G Protein–Coupled Estrogen Receptor Agonist Improves Cerebral Microvascular Function After Hypoxia/Reoxygenation Injury in Male and Female Rats

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Background and Purpose—Reduced risk and severity of stroke in adult females are thought to depend on normal levels of endogenous estrogen, which is a known neuro- and vasoprotective agent in experimental cerebral ischemia. Recently, a novel G protein–coupled estrogen receptor (GPER, formerly GPR30) has been identified and may mediate the vasomotor and protective effects of estrogen. However, the signaling mechanisms associated with GPER in the cerebral microcirculation remain unclear. We investigated the mechanism of GPER-mediated vasoreactivity and also its vasoprotective effect after hypoxia/reoxygenation (H/RO) injury.

Methods—Rat cerebral penetrating arterioles from both sexes were isolated, cannulated, and pressurized. Vessel diameters were recorded by computer-aided videomicroscopy. To investigate vasomotor mechanism of the GPER agonist (G-1), several inhibitors with or without endothelial impairment were tested. Ischemia/reperfusion injury was simulated using H/RO. Vasomotor responses to adenosine triphosphate after H/RO were measured with or without G-1 and compared with controls.

Results—G-1 produced a vasodilatory response, which was partially dependent on endothelium-derived nitric oxide (NO) but not arachidonic acid cascades and endothelial hyperpolarization factor. Attenuation of G-1-vasodilation by the NO synthase inhibitor and endothelium-impairment were greater in vessels from female than male animals. G-1 treatment after H/RO injury fully restored arteriolar dilation to adenosine triphosphate compared with controls.

Conclusions—GPER agonist elicited dilation, which was partially caused by endothelial NO pathway and induced by direct relaxation of smooth muscle cells. Further, GPER agonist restored vessel function of arterioles after H/RO injury and may play an important role in the ability of estrogen to protect the cerebrovasculature against ischemia/reperfusion injury. *(Stroke. 2013;44:779-785.)*

Key Words: brain hypoxia ■ GPER protein ■ recovery of function ■ sex differences

Stroke presents sex differences in terms of disease risk and outcome.1 Lower risk and severity of ischemic stroke in women is thought to depend on normal endogenous levels of estrogen, which is a known neuro- and vasoprotective agent in experimental cerebral ischemia.2 Estrogen has a rapid vasodilatory effect in the systemic circulation, and it was thought that the effect has been mediated via the activation of 2 classic nuclear receptors: estrogen receptor-α (ERα) or -β (ER-β).3 Recently, a novel G protein–coupled estrogen receptor (GPER, formerly GPR30), was identified to bind estrogen and mediate rapid nongenomic signaling events.4 Furthermore, GPER expressed in human arteries and veins may mediate the acute vasodilatory effect of estrogen.5 However, the vasoactive effects associated with GPER and its signaling mechanisms in the cerebral microcirculation remain unclear.

Cerebral ischemia and reperfusion (I/R) is well known to induce early vascular abnormalities, including hyperemia, delayed hypoperfusion, and markedly depressed responsiveness to endothelium-mediated vasodilators such as acetylcholine.6,7 Numerous mechanisms causing the vessel dysfunction during I/R are suggested, including decreased nitric oxide (NO) availability,8 potassium channel inhibition,9 and increased production of reactive oxygen species (ROS).10 Chronic estrogen treatment can improve microvascular dysfunction after experimental cerebral I/R, possibly via preserving cGMP-dependent vasodilation11 or by reducing oxidative stress.12 The purpose of the present study was to elucidate the mechanism of GPER-mediated vasoreactivity in cerebral microcirculation and also its vasoprotective effect after hypoxia and reoxygenation (H/RO) injury.

Materials and Methods

Experimental protocols in the present study were approved by the Washington University Advisory Committee for Animal Resources.

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Vessel Isolation and Cannulation

The techniques used in this study for the dissection and cannulation of intracerebral arterioles were adopted from published methods and are described in detail in the online-only Data Supplement.

Hypoxia and Reoxygenation Injury

To simulate I/R we applied a method of H/RO. To induce H/RO, pial sheaths were incubated for 1 hour in the hypoxic bath (PO2 < 2%) and then transferred to the normoxic bath (PO2 of 21%) to induce reoxygenation. Vessels from pial sheaths incubated for 1 hour in the normoxic bath served as time controls. A detailed method of H/RO is described in the online-only Data Supplement.

