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 mice back-crossed to the C57BL6/J strain for several generations. C57BL6/J mice were used as wild-type controls. Mice were anesthetized with isoflurane (maintenance 2%), 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. Microvessels retained on this mesh were washed, eluted from the mesh, and collected by centrifugation. Purity of preparations was >95%. 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 preparations 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 fluorophere ethidium bromide that intercalates in double-stranded DNA. Brain microvessels were incubated in assay medium (DMEM without Phenol Red, 0.5% FBS, and 2 mmol/L HEPES) containing Ang II (100 nM) or vehicle for 1 hour. In some studies, vessels were preincubated for 30 minutes with the NO donor S-nitroso-N-acetylpenicillamine (SNAP) (10 mmol/L) or tiron (10 mmol/L) as 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. In separate mice, CBF responses to hypercapnia (arterial PCO2 60 mm Hg) and superfusion with the NO donor SNAP (50 μmol/L) 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 littermates. After baseline CBF responses were recorded, Ang II was infused intravenously as described above, and functional hyperemia tested again 30 minutes later.
NADPH Oxidase Peptide Inhibitor

The NADPH oxidase peptide inhibitor gp91ds-tat (TAMRA-GGGYGRKKRRQRRRCSTRIRRQSR-NH2) and the scrambled sequence (sgp91ds-tat; TAMRA-GGGYGRKKRRQRRRCSTRIRRQSR-NH2) 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.

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 (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. 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, 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 (ROS) responsible for the Ang II–induced attenuation of functional hyperemia. Effect of Ang II on the increase in CBF produced by hypercapnia (E) or SNAP (F). *P<0.05 from vehicle; ANOVA and Tukey test; n=5 per group.

**Figure 2.** Effect of intravenous infusion of Ang II (AII), with and without neocortical superfusion of PEG-SOD (SOD); 500 U/mL, on AP (A), resting CBF (B), and on the increase in CBF produced by whisker stimulation (C). D, Stability of the effect of Ang II on functional hyperemia. Effect of Ang II on the increase in CBF produced by hypercapnia (E) or SNAP (F). *P<0.05 from vehicle; ANOVA and Tukey test; n=5 per group.

**PEG-SOD Reverses the Effect of Sustained Administration of Ang II on Functional Hyperemia**

Sustained administration of Ang II but not phenylephrine for 7 days attenuates the elevation in CBF produced by whisker stimulation. To determine whether such attenuation is also reversed by topical application of PEG-SOD, we studied mice in which Ang II was infused systemically for 7 days using osmotic minipumps (n=5 per group). As in mice receiving Ang II acutely, PEG-SOD reversed attenuation in functional hyperemia induced by Ang II (Figure 3E and 3F).

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

We used gp91-null mice to test the hypothesis that a gp91\textsuperscript{flx}–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±2%; gp91\textsuperscript{−/−} 28±2%; P>0.05), acetylcholine (10 μmol/L; wild-type 25±3%; gp91\textsuperscript{−/−} 27±1%; P>0.05), and hypercapnia (Pco\textsubscript{2} 50 to 60 mm Hg; wild-type 77±8%; gp91\textsuperscript{−/−} 75±3%; P>0.05), as well as the CBF reduction produced by hypercapnia (Pco\textsubscript{2} 20 to 30 mm Hg; wild-type −23±2%; gp91\textsuperscript{−/−} −20±4%; P>0.05) did not differ between gp91-null mice and wild-type littermates. Contrary to a previous report in awake mice,\textsuperscript{17} AP did not differ between wild-type and gp91\textsuperscript{−/−} 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.\textsuperscript{28}

**Ang II Does Not Increase ROS Production in Cerebral Microvessels of gp91-Null Mice**

Hydroethidine microfluorography was used to determine whether Ang II increases ROS production in isolated cerebral microvessels and, if so, whether such increase is mediated by a gp91\textsuperscript{flx}–containing NADPH oxidase. The ROS signal was slightly higher in gp91-null mice than in wild-type mice, but 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 H\textsubscript{2}O\textsubscript{2}, peroxides, and peroxynitrite,\textsuperscript{32} 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).

**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 gp91\textsuperscript{flx} and the peptide inhibitor gp91ds-tat were then used to investigate whether a gp91\textsuperscript{flx}–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 gp91\textsuperscript{flx} and AT\textsubscript{1} 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 AT\textsubscript{1} 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...
tissue fixation prevented their recognition by the antibody, recent immunocytochemical studies also failed to find AT₁ receptors in rat cerebral smooth muscles. The apparent lack of AT₁ 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 AT₁ 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. 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 β-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.

Acknowledgments

This work was supported by grants from the National Institutes of Health (HL18974, NS37853, and NS38252). C.I. is recipient of a Javits Award from National Institute of Neurological Disorders and Stroke, National Institutes of Health. Losartan was a generous gift of Merck and DuPont. We thank Dr Robert C. Speth for providing the AT1 receptor antibody.

