Angiotensin II Impairs Neurovascular Coupling in Neocortex Through NADPH Oxidase–Derived Radicals

Ken Kazama, Josef Anrather, Ping Zhou, Helene Girouard, Kelly Frys, Teresa A. Milner, Costantino Iadecola

Abstract—Angiotensin II (Ang II) exerts detrimental effects on cerebral circulation, the mechanisms of which have not been elucidated. In particular, Ang II impairs the increase in cerebral blood flow (CBF) produced by neural activity, a critical mechanism that matches substrate delivery with energy demands in brain. We investigated whether Ang II exerts its deleterious actions by activating Ang II type 1 (AT₁) receptors on cerebral blood vessels and producing reactive oxygen species (ROS) through NADPH oxidase. Somatosensory cortex CBF was monitored in anesthetized mice by laser-Doppler flowmetry. Ang II (0.25 μg/kg per minute IV) attenuated the CBF increase produced by mechanical stimulation of the vibrissae. The effect was blocked by the AT₁ antagonist losartan and by ROS scavenger superoxide dismutase or tiron and was not observed in mice lacking the gp91phox subunit of NADPH oxidase or in wild-type mice treated with the NADPH oxidase peptide inhibitor gp91ds-tat. Ang II increased ROS production in cerebral microvessels, an effect blocked by the ROS scavenger Mn(III)tetrakis (4-benzoic acid) porphyrin and by the NADPH oxidase assembly inhibitor apocynin. Ang II did not increase ROS production in gp91-null mice. Double-label immunoelectron microscopy demonstrated that AT₁ and gp91phox immunoreactivities were present in endothelium and adventitia of neocortical arterioles. Collectively, these findings suggest that Ang II impairs functional hyperemia by activating AT₁ receptors and inducing ROS production via a gp91phox containing NADPH oxidase. The data provide the mechanistic basis for the cerebrovascular dysregulation induced by Ang II and suggest novel therapeutic strategies to counteract the effects of hypertension on the brain. (Circ Res. 2004;95:1019-1026.)

Key Words: cerebral circulation ■ hypertension ■ somatosensory activation ■ gp91-null mice ■ laser-Doppler flowmetry

The functional and structural integrity of the brain depends on a continuous blood supply commensurate to its changing energy needs.¹ Thus, if a brain region is activated, cerebral blood flow (CBF) to that region increases to match the increased energy demands and to remove potentially deleterious byproducts of cellular metabolism.² This phenomenon, termed functional hyperemia, is crucial to maintain the homeostasis of the cerebral microenvironment, and its alteration leads to brain dysfunction and disease.³ Hypertension has profound effects on the brain and its circulation.⁴ Whereas hypertension alters the structure of cerebral blood vessels, it also disrupts regulation of CBF.⁵ These alterations are believed to underlie the cognitive impairment and brain damage associated with hypertension.⁶⁻⁷ Angiotensin II (Ang II) has emerged as a critical factor in the deleterious cerebrovascular effects of hypertension.⁸ Ang II produces cerebrovascular remodeling, promotes vascular inflammation, and impairs CBF regulation.⁹⁻¹¹ Importantly, Ang II attenuates the CBF increase produced by activation of the mouse somatosensory cortex.¹² Such impairment in functional hyperemia is not related to the associated elevation in arterial pressure (AP) or to actions of Ang II on neural activity.¹² Thus, Ang II may interfere with critical neurovascular processes that link increased neural activity to CBF.

The mechanisms of the Ang II-induced alteration in functional hyperemia have not been elucidated. Ang II exerts some of its cellular effects via Ang II type 1 (AT₁) receptors through production of reactive oxygen species (ROS) by the enzyme NADPH oxidase.¹³⁻¹⁴ However, it is not known whether the mechanisms by which Ang II disrupt functional hyperemia involve NADPH oxidase–derived ROS. Furthermore, although NADPH oxidase is found in brain,¹⁵ it remains to be established whether this enzyme is present in cerebral resistance arterioles with AT₁ receptors.

