TRANSITIONAL PHYSIOLOGY

Chronic angiotensin II AT1 receptor blockade increases cerebral cortical microvessel density

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Munzenmaier, Diane H., and Andrew S. Greene. Chronic angiotensin II AT1 receptor blockade increases cerebral cortical microvessel density. Am J Physiol Heart Circ Physiol 290: H512–H516, 2006. First published September 30, 2005; doi:10.1152/ajpheart.01136.2004.—Angiotensin II is known to stimulate angiogenesis in the peripheral circulation through activation of the angiotensin II type 1 (AT1) receptor. This study investigated the effect of angiotensin receptor blockade on cerebral cortical microvessel density. Rats (6–7 wk old, n = 5–17) were instrumented with femoral arterial and venous indwelling catheters for arterial blood pressure measurement and drug administration. Rats were treated for 3 or 14 days with the AT1 receptor blocker losartan (50 mg/day in drinking water) or vehicle. Brains were sectioned and immunostained for CD31, and microvessel density was measured. Treatment with losartan for 3 or 14 days resulted in a slight decrease in mean arterial blood pressure (3 days, 92 ± 1 mmHg; and 14 days, 99 ± 2 mmHg) compared with vehicle (109 ± 3 and 125 ± 4 mmHg, respectively). A furosemide + captopril 14-day treatment group was added to control for the blood pressure change (96 ± 3 mmHg). Microvessel density increased in groups treated with losartan for 14 days (429 ± 13 vessels/mm²) compared with vehicle (383 ± 11 vessels/mm²), but did not change with furosemide + captopril (364 ± 7 vessels/mm²). Thus AT1 receptor blockade for 14 days resulted in increased cerebral microvessel density in a blood pressure-independent manner.

angiotensin receptor blockers; hypertension; microvascular growth

IT IS WELL RECOGNIZED THAT ANG II modulates vascular growth. ANG II exerts these actions through two receptor subtypes with opposing functions. In addition to its actions in promoting vasoconstriction and fluid conservation, ANG II type 1 (AT1) receptor activation is known to stimulate vascular growth (21) and microvascular angiogenesis (10) in nonneural tissues such as skeletal and cardiac muscle, whereas ANG II type 2 (AT2) receptor activation was recently shown to antagonize these actions (6, 10, 14, 15, 18). However, less is known about the actions of ANG II on vascular growth in the brain.

Functional opposition between the two ANG II receptors has led to many recent pharmaceutical advances. Specific antagonists for the AT1 receptor (e.g., losartan, candesartan, and irbesartan), known as a drug class as angiotensin receptor blockers (ARBs), are gaining wide use as antihypertensive agents. These drugs act by suppressing the hypertensive AT1 receptor-mediated actions of ANG II while preserving AT2 receptor-mediated vasorelaxation, making them theoretically optimal therapeutic compounds for the treatment of high blood pressure. Although ARBs are highly effective in reducing blood pressure, they do not appear to be more efficacious than other antihypertensive agents. However, recent studies indicate that ARBs confer additional benefits not directly related to their blood pressure-lowering actions (8). The recent Losartan Intervention For Endpoint reduction in hypertension study (LIFE study) was a double-masked, randomized, parallel-group trial with 9,193 participants over 4 years (2). Hypertensive participants were chronically treated with either losartan or atenolol. Although no differences in mean arterial blood pressure existed between treatment groups, the losartan group showed a 24.9% reduction in relative risk for stroke compared with the atenolol group. Recent evidence from animal studies has indicated that ARBs have neuroprotective effects independent of their actions on lowering blood pressure. Several studies, including observations from our own laboratory (5), have shown that ARB administration protects against cerebral ischemia, resulting in reduced infarct size (3, 9, 20). Whether this apparent preservation of neuronal function is due purely to direct actions of AT1 blockade on neurons, or whether enhanced local circulation might be involved, has not yet been addressed. Thus there appear to be specific actions of losartan, and presumably AT1 receptor antagonists in general, that are stroke protective, separate from their actions on blood pressure reduction.

This purpose of this study was to investigate the action of chronic AT1 receptor blockade on cerebral microvascular growth. Normotensive rats were treated with losartan for up to 2 wk. Losartan administration resulted in increased cerebral cortical microvessel density indicative of cerebral angiogenesis. These effects were shown to be independent of the modest hypotensive actions of losartan administration in otherwise normotensive animals.

