Angiotensin II Produces Superoxide-Mediated Impairment of Endothelial Function in Cerebral Arterioles

Sean P. Didion, PhD; Frank M. Faraci, PhD

Background and Purpose—Angiotensin II (Ang II) produces oxidative stress in vascular cells in culture and in extracranial conduit arteries. The goal of this study was to examine the hypothesis that Ang II produces superoxide-mediated impairment of endothelial function in cerebral microvessels.

Methods—Diameter of cerebral arterioles (baseline diameter=104±3 µm) was measured with the use of a closed cranial window in anesthetized rabbits. Topical application of Ang II was used to avoid effects on arterial pressure.

Results—Ang II (0.1 to 1 µmol/L for 2 hours) had no effect on baseline diameter (change in diameter of −3±2% in response to 1 µmol/L Ang II) but produced concentration-dependent inhibition of vasodilatation to the endothelium-dependent agonist bradykinin. For example, 1 µmol/L Ang II inhibited responses to 1 nmol/L bradykinin by almost 80%. These inhibitory effects of Ang II were prevented by the superoxide scavenger 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron; 10 mmol/L) or diphenylene iodonium (DPI; 3 µmol/L), an inhibitor of NAD(P)H oxidase. Ang II did not inhibit vasodilatation in response to nitroprusside, an endothelium-independent vasodilator.

Conclusions—These findings are the first evidence that local Ang II produces superoxide-mediated vascular dysfunction in cerebral microvessels. The results with DPI suggest that the source of superoxide may be an NAD(P)H oxidase. (Stroke. 2003;34:2038-2042.)

Key Words: bradykinin ■ cerebral circulation ■ endothelium ■ reactive oxygen species ■ rabbits

In recent years, there has been renewed interest in the role of angiotensin II (Ang II) in vascular disease. In studies of extracranial blood vessels and vascular cells in culture, Ang II has been implicated as a major cause of oxidative stress, inflammation, and endothelial dysfunction as well as vascular hypertrophy and remodeling.1–4 For example, Ang II increases superoxide formation in cultured vascular muscle and endothelium and within the wall of intact vessels.5–8 We have found that superoxide is increased within the vessel wall and that endothelial dysfunction is present in animals made hypertensive using osmotic mini-pumps and in transgenic mice made hypertensive by expression of human renin and human angiotensinogen.9,10

Vasomotor effects of Ang II have been studied in the cerebral circulation, but the results of these studies have been quite divergent. For example, acute administration of Ang II has been reported to produce constriction,11–17 to have no effect,18 or to produce dilation19–21 on cerebral blood vessels. In contrast to these studies, nothing is known regarding the possibility that Ang II produces oxidative stress or impairment of endothelium-dependent relaxation in the cerebral circulation. Thus, the goals of the present study were to (1) examine the hypothesis that Ang II produces endothelial dysfunction in the cerebral circulation and (2) determine whether endothelial dysfunction in response to Ang II was mediated by superoxide. The approach used involved application of Ang II locally within a closed cranial window. This method has an advantage in that direct effects of the peptide on the cerebral microcirculation could be examined independent of changes in arterial pressure.

Materials and Methods

Animal Preparation

Experiments were performed on 41 New Zealand White rabbits (weight, 2.5 to 3.5 kg) that were anesthetized with pentobarbital sodium (40 mg · kg−1 · IV). Pentobarbital was supplemented regularly at approximately 10 mg · kg−1 · h−1. The trachea was cannulated, and the animals were ventilated mechanically with air and supplemental oxygen. Arterial blood gases were monitored and were stable throughout the experiment (PCO2=37±1 [mean±SE] mm Hg; Po2=110±1 mm Hg; pH 7.40±0.01). A femoral artery was cannulated for measurement of systemic pressure and to sample arterial blood. A femoral vein was cannulated for infusion of drugs. Rabbits were placed in a headholder, and a closed cranial window was placed over the parietal cortex as described previously.14–22 The cranial window was filled with artificial cerebrospinal fluid (CSF) warmed to 37°C. Diameter of pial arterioles were measured with the use of a microscope equipped with a television camera coupled to a video monitor. Images were recorded on videotape, and vessel diameters were measured later with an image analyzer. One cerebral arteriole was studied in each animal. Flushing the window with artificial CSF maintained at 37°C did not alter baseline diameter of arterioles.
Experimental Protocol