In this study, we investigated whether NADPH-derived ROS mediate the deleterious effects of Ang II on functional hyperemia. Using double-label immunoelectronmicroscopy, we found that the essential NADPH oxidase subunit gp91phox and AT₁ receptors are localized to the same cells of neocortical cerebral arterioles. Furthermore, using vibrissal stimulation as a model of neocortical activation, we found that NADPH oxidase–derived radicals are responsible for the...
attenuation in functional hyperemia produced by Ang II. These data provide novel insights into the mechanisms of the deleterious effects of Ang II on the cerebral circulation and suggest new treatment strategies for counteracting the cerebrovascular effects of hypertension.

Materials and Methods

General Surgical Procedures

All procedures were approved by the institutional animal care and use committee. Studies were conducted in 2- to 3-month-old C57BL/6J male mice (weight 20 to 30 g; The Jackson Laboratory; Bar Harbor, Me) or in gp91-null male mice16 back-crossed to the C57BL6/J strain for several generations. C57BL6/J mice were used as wild-type controls.17 Mice were anesthetized with isoflurane (maintenance 2%),18 intubated, and artificially ventilated (SAR-830; CWE Inc.). A femoral artery was cannulated for recording mean AP (MAP) and collecting blood samples. Rectal temperature was maintained at 37°C. After surgery, anesthesia was maintained only with urethane (750 mg/kg IP) and chloralose (50 mg/kg IP).18

Monitoring CBF

The parietal cortex was exposed (2×2 mm), the dura removed, and the site superfused with a modified Ringer’s solution (37°C; pH 7.3 to 7.4).18 CBF was monitored in the window with a laser-Doppler probe (Vasamedic). Outputs of the flowmeter and blood pressure transducer were connected to a computerized data acquisition system (MacLab). CBF was expressed as percentage increases relative to the resting level. Zero values were obtained after the heart was stopped by an overdose of isoflurane.

Cerebral Microvessel Preparation and ROS Measurement

The neocortices of C57BL/6J or gp91-null mice were dissected in medium 199, homogenized, centrifuged, resuspended, and the solution passed through a 40-μm nylon mesh.19 Microvessels retained on this mesh were washed, eluted from the mesh, and collected by centrifugation. Purity of preparations was >95%.19 A range of vessels spanning from capillaries (<10 μm diameter) to small arterioles/venules (50 to 100 μm diameter) is obtained with this procedure.19 ROS production was assessed by hydroethidine microfluorography. This method was chosen because it has been extensively validated in vascular preparations20 and is suitable to detect ROS production in small amounts of microvascular tissue.19 Hydroethidine (dihydroethidium) is cell permeable and is oxidized to become the fluorophore ethidium bromide that intercalates in double-stranded DNA.20 Brain microvessels were incubated in assay medium (DMEM without Phenol Red, 0.5% FBS, and 2 μmol/L hydroethidine) containing Ang II (100 nM) or vehicle for 1 hour. In some studies, vessels were preincubated for 30 minutes with the NADPH oxidase assembly inhibitor apocynin (100 μmol/L) or the ROS scavenger MnIII/tetrakis (4-benzoic acid) porphyrin (Mn-TBAP) (100 μmol/L). Fluorescent images of control and Ang II–stimulated vessels were captured with a Nikon E800 microscope equipped with a digital camera (Coolsnap; Roper Scientific). Images were postprocessed with IPLab (Scanalytics) as described previously.19 Usually, 20- to 40-vessel fragments with a total of 500 to 1000 nuclei were analyzed for each treatment group. All experimental groups were processed in the same session.