Experimental Procedures

After cannulation, pressurization without intraluminal flow, and development of spontaneous tone we tested the vessel response to pH 6.8 and pH 7.65. To investigate whether activation via GPER can regulate tone in cerebral arterioles, concentration–response curves to the selective GPER agonist, G-1 (GPR30-specific compound 1, 1 mmol/L–10 μmol/L), or vehicle (ethanol), were applied in the arterioles from both male and female rats. The response to each concentration was allowed to stabilize (10–15 minutes) before the next concentration was applied. To test the mechanism of vasoreactive effect in G-1 we used air embolism to inhibit endothelial function (confirmed by lack of dilation to adenosine triphosphate [ATP]), L-NNa (N-Nitro-o-arginine, 10 μmol/L) to inhibit endothelial NO production, indomethacin (10 μmol/L) to inhibit cyclooxygenase, 17-ODYA (17-octadecadiynoic acid, 10 μmol/L) to inhibit cytochrome P-450, ETYA (5,8,11,14-Eicosatetraynoic acid, 10 μmol/L) to inhibit cyclooxygenase and lipooxygenase, and high concentration BaCl2, (100 μmol/L) to generally inhibit potassium channels. To assess the inhibitory actions of the GPER antagonist in both intact and endothelial impairment vessels we pretreated with G15 (1 μmol/L) or its vehicle (dimethyl sulfoxide) in the G-1 response (1 mmol/L–5 μmol/L). Finally, to investigate molecular mechanisms involved the GPER response after endothelial impairment we applied the protein kinase A inhibitor Rp-8-CPT-cAMP (8- (4-Chlorophenylthio) adenosine- 5′- cyclic monophosphorothioate, 10 μmol/L) and Rp-8-Br-PET-cGMP (β- Phenyl- 1, N2- etheno- 8- bromo guanosine- 3′- 5′- cyclic monophosphorothioate, 10 μmol/L), and 100 μmol/L BaCl2 before testing the response to G-1.

The vessels with H/RO or normoxia for control developed spontaneous tone and responded to pH 6.8 and pH 7.65. We used ATP to test for endothelial function in the H/RO vessels because ATP induces endothelium-dependent dilation in cerebral arterioles. To test whether G-1 or superoxide dismutase mimetic Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) can restore endothelial function after H/RO, vessels subjected to H/RO were incubated 1 hour with G-1 or MnTBAP, and the dilations to ATP (100 μmol/L) were measured before and after incubation. Chemicals were obtained from Sigma-Aldrich (St. Louis, MO), Tocris (St. Louis, MO) and Cayman Chemical (Ann Arbor, MI).

Statistical Analysis

All data are presented as mean±SEM, with n representing the number of observations. Statistics were conducted on absolute vessel diameters. All data analysis was done using a statistical software package (InStatGraphPad Software, San Diego, CA). Differences were considered significant at P<0.05 and determined by analysis of variance ANOVA with a post hoc Student-Newman-Keuls test or paired Student t test where appropriate. The data are presented as percent maximal diameter to correct for the changed control diameter and was calculated the following formula: % maximum dilation = [(Dmax−DBaseline)/(Dmax−Dnorm)]*100, where Dnorm is maximal passive diameter of the vessel at 60 mm Hg before the development of spontaneous tone, and Dmax is the baseline diameter of the arteriole before stimulation with G-1 and Dafter are the arteriolar diameter after the stimulation. Statistical Analysis

Results

G-1 Induced Arteriolar Vasomotor Responses

Intact cerebral penetrating arterioles had a maximal passive diameter of 75.5±2.41 μm in male (n=22) and 80.7±2.03 μm in female (n=14), and spontaneous tone diameter of 54.59±2.19 μm in male and 59.86±1.85 μm in female (baseline, −28.1±0.96% and −25.9±0.90% of their maximal passive diameter in male and female, respectively).

In isolated cerebral penetrating arterioles, the GPER agonist G-1 induced vasodilation that was not significantly different between male and female rats, with the vehicle having no effect (Figure 1). Endothelial impairment after air embolism partially attenuated the response to G-1 in both male and female vessels (Figure 2A and 2B).

Mechanistic Studies

Pretreatment of intact arterioles with L-NNa (10 μmol/L) inhibited vasodilation to the same extent as endothelial impairment in both male and female vessels (Figure 2A and 2B). The attenuation of G-1–induced vasodilation was significantly larger in vessels from females compared with males (Figure 2C). We next investigated whether vasodilation of G-1 was dependent on the arachidonic acid cascades or endothelial hyperpolarization factor in male rats. Application of indomethacin (10 μmol/L), 17-ODYA (10 μmol/L), ETYA (10 μmol/L), and BaCl2 (100 μmol/L) did not affect the vasodilation (data not shown).