METHODS

Chronic instrumentation. Six- to seven-week-old male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were anesthetized with 5 mg/kg ketamine, 0.5 mg/kg xylazine, and 0.1 mg/kg acepromazine (im) and instrumented for chronic arterial pressure measurement and venous infusion. Femoral arterial and venous indwelling catheters were implanted, tunneled subcutaneously, and exited through the back. For each rat, polyurethane catheters (Micro-Rennahete, Braintree Scientific, Braintree, MA) were housed in a stainless steel spring (McMaster Carr, Elmhurst, IL) attached to a polysulfone button (Instech, Plymouth Meeting, PA) that was sutured

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to the back with 4-0 silk. The spring was tethered to a swivel at the center of the cage so that catheters passed through the ceiling of the cage and the rat was able to move about the cage freely. Rats were allowed to recover from surgery for 3–4 days. Experimental protocols were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin in accordance with National Institutes of Health guidelines for the use of animals.

**Drug treatments.** Rats (n = 5–17/group) were given drinking water ad libitum with or without dissolved losartan. Water consumption was monitored, and losartan concentration was titrated to maintain a losartan intake of 50 mg·kg⁻¹·day⁻¹. Some groups received infusion of ANG II (20 ng·kg⁻¹·min⁻¹), captopril (100 mg·kg⁻¹·day⁻¹), or captopril (10 mg·kg⁻¹·day⁻¹) + furosemide (60 mg·kg⁻¹·day⁻¹) through the venous catheters at a rate of 0.5 ml/h. Rats not receiving intravenous drug delivery received saline infusion at the same rate.

**Blood pressure monitoring.** Systolic and diastolic blood pressures were measured from femoral arterial catheters and recorded every second for 1–3 h each morning. Mean arterial blood pressure was calculated from raw data. Control blood pressure measurements were taken for 3 days after recovery. Treatment blood pressure measurements were taken on every treatment day.

**Plasma ANG II measurement.** At the end of treatment, 1 ml of arterial blood was drawn into chilled tubes containing peptidase inhibitors. Samples were centrifuged, and plasma was stored at −80°C until extracted. Plasma ANG II was measured by HPLC and RIA as previously reported (12).

**Vessel density quantitation.** Brains were sectioned coronally in 4-µm thicknesses starting 4 mm rostral to bregma and progressing caudally. The sections were made at 17°C on a Leico Cryocut 1800 cryostat (Reichert-Jung, Heidelberg, Germany) and allowed to dry on microscope slides. Sections were fixed in cold acetone (−20°C) for 10 min. Immunohistochemistry was done with the Vectastain Elite avidin-biotin complex (ABC kit) (Vector Laboratories, Burlingame, CA) and the Dako Universal Staining System (Dako, Carpinteria, CA). Endogenous peroxidase activity was quenched with 1.5% H₂O₂ for 10 min. Slides were blocked for 30 min with 1% horse serum in Tris-buffered saline and incubated with avidin for 15 min and biotin for 15 min. Sections were then treated with primary anti-CD31 (platelet endothelial cell adhesion molecule (PECAM) 1) antibody (Chemicon: 1:400 in 3% horse serum) for 1 h. Slides were incubated with secondary biotin-conjugated anti-mouse antibody for 30 min and with ABC reagent for 30 min and treated with diaminobenzidine for 4 min. Slides were mounted with gelvatol and dried overnight. Sections were imaged on a Nikon Eclipse E400 microscope with a ×10 objective. Five adjacent 1-mm² images were taken from each of three sections in the frontal cortex region and the cingulated cortex, for a total of fifteen images used for automated vessel counting and quantitated with Metamorph software (version 4.6; Universal Imaging, Downingtown, PA). The average vessel density of the 15 images was used for the vessel density measurement for each rat.

**Statistics.** All data are expressed as means ± SE. Significance was determined by ANOVA and Dunnett’s test for comparison to control.

**RESULTS**

The goal of this study was to determine the effect of chronic angiotensin receptor blockade on cerebral microvascular density. In an attempt to ensure that drug treatments did not result in hemodynamic alterations that might indirectly affect vascular growth, systemic arterial blood pressures were taken daily throughout the treatment period. Blood pressures are reported as an average of the measurements from all treatment days (Table 1). Losartan treatment decreased blood pressure slightly throughout the treatment periods. Because blood pressure did decrease during losartan infusion, a group receiving furosemide and captopril was added to serve as a blood pressure control. This control was designed to reduce blood pressure by the same amount that losartan treatment did in an ANG II-independent manner; plasma ANG II concentrations in this group were held at control levels through inhibition of angiotensin-converting enzyme (ACE) by low-dose captopril. As expected, plasma ANG II levels increased significantly in all groups receiving losartan.