Electron Microscopy

C57BL/6J mice (n = 6) and Sprague-Dawley rats (n = 6; 250 to 325 g; Taconic Farms; Chatham, NY) were anesthetized (sodium pentobarbital, 150 mg/kg IP) and their brains perfused fixed with 3.75% acrolein and 2% paraformaldehyde in phosphate buffer.21 Coronal sections (40-μm thick) were cut through the somatosensory cortex on a Vibratome and processed for the dual localization of gp91phox and AT, receptors as described previously.21 Briefly, sections were incubated in a cocktail of goat anti-gp91 antibody (1:200 dilution; Santa Cruz Biotechnology)15 and rabbit anti-AT, antibody (1:500 dilution; provided by Dr R.C. Speth, University of Mississippi School of Pharmacy)22 in Tris saline containing 0.1% BSA. For gp91phox immunoreactivity, sections were incubated sequentially in: (1) anti-goat IgG conjugated to biotin (Vector Laboratories) diluted 1/400 in TS containing 0.1% BSA; (2) peroxidase-avidin complex (Vectastain Elite kit) in TS; and (3) 3,3’-diaminobenzidine (Aldrich Chemical) and hydrogen peroxide in TS.23 For AT, receptor immunoreactivity, sections were incubated in anti-rabbit IgG conjugated to 1-nm gold particles (AuroProbe One; Amersham). Sections were rinsed in PBS, postfixed in glutaraldehyde, and rinsed again. Gold particles were intensified by treatment with silver solution (Electron Microscopy Sciences [EMS]).24 Sections then were postfixed in 2% osmium tetroxide, dehydrated, and embedded in EMBed (EMS). Additional sections were processed for the single localization of gp91phox as described above. For this, the gp91phox antibody was diluted 1:100, and the secondary anti-goat IgG was conjugated to 1-nm gold particles (EMS). Ultrathin sections (70-nm thick) were taken from surface of the cortex as described previously.23 Sections were counterstained with Reynolds’s lead citrate and uranyl acetate and imaged with a digital camera (Advanced Microscopy Techniques, software version 3.2) on a Philips Tecnai Biotwin transmission electron microscope.

Implantation of Osmotic Minipumps for Sustained Delivery of Ang II

Osmotic minipumps containing saline or Ang II were implanted subcutaneously in mice under isoflurane anesthesia as described previously.12 Systolic AP and heart rate were monitored daily in awake mice using tail-cuff plethysmography. Mice were accustomed to the procedure for 4 days before pump implantation. Ang II was delivered at a rate of 2.74 mg/kg per day. Seven days after implantation, mice were anesthetized and instrumented for assessment of cerebrovascular reactivity by laser-Doppler flowmetry as described below.

Experimental Protocols

Effect of Ang II on Functional Hyperemia With or Without Topical Application of ROS Scavengers

After stabilization of MAP and blood gases (supplemental Table I, available in the online data supplement at http://circres.ahajournals.org), the whisker-barrel cortex was activated for 60 seconds by stroking the contralateral facial whiskers,18 and the evoked changes in CBF were recorded. After a stable response was achieved, Ang II (Ang II acetate; Sigma) was administered intravenously. The Ang II infusion was adjusted to elevate MAP by 20 to 25 mm Hg gradually over 10 to 15 minutes until a stable increase was obtained. At this time, the infusion rate was 0.25±0.02 μg/kg per minute, which produces elevations in plasma Ang II within the upper range of that produced by endogenous activation of the renin-angiotensin system in rodents.26 The response to whisker activation was tested again after 30 minutes of Ang II infusion. Next, polyethylene glycol–superoxide dismutase (PEG-SOD; 500 U/mL) or tiron (10 mmol/L) was superfused on the somatosensory cortex, and the response was tested again 30 minutes later. PEG-SOD is more diffusible than SOD and attenuates Ang II–induced ROS production in isolated vessels.27 In separate mice, CBF responses to hypercapnia (arterial PCO2–50 to 60 mm Hg) and superfusion with the NO donor S-nitroso-N-acetylpenicillamine (SNAP; 50 μL) were tested. A similar experimental protocol was used in mice in which Ang II was administered for 7 days using osmotic minipumps.

Effect of Ang II on Functional Hyperemia in gp91-Null Mice

These experiments were performed in mice deficient in the NADPH oxidase subunit gp91phox and wild-type littersmates. After baseline CBF responses were recorded, Ang II was infused intravenously as described above, and functional hyperemia tested again 30 minutes later.