The GPER antagonist G15 10 μmol/L caused a small dilation in both control and denuded vessels from male rats, but this was not different from vehicle (Figure 3A). Pretreatment with the GPER antagonist G15 (1 μmol/L) attenuated the G-1 response in intact vessels from male rats (Figure 3B) but not in endothelium-impaired vessels (Figure 3C). The residual vasodilation of G-1 after endothelial impairment vessels from male rats were not altered by Rp-8-CPT-cAMP (10 μmol/L, inhibitor of cAMP-dependent protein kinase type I and type II), Rp-8-Br-PET-cGMP (10 μmol/L, inhibitor of cGMP-dependent protein kinase G) and Rp-8-Br-PET-cGMP (10 μmol/L, inhibitor of cGMP-dependent protein kinase G).

Figure 1. Vasodilatory responses to increasing concentration of the G protein–coupled estrogen receptor (GPER) agonist, G-1, in cerebral penetrating arterioles from male and female rats. *P<0.05 vs vehicle.
cGMP-dependent protein kinases), and BaCl₂ (100 μmol/L, general potassium channel inhibitor; data not shown).

**H/RO and G-1 Mediated Vasomotor Responses**

The next experiments were carried out to study the vasoprotective effect of G-1 after H/RO. The H/RO vessels had a maximal passive diameter of 78.29±3.11 μm in male (n=14) and 78.79±3.00 μm in female (n=14), and spontaneous tone diameter of 57.14±2.34 μm in male and 55.64±2.15 μm in female (baseline, -37.17±0.90% and -41.75±1.21% of their maximal passive diameter in male and female, respectively) (Figure 4). The normoxic vessels had a maximal passive diameter of 77.14±4.70 μm in male (n=7) and 77.00±3.91 μm in female (n=5), and spontaneous tone diameter of 60.43±4.05 μm in male and 58.40±3.04 μm in female (baseline, −27.99±1.68% and −24.17±0.70% of their maximal passive diameter in male and female). The difference in spontaneous tone diameter of the vessels was not statistically significant. Further studies are needed to elucidate the mechanisms underlying these differences and to determine the role of GPER in vasoprotection.

**Figure 2.** Vasodilatory responses to increasing concentration of G-1 before or after L-NNA (10 μmol/L, endothelial nitric oxide synthase inhibitor) or endothelial impairment caused by air embolus. A, male rats. B, Female rats. C, Comparison between male and female rats. *P<0.05 vs L-NNA and endothelial impairment and male vs female.

**Figure 3.** A, Vasomotor responses to increasing concentration of the G protein-coupled estrogen receptor (GPER) antagonist, G15, in cerebral penetrating arterioles from male rats. B, Vasodilation response to increasing concentration of G-1 with or without G15 (1 μmol/L) in intact cerebral penetrating arterioles from male rats. C, Vasodilatory responses to increasing concentration of G-1 with or without G15 (1 μmol/L) in endothelial impaired cerebral penetrating arterioles from male rats. NS indicates no significant difference; *P<0.05 vs with G15.
The H/RO vessels developed significantly more tone compared with the normoxic vessels, but the pH response to either alkaline (pH 7.65) or acidic (pH 6.8) did not differ between H/RO and normoxic vessels (data not shown). After H/RO, vessels from female animals developed significantly greater spontaneous tone than male vessels (Fig. 4). After H/RO, dilation to G-1 at 10 and 100 nmol/L was reduced in vessels from both male and female animals compared with normoxic vessels (Figure 5A). We used extraluminal application of ATP to test for endothelium-dependent vasodilation before and after H/RO. In normoxic male vessels the ATP (100 μmol/L)-induced relative maximum dilation was 37.20±2.22%, which was reduced to 2.68±1.65% after H/RO (n=5, respectively).

Similarly, in female vessels dilation to ATP was reduced from 32.32±2.67% at normoxia to 0.91±0.91% (n=5, respectively), indicating that H/RO injury caused loss of endothelium-dependent dilation to ATP (Figure 5B and 5C). To test whether G-1 is vasoprotective we incubated the H/RO-injured vessels with G-1 of 100 nmol/L, a concentration that does not affect arteriolar diameter per se. In H/RO-injured vessels, G-1-treated vasodilation to ATP (100 μmol/L) were 33.28±2.51% in male (n=5) and 25.65±1.73% in female (n=5), and nontreated (time control) vasodilation to ATP (100 μmol/L) were 2.13±1.37% in male (n=5) and 1.14±1.14% in female (n=5; Figure 5B and 5C). G-1 significantly improved the ATP-vasodilation with H/RO in both male and female vessels similar to pre-H/RO dilations. Because it is hypothesized that G-1 may act as a reducer of oxidative stress by scavenging ROS,18,19 we also tested the oxygen radical scavenger MnTBAP, which fully restored dilation to 100 μmol/L ATP in H/RO injured vessels from male rats (see the online-only Data Supplement).