Seven groups of animals were studied (the n for each group is shown in Results). In each group, arteriolar diameter was measured under control conditions and after filling the window with artificial CSF containing the agonist to be studied (bradykinin or nitroprusside). Bradykinin is known to produce endothelium-dependent dilatation of cerebral arterioles.\textsuperscript{23,24} Nitroprusside is an endothelium-independent vasodilator. After application of the agonist, the cranial window was flushed several times, allowing cerebral arterioles to return to baseline diameter. After this initial measurement of vascular responses, the cranial window was treated with vehicle (the time control group) or Ang II (0.1 or 1 μmol/L in separate groups) for 2 hours. The cranial window was flushed with fresh CSF or Ang II–containing CSF in the vehicle and Ang II groups, respectively, every 15 minutes of the 2-hour pretreatment protocol and during subsequent application of agonists. The concentrations of Ang II and the time course for these experiments were based on previous studies in cultured cells and in extracranial arteries in vitro.\textsuperscript{5–8}

Finally, a second measurement of vascular responses was then made with the use of bradykinin or nitroprusside (done in the presence of Ang II or vehicle). In some groups, the second application of bradykinin was performed in the presence of 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron; 10 mmol/L), a scavenger of superoxide. We have shown previously that this concentration of Tiron is approximately as efficacious as polyethylene glycol–superoxide dismutase at inhibiting increases in superoxide in blood vessels.\textsuperscript{25}

An important source of Ang II–induced superoxide within blood vessels is thought to be a NAD(P)H oxidase. Thus, we also examined effects of diphenylene iodonium (DPI; 3 μmol/L), an inhibitor of flavin-containing enzymes and the most commonly used inhibitor of NAD(P)H oxidase activity.

Statistical Analysis

For comparison of vessel diameter under control conditions and during administration of an inhibitor, statistical analysis was performed with the use of paired \( t \) tests. All values are expressed as mean±SE. A probability value <0.05 was considered significant.

Results

Baseline diameter of cerebral arterioles was similar in the different groups and averaged 104±3 μm under control conditions (n=41). Mean arterial pressure was 79±1 mm Hg under baseline conditions and was not affected by application of Ang II into the cranial window. Similarly, arterial pressure was not affected by local application of bradykinin or nitroprusside or the inhibitors.

Ang II Inhibited Endothelial Function

Bradykinin produced reproducible dilatation of cerebral arterioles. In time control experiments, the maximum change in diameter in response to bradykinin (1 and 10 mmol/L) was 15±4 and 35±2 μm during the first application and 16±2 and 37±2 μm during the second application, respectively (n=5). Consistent with previous studies in rats and rabbits,\textsuperscript{20,21} application of Ang II into the cranial window produced initial vasodilatation. However, this response was transient, and baseline diameter of cerebral arterioles at the end of the 2-hour treatment with Ang II was not significantly different from control. For example, a change in arteriolar diameter of −3±2% (101±5 versus 98±5 μm; n=12) occurred after treatment with 1 μmol/L Ang II. In time control experiments, a stable baseline diameter was observed over this time period (data not shown).

Dilatation of cerebral arterioles in response to bradykinin (1 nmol/L) was inhibited by approximately 55% after a 2-hour treatment with 0.1 μmol/L Ang II (Figure 1). The higher concentration of Ang II (1 μmol/L) produced further inhibition of vasodilator responses to bradykinin (Figure 2). In contrast to responses to bradykinin, Ang II (1 μmol/L) did not alter dilatation of cerebral arterioles in response to nitroprusside (Figure 3). These findings indicate that Ang II produces concentration-dependent and selective inhibition of responses of cerebral arterioles to bradykinin.

Tiron, a Scavenger of Superoxide, Prevented Inhibitory Effect of Ang II on Endothelial Function

Baseline diameter of cerebral arterioles was not altered by Tiron (10 mmol/L; 101±3 versus 101±4 μm). Tiron completely prevented the inhibitor effects of Ang II (1 μmol/L) on vasodilator responses to bradykinin (Figure 4).