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NADPH Oxidase Peptide Inhibitor

The NADPH oxidase peptide inhibitor gp91ds-tat (TAMRA-GGGGGRKKRRQRRRCSTRIRRQLRQRRR-NH2) and the scrambled sequence (sgp91ds-tat; TAMRA-GGGGGRKKRRQRRRCSTRIRRQLRQRRR-NH2; BioSynthesis) were used as described previously. This peptide includes part of the HIV–tat sequence (YGRKKRRQRRR) to facilitate entry into cells and interferes with NADPH oxidase assembly. The peptides were synthesized with a fluorescent tag (carboxytetramethylrhodamine; TAMRA). The GGGG sequence was introduced to minimize allosteric effects of the fluorescent tag. CBF was assessed 40 minutes after gp91ds-tat (1 μmol/L) or sgp91ds-tat (1 μmol/L) superfusion. The penetration of the peptide in the cortex was verified after the experiments by fluorescence microscopy.

Data Analysis

Data in text and figures are expressed as means±SE. Two-group comparisons were analyzed by the two-tailed Student t test. Multiple comparisons were evaluated by ANOVA and Tukey test. P values <0.05 were considered statistically significant.

Results

AT1 Receptors and gp91phox Are Present in Cortical Cerebral Arterioles

We used single- and dual-label immunoelectron microscopy to determine whether AT1 receptors and gp91phox are present within cells comprising the wall of cerebral arterioles. Rats and mice were studied. Analysis of the pial surface of the cerebral cortex revealed arterioles (diameter 80 to 100 μm) that contained AT1 and gp91phox immunoreactivities in the endothelial cell cytoplasm and in the adventitia (Figure 1A through 1C). AT1 receptor labeling was dispersed throughout the endothelial cell cytoplasm (Figure 1A and 1C), whereas gp91phox labeling was usually present in discrete patches (Figure 1B and 1C). Occasionally, AT1 receptor and gp91phox immunoreactivities were on the plasma membrane of endothelial cells (Figure 1A through 1C). No immunolabeling for gp91phox or AT1 receptors was observed in smooth muscle cells (Figure 1A and 1B). In cerebral capillaries, immunoreactivity for gp91phox was observed in endothelial cells (data not shown), perivascular astrocytes, and pericytes (Figure 1D), but these cells were without detectable immunolabeling for AT1 receptor (Figure 1C and 1D).

ROS Scavengers Reverse the Effect of Acute Administration of Ang II on Functional Hyperemia

The ROS scavengers PEG-SOD and tiron were used to test the hypothesis that ROS are involved in the Ang II–induced attenuation of functional hyperemia. In untreated C57BL/6J mice (n=5 per group), whisker stimulation increased CBF in the somatosensory cortex (Figure 2). Systemic administration of Ang II elevated MAP and attenuated the increase in CBF produced by whisker stimulation but did not affect resting CBF (Figure 2A through 2C). The attenuation was reversed by topical application of the AT1 receptor blocker losartan (5 μmol/L; CBF increase: Ringer 25±2%; Ang II 15±1%; losartan 25±2%; Ang II+losartan 23±1%; P>0.05 from Ringer; n=5 per group). Superfusion of the somatosensory cortex with PEG-SOD (500 U/mL; n=5 per group) reversed the attenuation produced by Ang II (Figure 2C). Ang II did not attenuate the increase in CBF produced by hypercapnia (Pco2 50 to 60 mm Hg) or by the NO donor SNAP (50 μmol/L; Figure 2E and 2F). We demonstrated previously that free radical scavengers do not alter resting CBF and functional hyperemia.18,26 To rule out the possibility that the SOD-induced reversal of the attenuation induced by Ang II was a consequence of loss of efficacy of the peptide,29 the effect of Ang II was tested 30 and 60 minutes after the start of the infusion (n=5 per group). As illustrated in Figure 2D, the effect of Ang II remained stable during this period. We then examined the effect of tiron on the attenuation in functional hyperemia produced by Ang II (n=5 per group). As with PEG-SOD, tiron (10 mmol/L) reversed the effect of Ang II (Figure 2C) without affecting resting CBF and the increase in CBF produced by SNAP (Figure 3D).
We used gp91-null mice to test the hypothesis that a gp91phox-containing NADPH oxidase is involved in the synthesis of the ROS responsible for the Ang II–induced attenuation of functional hyperemia. First, we investigated whether the ROS signal was present in cerebral microvessels and, if so, whether such increase is mediated by a gp91phox-containing NADPH oxidase. The ROS signal was demonstrated that Ang II increases ROS production and that the effect did not reach statistical significance (P>0.05; Figure 5). In wild-type mice (n=5 to 8 per group), Ang II increased ROS production, an effect that was attenuated by pretreatment with the ROS scavenger Mn-TBAP or the NADPH oxidase assembly inhibitor apocynin (100 μmol/L; Figure 5A). In contrast, in gp91-null mice (n=4 per group), angiotensin failed to increase ROS production (Figure 5B). In wild-type and gp91-null mice, the ROS scavenger Mn-TBAP (100 μmol/L) reduced ROS production below basal levels (Figure 5). To complement the data obtained with hydroethidine, 2’,7’-dichlorodihydrofluorescein diacetate (DCF-DA), a marker of H2O2, peroxides, and peroxynitrite, was also used. In agreement with the hydroethidine data, DCF-DA demonstrated that Ang II increases ROS production and that the effect is blocked by apocynin (supplemental Figure I).

