidation of the intracellular mechanisms by which interaction of ANG II with the AT1 receptor preserves the mechanisms of vascular relaxation in skeletal muscle resistance arteries of animals on a high-salt diet is clearly a promising area for further investigation.

In summary, the results of the current study provide additional evidence that the impaired responses to vasodilator stimuli occurring as a result of elevations in dietary salt intake are due to a suppression of ANG II occurring in response to high-salt diet. Prevention of the reduction in the circulating levels of ANG II and maintenance of the interaction of ANG II with its AT1 receptor subtype appear to restore vascular relaxation either by maintaining membrane receptor function or G protein function/receptor coupling in the vascular smooth muscle cells. These findings suggest that circulating ANG II may have a protective effect on normal vascular function and may play a role in the normal maintenance of intracellular signaling mechanisms in the vascular smooth muscle cells of resistance arteries.

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