Cerebral vessel density was measured by an automatic method for counting microvessels immunostained for expression of PECAM-1, an endothelial cell-specific marker. Typical images of brain sections after 2 wk of vehicle and losartan treatment are shown in Fig. 1. Negative controls carried out in the absence of primary antibody resulted in the absence of discernible staining (Fig. 1A).

Cerebral cortical microvessel density was quantitated in all groups receiving ANG II receptor blockade at the end of the 3- or 14-day infusion period, and the results are shown in Fig. 2. Vessel density increased significantly after the 14-day losartan treatment compared with vehicle infusion. Preliminary studies using a lower dose of losartan (10 mg·kg⁻¹·day⁻¹) also resulted in an increased vessel density. However, the vascular changes were greater and more consistent with the higher dose (50 mg·kg⁻¹·day⁻¹), so all losartan groups included in this study received the higher dose.

In the blood pressure control group (furosemide + captopril; Fig. 3), vessel density was not different from the vehicle-infused control, indicating that the blood pressure change alone was not responsible for the change in vessel density observed in the losartan treatment group. To further address indirect actions of losartan treatment, rats were treated with high-dose ANG II to determine whether elevated circulating ANG II levels, as resulted from losartan treatment, could be responsible for the cerebral angiogenesis observed. Cerebral microvessel density with ANG II infusion was not different from that observed in vehicle-infused rats. Another group was treated with the ACE inhibitor captopril in an attempt to mimic the effects of AT₁ receptor blockade on vessel density. Again, no vessel density alterations were observed in these animals, indicating that the increase in vessel density seen with losartan treatment is due to specific AT₁ receptor blockade and the associated increase in circulating ANG II and not to generalized ANG II inhibition.

<table>
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<tr>
<th>Treatment Group</th>
<th>Treatment Period, days</th>
<th>Treatment MAP, mmHg</th>
<th>n</th>
<th>Plasma ANG II, pg/ml</th>
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<tr>
<td></td>
<td>14</td>
<td>98.6±2.3*</td>
<td>10</td>
<td>86.9±3.0*</td>
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<tr>
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<td>7</td>
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<td>5</td>
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Values are means ± SE for n rats. Mean arterial blood pressure (MAP) was measured daily and averaged over the 3- or 14-day treatment period. *P < 0.05 compared with vehicle group of same treatment period.
DISCUSSION

These studies demonstrated that administration of the AT1 receptor antagonist losartan for 14 days increased cerebral cortical microvessel density in Sprague-Dawley rats. Arterial blood pressure measured throughout the 2-wk period decreased slightly in the groups treated with losartan compared with control. Despite the assumption of cerebral autoregulation within the measured range of systemic arterial pressure observed in this study, a group was added as a blood pressure control. Rats treated with furosemide and captopril showed a similar reduction in blood pressure but did not exhibit cerebral vessel density alterations. These data indicate that chronic AT1 receptor blockade stimulates cerebral angiogenesis in a blood pressure-independent manner.

Losartan administration resulted in elevated circulating plasma ANG II levels in this study because of interruption of negative feedback, thereby enhancing AT2 receptor activation. The observation of increased vessel growth under these conditions is paradoxical because of the known vascular growth-inhibitory properties of the AT2 receptor (6, 10, 14, 15, 18). This would suggest either that the functional attributes of the two major ANG II receptor subtypes are somehow reversed in the brain vasculature or that the response is mediated by an indirect action of losartan (i.e., an action not directly targeting vascular growth), such as hemodynamic alterations in the brain.

Losartan is known to freely cross the blood-brain barrier. Although the observed actions of losartan are assumed to be due to blockade of vascular receptors, brain cell receptor involvement cannot be ruled out. Little evidence exists to suggest reversed receptor function in the brain in reference to cellular or vascular growth. ANG II activation has been impli-

![Fig. 1](image1.png)

**Fig. 1.** Visualization of cerebral microvessels for measurement of vessel density alterations. Brain sections were immunostained for CD31 (platelet endothelial cell adhesion molecule 1) to specifically stain cerebral microvessels. **A:** negative control in which the section was treated with secondary antibody in the absence of the primary antibody. Also shown are typical images taken from a vehicle-infused rat (B) and from a rat treated with oral losartan for 2 wk (C). Bar = 200 μm.