### Role of NADPH Oxidase in the Effect of Ang II on Functional Hyperemia

We used gp91-null mice to test the hypothesis that a gp91phox-containing NADPH oxidase is involved in the synthesis of the ROS responsible for the Ang II–induced attenuation of functional hyperemia. First, we investigated whether the reactivity of the cerebral circulation was altered in gp91-null mice (n=5 per group). Thus, the increase in CBF produced by whisker stimulation (wild-type 28±1%; gp91/−/− 28±2%; P>0.05), acetylcholine (10 μmol/L; wild-type 25±3%; gp91/−/− 27±1%; P>0.05), and hypercapnia (Pco2 50 to 60 mm Hg; wild-type 77±8%; gp91/−/− 75±3%; P>0.05), as well as the CBF reduction produced by hypercapnia (Pco2 20 to 30 mm Hg; wild-type −23±2%; gp91/−/− −20±4%; P>0.05) did not differ between gp91-null mice and wild-type littermates. Contrary to a previous report in awake mice, AP did not differ between wild-type and gp91/−/− mice (Figure 4A), a finding attributable to effects of anesthesia. We then studied the effect of Ang II on functional hyperemia. Unlike wild-type mice (n=5), in gp91-null mice (n=5), Ang II elevated MAP (Figure 4A) but did not attenuate the CBF increase (Figure 4E). Similarly, in wild-type mice, superfusion with the peptide inhibitor gp91ds-tat but not its scrambled version sgp91ds-tat prevented the attenuation in functional hyperemia (Figure 4F). gp91ds-tat does not alter resting CBF and cerebrovascular reactivity in this preparation.

### Discussion

We demonstrated that ROS scavengers reverse the attenuation of functional hyperemia produced by acute or chronic (7-day) administration of Ang II, suggesting that ROS are responsible for the alteration in neurovascular coupling induced by this peptide. Mice lacking gp91phox and the peptide inhibitor gp91ds-tat were then used to investigate whether a gp91phox-containing NADPH oxidase was a source of the ROS. We found that Ang II did not attenuate functional hyperemia is gp91-null mice and in wild-type mice treated with gp91ds-tat. Furthermore, using double-label immunoelectronmicroscopy, we discovered that gp91phox and AT1 receptor immunoreactivities are present in cerebral resistance arterioles wherein they are localized in the same cell and in close proximity. We then used hydroethidine microfluorography to determine whether Ang II leads to NADPH oxidase–dependent ROS production in cerebral microvessels. We found that Ang II induces a robust ROS production in these vessels, an effect attenuated by the free radical scavenger Mn-TBAP and by the NADPH oxidase assembly inhibitor apocynin. Importantly, Ang II failed to increase ROS production in microvessels of gp91-null mice. Collectively, these findings provide evidence that the deleterious effects of Ang II on functional hyperemia depend on AT1 receptor activation.
and ROS production by a gp91phox-containing NADPH oxidase.