References


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 and Costantino Iadecola

*Circ Res.* 2004;95:1019-1026; originally published online October 21, 2004; doi: 10.1161/01.RES.0000148637.85595.c5
*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/95/10/1019

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2004/11/02/95.10.1019.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* 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 *Circulation Research* is online at:
http://circres.ahajournals.org//subscriptions/
## Table 1 (supplemental material)

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

<table>
<thead>
<tr>
<th></th>
<th>MAP (mmHg)</th>
<th>paCO2 (mmHg)</th>
<th>paO2 (mmHg)</th>
<th>pH</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SOD-Whisker and SNAP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>34.1±0.9</td>
<td>134.7±3.0</td>
<td></td>
<td>7.33±0.01</td>
<td>5</td>
</tr>
<tr>
<td>Ang II</td>
<td>33.4±0.4</td>
<td>136.0±1.4</td>
<td></td>
<td>7.36±0.01</td>
<td>4</td>
</tr>
<tr>
<td>Ang II+SOD</td>
<td>34.3±0.4</td>
<td>138.0±1.3</td>
<td></td>
<td>7.33±0.02</td>
<td>3</td>
</tr>
<tr>
<td><strong>SOD-Hypercapnia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>77.7±1.2</td>
<td>56.0±0.6*</td>
<td>127.1±1.2</td>
<td>7.12±0.01*</td>
<td>5</td>
</tr>
<tr>
<td>Ang II</td>
<td>100.0±3.2*</td>
<td>57.6±0.5*</td>
<td>129.4±2.3</td>
<td>7.09±0.02*</td>
<td>3</td>
</tr>
<tr>
<td>Ang II+SOD</td>
<td>98.3±2.8*</td>
<td>57.3±0.8*</td>
<td>127.0±2.6</td>
<td>7.10±0.01*</td>
<td>6</td>
</tr>
<tr>
<td><strong>Ang II Effect Stability</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>82.5±1.1</td>
<td>34.1±0.6</td>
<td>132.0±2.0</td>
<td>7.35±0.01</td>
<td>5</td>
</tr>
<tr>
<td>Ang II 30 min</td>
<td>101.7±1.7*</td>
<td>35.0±0.6</td>
<td>132.6±2.6</td>
<td>7.33±0.01</td>
<td>6</td>
</tr>
<tr>
<td>Ang II 60 min</td>
<td>101.0±1.0*</td>
<td>34.1±0.5</td>
<td>130.3±1.8</td>
<td>7.33±0.01</td>
<td>8</td>
</tr>
<tr>
<td><strong>Tiron-Whisker and SNAP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>34.7±0.9</td>
<td>125.1±1.8</td>
<td></td>
<td>7.33±0.01</td>
<td>8</td>
</tr>
<tr>
<td>Ang II</td>
<td>34.2±0.6</td>
<td>128.7±2.5</td>
<td></td>
<td>7.35±0.01</td>
<td>5</td>
</tr>
<tr>
<td>Ang II+Tiron</td>
<td>35.0±1.0</td>
<td>129.3±2.2</td>
<td></td>
<td>7.33±0.01</td>
<td>2</td>
</tr>
<tr>
<td><strong>Osmotic minipumps</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SOD-Whisker</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>34.7±1.1</td>
<td>133.0±4.9</td>
<td></td>
<td>7.36±0.02</td>
<td>5</td>
</tr>
<tr>
<td>Ang II</td>
<td>34.2±1.3</td>
<td>131.2±7.7</td>
<td></td>
<td>7.37±0.01</td>
<td>5</td>
</tr>
<tr>
<td>Ang II+SOD</td>
<td>35.4±1.1</td>
<td>131.3±3.2</td>
<td></td>
<td>7.38±0.01</td>
<td>5</td>
</tr>
</tbody>
</table>

**gp91 nulls**

**gp91+/+**
<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Fig. 4</th>
<th>Value ± SE</th>
<th>Value ± SE</th>
<th>Value ± SE</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>gp91/−−</td>
<td>Vehicle</td>
<td>Fig. 4</td>
<td>33.3±0.4</td>
<td>134.4±3.3</td>
<td>7.34±0.01</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Ang II</td>
<td></td>
<td>33.4±0.5</td>
<td>127.8±2.3</td>
<td>7.32±0.02</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>Fig. 4</td>
<td></td>
<td>33.7±0.4</td>
<td>134.5±2.5</td>
<td>7.33±0.01</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Ang II</td>
<td></td>
<td>33.4±0.3</td>
<td>131.0±1.9</td>
<td>7.34±0.01</td>
<td></td>
</tr>
</tbody>
</table>

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.