![Fig. 2](image2.png)

**Fig. 2.** Cerebral microvessel density quantitation with ANG II receptor blockade. Cerebral cortical microvessel density measurements were determined by averaging the vessel counts from fifteen 1-mm² cortical images. Group treated with losartan (Los) for 2 wk exhibited increased microvessel density compared with group treated with vehicle (VEH); n = 8–17. *P < 0.05 compared with vehicle group of same treatment period.

![Fig. 3](image3.png)

**Fig. 3.** Cerebral microvessel density quantitation with renin-angiotensin system manipulation. Cerebral vessel density after 2-wk treatment with vehicle, losartan, ANG II, captopril (CAP), or captopril + furosemide (CAP + FURO) combination is shown; n = 8–10. *P < 0.05 compared with vehicle group.
cated in brain tumor growth and tumor angiogenesis (4, 13). Glioma cells have been shown to express the AT1 receptor. Rivera et al. (13) demonstrated that rats with experimentally induced gliomas treated with oral losartan for 30 days had both reduced tumor growth and reduced angiogenesis associated with the tumor. Whether the actions of losartan in these results were to inhibit glioma cell growth, blood vessel growth, or both is not clear. However, these results suggest that the AT1 receptor acts to stimulate cell proliferation and angiogenesis in the brain as it does in nonneural tissues. No evidence exists to indicate that blockade of AT1 receptor in the brain would have proproliferation actions, either in the parenchyma or the vasculature.

Data regarding the role of ANG II on vessel growth in the normal brain are limited. Both the AT1 and AT2 receptor subtypes have been shown to be expressed in the cerebral vasculature, with AT2 being the more prevalent subtype in the fetus and AT1 being more prevalent in the adult (16, 17). A third receptor subtype, ANG II type 4 (AT4), has been shown to be localized in many regions of the brain and has been suggested to be involved in cellular proliferation (11) among a plethora of other actions (22). Although there is some evidence that the AT4 receptor might be present in aortic (1) and coronary (7) endothelial cells, it is not clear whether this subtype is expressed in blood vessels in the brain (19). If cerebral microvessel endothelial cells express AT4 receptors and they function to stimulate cell proliferation pathways, it might explain how blockade of AT1 (and concomitant stimulation of AT2) could result in cerebral angiogenesis. Blockade of both AT1 and AT2 receptors could further enhance AT4 stimulation, leading to a greater increase in vessel density with dual blockade. Preliminary data from our laboratory are consistent with this possibility; however, further investigation is required to test this provocative hypothesis.

Losartan administration might have had indirect actions on angiogenesis because of potential local hemodynamic alterations caused by chronic losartan administration. It is feasible that AT1 blockade (and the resulting increase in plasma ANG II) might result in cerebral vasodilation due to selective and enhanced activation of AT2 receptors. However, this would not be a probable stimulus for angiogenesis because local blood flow would be increased. Also, it is unlikely that local hemodynamic changes, if any, would be maintained chronically because of metabolic autoregulation. Although this possibility cannot be entirely ruled out without measurement of local blood flow throughout the treatment period, it does not constitute a likely mechanism for this observation.

Decreased cerebral blood flow could be a stimulus for angiogenesis, so it was critical in these studies that the slight decrease in blood pressure observed in the losartan-treated animals was controlled. Autoregulation should maintain cerebral circulation at optimal levels with the mean arterial blood pressure on angiogenesis. Treatment with furosemide and low-dose captopril was used to control for blood pressure in an ANG II-independent manner. Furosemide administration alone acts to reflexively increase circulating ANG II to very high levels. The addition of a low dose of captopril reduced ANG II close to control levels without completely blocking production altogether. These rats showed no increase in vessel density, confirming that the response was due to the actions of losartan, not to the decrease in mean arterial blood pressure resulting from losartan administration.

Finally, groups were added in which the renin-angiotensin system was manipulated to ensure that the results from losartan treatment were not due to generalized ANG II blockade or due to the elevated circulating ANG II levels that result from chronic losartan administration. Rats receiving high-dose ANG II or captopril infusion for 14 days did not exhibit alterations in vessel density compared with vehicle infusion, suggesting specific actions of AT1 receptor blockade and/or unmasking of non-AT1 ANG II receptor subtypes in the cerebral microcirculation.

The results of this study indicate that treatment with losartan for 2 wk increases cerebral microvessel density in rats in a blood pressure-independent manner. These data reveal an unexpected, but intriguing, outcome of chronic ARB administration that could help to explain the stroke-protective actions of chronic AT1 receptor blockade. This study adds to the recent mounting evidence in support of ARB therapy in the treatment of hypertension.

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GRANTS

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