The findings of the present study cannot be attributed to differences in physiological parameters because AP and blood gases were carefully controlled and did not differ among the groups of mice studied. Furthermore, the effects of SOD or tiron on the attenuation of functional hyperemia cannot be attributed to a time-dependent loss of efficacy of Ang II because the effect of Ang II was stable over the experimental period. On the other hand, the increase in ROS production by Ang II in microvessels cannot be an artifact of the hydroethidine technique because the ROS increase was attenuated by Mn-TBAP and by the NADPH oxidase assembly inhibitor apocynin. Furthermore, the concordance between the data with hydroethidine and DCF-DA attests to the validity of ROS assessment in this preparation.

We found that gp91phox immunoreactivity is present in endothelial cells and adventitia of cerebral resistance arterioles. This finding represents the first ultrastructural localization of gp91phox to specific cell types in blood vessels. The lack of localization to smooth muscle cells was anticipated on the basis of less precise light microscopic and molecular evidence in systemic vessels, although gp91phox was reported in cultured smooth muscles from human arteries. The intracellular localization of gp91phox is in agreement with confocal data in endothelial cells and our own data in neurons and represents a major difference with the neutrophil oxidase, in which gp91phox is localized mainly to the cell membrane. However, because we used cell-permeable ROS scavengers, our experiments do not provide insight into whether the NADPH oxidase–derived ROS that mediate the vascular dysfunction are intracellular or extracellular. We also provided the first ultrastructural demonstration in any vascular district that gp91phox and AT1 receptors are present in the same cells and in close proximity. These observations not only provide the structural basis for the interaction between AT1 receptors and NADPH oxidase in cerebrovascular cells, but also represent the cellular underpinnings for the cerebrovascular effects of Ang II observed in the present study. Furthermore, although AT1 receptors and gp91phox immunoreactivities were rarely overlapping, their intracellular proximity provides the structural basis for their interaction.

Our electron microscopic findings that AT1 receptors and gp91phox immunoreactivities are located in endothelium and adventitia are consistent with several studies demonstrating that in large systemic arteries, Ang II induces ROS production predominantly in the adventitia and the endothelium. We also provided evidence concerning the cellular localization of AT1 receptors in cerebral resistance arterioles. Although AT1 receptors were described previously in cerebral arteries using receptor autoradiography, this technique does not have sufficient resolution to permit the localization to a specific cell type. We did not find AT1 receptors in smooth muscle cells of cerebral arterioles. Although we cannot rule out that their density in smooth muscle cells is below the detection threshold of immunoelectron microscopy or that

Figure 3. Effect of Ang II (All), with and without neocortical superfusion of tiron (10 mmol/L) on MAP (A), resting CBF (B), and on the CBF increase produced by whisker stimulation (C) or SNAP (D). (*P<0.05 from vehicle; ANOVA and Tukey test; n=5 per group.) E, Effect of chronic infusion (7 day) of Ang II on MAP. F, Effect of SOD on the attenuation of functional hyperemia in mice with chronic infusion (7 days) of Ang II. (*P<0.05 from respective saline group; t test; n=5 per group).
tissue fixation prevented their recognition by the antibody, recent immunocytochemical studies also failed to find AT\(_1\) receptors in rat cerebral smooth muscles. The apparent lack of AT\(_1\) receptors in cerebral smooth muscle cells is consistent with the finding that in cerebral arterioles, the constriction by Ang II is endothelium dependent. However, considering the possible sources of error, this issue needs to be addressed in future studies.