DPI (3 μmol/L; n=6) had no significant effect on baseline diameter of cerebral arterioles (111±3 versus 115±5 μm) but prevented the inhibitory effects of Ang II (1 μmol/L) on vasodilator responses to bradykinin. For example, bradykinin (10 nmol/L) dilated cerebral arterioles by 35±4 μm in the absence and 33±4 μm in the presence of DPI after treatment with Ang II (1 μmol/L). DPI treatment (in the absence of Ang II) had no effect of responses to bradykinin (data not shown).

Discussion

There are 2 major new findings in this study. First, local application of Ang II produced concentration-dependent inhibition of dilatation of cerebral arterioles in response to
bradykinin in vivo. In contrast, vasodilatation in response to nitroprusside was not affected, indicating that effects of Ang II were selective. Second, Tiron prevented inhibitory effects of Ang II on responses of cerebral arterioles to bradykinin. Inhibitory effects of Ang II on responses of cerebral arterioles to bradykinin were also prevented by DPI. To our knowledge, this is the first study to examine effects of Ang II on endothelial function in the cerebral circulation (in vivo or in vitro). Our findings support the concept that Ang II produces oxidative stress and impairment of endothelial function in cerebral microvessels in vivo. Importantly, these effects were due to direct actions of Ang II. The results with DPI suggest that the source of superoxide after stimulation with Ang II may be an NAD(P)H oxidase, which is consistent with previous studies in extracranial vessels or vascular cells in culture. Initial studies suggest than an NAD(P)H oxidase is expressed in cerebral blood vessels under normal conditions.

Ang II and Extracranial Blood Vessels

Multiple lines of evidence, both basic and clinical, suggest that Ang II plays a major role in several forms of vascular disease. Effects of Ang II on blood vessels (or components of the vessel wall) include increasing oxidative stress and formation of reactive oxygen species (ROS), activation of nuclear factor-κB and other transcription factors, inflammation, and endothelial dysfunction, as well as vascular hypertrophy and remodeling.1–3

In relation to oxidative stress, Ang II increases levels of reactive oxygen species in vascular cells in culture and in intact arteries from humans. The range of concentrations of Ang II that produce these effects (up to 10^{-6} mol/L) and the time course over which the effect occurs in vitro are similar to those in the present study performed in vivo.

In vivo, we and others have demonstrated that systemic infusion of Ang II with the use of osmotic mini-pumps for approximately 1 week increases superoxide and arterial pressure and impairs endothelial function in aorta. In a more chronic model, superoxide is increased and endothelial dysfunction is present in transgenic mice made chronically hypertensive by life-long expression of human renin and human angiotensinogen.

Ang II and the Cerebral Circulation

Chronic hypertension is a risk factor for carotid artery disease and stroke, and Ang II is known to contribute to some forms of hypertension. However, the roles of Ang II in changes in cerebral vascular structure and function during hypertension are poorly defined. Recent evidence suggests that Ang II produces vascular remodeling but not hypertrophy in cerebral arterioles during chronic hypertension.29

Acute administration of Ang II produces constriction of large cerebral arteries and cerebral arterioles in some species but can also produce dilatation of cerebral arterioles. In the present study Ang II produced transient dilatation of cerebral arterioles, consistent with previous studies, but had no significant effect on arteriolar diameter after 2 hours of treatment.

Before the present study, nothing was known regarding the possibility that Ang II may produce endothelial dysfunction in the cerebral circulation. Our results suggest that Ang II produces oxidative stress and impairment of responses of cerebral microvessels to an endothelium-dependent stimulus. Importantly, this effect of Ang II occurred independent of increases in arterial pressure. Thus, although Ang II is a powerful pressor agent, the present results indicate that Ang II can produce relatively rapid vascular dysfunction by a local mechanism that is not due to hypertension.

Although many studies have shown that Ang II–dependent hypertension is associated with increases in superoxide and endothelial dysfunction, very few have examined direct effects of Ang II independent of the pressor effects of the peptide. Wattanapitayakul et al recently reported that systemic administration of a subpressor dose of Ang II for 3 days caused formation of peroxynitrite and impairment of endothelial function in aorta in vitro. Our findings are consistent with this concept but extend it by suggesting that Ang II can produce superoxide-mediated impairment of endothelial function in a key microvascular bed in vivo.