There are several potential mechanisms by which Ang II–generated ROS could alter neurovascular coupling. Although ROS could impair functional hyperemia by attenuating neural activity, this possibility does not seem plausible because Ang II does not alter the field potentials evoked by whisker stimulation. Therefore, it is more likely that Ang II acts at the vascular level. This possibility is supported by the findings that AT\(_1\) receptors and gp91phox are present in the same vascular cells, and that Ang II induces NADPH oxidase–dependent ROS production in cerebral microvessels. ROS could impair vascular function by scavenging NO to form peroxynitrite. Although this process removes the NO available for vasodilation, peroxynitrite produces DNA damage and activation of the DNA repair enzyme poly-ADP ribose polymerase, which, in turn, can impair vascular function by depleting essential energy substrates. Peroxynitrite also inhibits prostacyclin and mitochondrial SOD, leading, respectively, to impairment of vasodilation and further oxidative stress. In addition, ROS inactivate tetrahydrobiopterin, an essential cofactor for NO synthase (NOS). Loss of tetrahydrobiopterin not only reduces NO synthesis, but also produces “uncoupling” of NOS, whereby the enzyme produces superoxide instead of NO. Therefore, inactivation of NO, an agent that is involved in the increase in CBF produced by functional hyperemia, could account for the effect of Ang II–derived ROS on neurovascular coupling. However, it is of interest that Ang II did not attenuate the CBF response to the NO donor SNAP. This finding is in agreement with previous reports indicating that ROS attenuate the vasodilation produced by endogenous NO more readily than that produced by exogenous NO.

Hypertension has profound effects on the brain. Relatively mild elevations in AP alter cognition, lead to silent brain infarcts, and increase the incidence of ischemic and hemorrhagic strokes. Data from large clinical trials suggest that the renin-angiotensin system may play a critical role in these deleterious effects of hypertension. Although more studies are needed to clarify the specific role of Ang II in these alterations, the findings of the present study provide an additional mechanism for the deleterious actions of Ang II on the brain. Functional hyperemia is a homeostatic mechanism that assures that the energetic demands of the active brain are met at all times. Disturbances in this process impair brain function and may underlie the cognitive impairment associated with hypertension and accumulation of \(\beta\)-amyloid in brain. In addition, Ang II impairs endothelial-dependent vasodilation and cerebrovascular autoregulation and induces a proinflammatory state. The combined effect of these cerebrovascular alterations is likely to play a role in the increased susceptibility to cerebral ischemic injury induced
by Ang II. Because some of these effects depend on ROS, early therapy with antioxidants could counteract the deleterious consequences of Ang II on neurovascular regulation. However, considering the species difference in sensitivity to Ang II, these observations in mice need to be confirmed in other species as well. Nevertheless, the mechanistic insights provided by the present results enhance our understanding of the powerful effects of Ang II on the regulation of the cerebral circulation during neural activity.

In conclusion, we have demonstrated that Ang II induces profound alterations of the mechanisms linking synaptic activity to blood flow in the brain. This alteration is abrogated by ROS scavengers or by a peptide inhibitor of NADPH oxidase and is not observed in mice lacking the gp91phox subunit of NADPH oxidase. Whereas AT1 receptors and gp91phox are present in endothelial cells and adventitia of resistance arterioles, Ang II induces NADPH oxidase–dependent ROS production in isolated cerebral microvessels. Collectively, these findings provide evidence that the deleterious effects of Ang II on neurovascular coupling are mediated by vascular production of NADPH oxidase–derived ROS. Whereas these observations expand our understanding of the deleterious effects of hypertension on the brain, they unveil novel treatment strategies targeting NADPH oxidase–dependent ROS production in cerebral blood vessels.

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References


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Table 1 (supplemental material)

MAP and blood gases in the mice in which CBF was studied

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**gp91 nulls**

**gp91+/*
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Values are mean ± SE; * p<0.05 from respective control, Analysis of variance and Tukey’s test.
Effect of Ang II on ROS production assessed by the oxidation-sensitive fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA). Brain microvessels were isolated and processed as described in the Methods. Vessels were seeded on chamber slides and incubated in phenol-free D-MEM containing 20 µM DCF-DA for 30 minutes before Ang II (100 nM) was added. After 1 hour of incubation, vessels were washed and analyzed as described in the Methods. Data were evaluated by the analysis of variance. Ang II increased the fluorescent signal (RFU) and the effect was blocked by apocynin (apo). These data support the findings of the experiments in which hydroethidine was used to assess ROS production (fig. 5). *p<0.05 analysis of variance. n=5 experiments/group.