It is interesting that baseline diameter of cerebral arterioles was not altered even though superoxide levels appear to be increased. This observation is consistent with previous work in which diabetes, as well as nicotine and alcohol, produced superoxide-mediated impairment of endothelial function even without changes in baseline diameter of vessels. Scavengers of superoxide had no effect on baseline vessel diameter in those studies or in the present study. The explanation for these findings is not clear, but this might occur if the level of superoxide needed to inhibit endothelial function is less than the concentration needed to alter baseline vascular tone.
Previous studies from our laboratory and others have suggested that superoxide can have complex effects on cerebral vascular tone (both vasodilator and vasoconstrictor effects) depending on concentration and perhaps subcellular location. Thus, the lack of change in baseline diameter might also reflect a balance between vasoconstrictor and vasodilator effects of superoxide.

Previous studies have shown (in several species including the rabbit) that dilatation of cerebral arterioles in response to bradykinin is dependent on activity of cyclooxygenase and mediated by ROS, including hydrogen peroxide. In this regard, it is noteworthy that Tiron, a scavenger of superoxide, prevented inhibitory effects of Ang II on bradykinin responses. We have previously shown that Tiron does not inhibit dilation of cerebral arterioles to bradykinin. Tiron is known to promote the formation of hydrogen peroxide as it scavenges superoxide. Thus, because hydrogen peroxide appears to be the mediator of responses to bradykinin, one would not expect Tiron to inhibit responses of cerebral arterioles to bradykinin. This finding is consistent with previous findings that cerebral vasodilator responses to bradykinin are blocked by catalase but augmented by superoxide dismutase, suggesting that responses to bradykinin are mediated by hydrogen peroxide.

It is interesting that Ang II produces superoxide-mediated impairment of a vascular response that is mediated by ROS (bradykinin-induced dilatation in cerebral arterioles). There is precedence for such a conclusion and mechanism. For example, overexpression of amyloid precursor protein (APP) in a transgenic mouse or topical application of amyloid-β peptide inhibits cerebral microvascular responses to bradykinin. APP and amyloid-β peptide are known to stimulate formation of superoxide, and overexpression of superoxide dismutase-1 prevents APP-induced impairment of bradykinin responses. Thus, although the mechanisms of these complex interactions are not fully understood, the present study and previous work suggest that superoxide and ROS can impair cerebral microvascular responses that are mediated by ROS.

**Functional Implications**

The present data suggest that exogenously applied Ang II, over a relatively short time period (2 hours), produces superoxide-mediated impairment of endothelial function in cerebral arterioles. It is important to note that in the present study, 100 nmol/L Ang II produced endothelial dysfunction in 2 hours. Previous work suggests that effects of Ang II in vessels are both concentration and time dependent. Thus, it is plausible that even lower concentrations of Ang II will increase superoxide and produce dysfunction of vessels exposed to the peptide for longer periods of time (such as during pathophysiological conditions).

Could endogenous Ang II produce similar effects? Multiple lines of evidence suggest that Ang II can be produced in brain. In stroke-prone spontaneously hypertensive rats that have endothelial dysfunction, local inhibition of angiotensin-converting enzyme restores endothelial function (responses to bradykinin and A23187) toward normal. Studies of focal ischemia in genetically altered mice suggest that reductions in cerebral blood flow in the ischemic core and penumbra are in part mediated by Ang II. Thus, endogenous Ang II appears to be a mediator of vascular dysfunction under some pathophysiological conditions. Unfortunately, relatively little is known regarding levels of Ang II in CSF, and, more importantly, within tissue and vessels, under normal conditions or in pathological states.

In conclusion, previous studies have suggested that superoxide impairs endothelial function in the carotid artery and/or cerebral circulation in several disease models, including inflammation, diabetes, ischemia, Alzheimer disease, and brain injury. The present findings extend this information and suggest that local increases in Ang II levels, produced by physiological or pathophysiological conditions, have the potential to produce superoxide-mediated endothelial dysfunction.

**Acknowledgments**

This work was supported by National Institutes of Health grants HL-62984, HL-38901, and NS-24621 Dr. Didion was supported by DK-25295 and a National Scientist Development Grant from the American Heart Association (0230327N). The authors thank Keith Breese for excellent technical assistance.

**References**


Angiotensin II Produces Superoxide-Mediated Impairment of Endothelial Function in Cerebral Arterioles
Sean P. Didion and Frank M. Faraci

Stroke. 2003;34:2038-2042; originally published online June 26, 2003;
doi: 10.1161/01.STR.0000081225.46324.AA
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/34/8/2038

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/