**Discussion**

The major findings of the present study are as follows: (1) The GPER agonist, G-1, induces significant vasodilation in cerebral penetrating arterioles from both male and female animals; (2) Vasodilation induced by G-1 is partially attenuated after endothelial impairment or endothelial nitric oxide synthase (eNOS) inhibition in vessels from both genders; (3) The attenuation of G-1-induced vasodilation is greater in arterioles from female animals compared with males; (4) Pretreatment with G-1 restores endothelium-dependent dilation to ATP after H/RO injury in vessels from both sexes. Collectively, these results suggest that activation of GPER elicits dilation of the penetrating arteriole via release of endothelium-derived NO and also causes direct smooth muscle cell-dependent dilation. Importantly, low vasoactive concentration of GPER agonist G-1 restores endothelial-dependent dilation to ATP after H/RO injury.

**Figure 4.** Spontaneous tone with or without hypoxia/reoxygenation (H/RO) in cerebral penetrating arterioles from male and female rats. Although tone development was not different between sexes at control (NS), H/RO significantly increased tone (*P<0.05) with vessels from females developing a greater tone compared with vessels from males (*P<0.05).

**Figure 5.** A, Vasodilatory response to increasing concentration of G-1 with or without hypoxia/reperfusion (H/RO) in cerebral penetrating arterioles from male and female rats. B and C, Vasodilation response to 100 μmol/L concentration of adenosine triphosphate (ATP) after H/RO and treatment of G-1 (subthreshold concentration 100 nmol/L) in cerebral penetrating arterioles from male and female rats. *P<0.05 vs control (normoxia).
Selective GPER Agonist G-1

This study found that selective GPER activation, application of G-1, elicits dilation in male and female rat intracerebral arterioles. Several studies demonstrated similar dilations in response to G-1 in vessels including aorta, carotid, and mesenteric arteries. In the systemic circulation, acute G-1 administration induces lower arterial pressure in normotensive rats and chronic G-1 treatment reduces blood pressure in ovariectomized female hypertensive mRen2.Lewis rats. G-1 is a specific agonist for GPER because G-1-mediated vasorelaxation was absent in mice deficient of GPER. In addition, G-1 binds at a similar affinity as estradiol (Kd \( \approx 10 \) nmol/L) but essentially shows no binding at ER\( \alpha \) or ER\( \beta \). Therefore, G-1 is suitable to elucidate the signaling mechanism of GPER to study the vasoreactivity of estrogen.

Vasodilatory Mechanisms of GPER Activation and Gender Differences of Vascular Function

It is well known that classical estrogen receptors, ER\( \alpha \) and ER\( \beta \), contribute to various signaling events in healthy and diseased vessels. Estrogen promotes endothelium-dependent relaxation or dilation by inducing the production/activity of NO, prostacyclin, and hyperpolarizing factor and inhibits the mechanism of vascular smooth muscle contraction. Conversely, very little is known about the role of the novel estrogen receptor, GPER, in regulating cerebral vascular tone. To determine whether GPER also mediates vasodilation via similar mechanisms, we investigated GPER agonist-induced responses in both intact and endothelium-impaired vessels. The present experiments demonstrated that GPER agonist elicits dilation partially dependent on endothelium-derived NO but not arachidonic acid cascades and endothelial hyperpolarization factor and inhibits the mechanism of vascular smooth muscle contraction.

Smooth Muscle Cells Dependent Vasodilation of G-1

We observed a residual vasodilation in response to G-1 in endothelial-impaired vessels, indicating a direct vasodilatory effect of G-1 on vascular smooth muscle cells. We sought to elucidate the molecular pathways involved in G protein coupling. However, we found that neither inhibiting protein kinase A nor protein kinase G reduced the residual vasodilation. This residual vasodilation indicates that GPER has a potential role for direct signaling on vascular smooth muscle cells in the cerebral microcirculation. Inese et al. showed that GPER in mice is expressed in endothelial cells of small systemic arteries; however, it is only expressed in the smooth muscle cells of cerebral vessels. Haas et al. also demonstrated that GPER is expressed in the smooth muscle cells of human arteries and veins. In addition, GPER mRNA and protein are found in smooth muscle of coronary arteries, and G-1 relaxes endothelium-denuded coronary arteries by activation of large-conductance calcium-sensitive potassium (BK\(_{Ca}\)) but not signaling of NO. However, the present study demonstrates that general inhibition of smooth muscle potassium channels did not attenuate the residual dilation of the endothelium-impaired cerebral arterioles. In contrast, some studies have demonstrated GPER immunostaining in both the endothelial and smooth muscle cells of rat aorta and carotid, although G-1 vasorelaxation was completely endothelium-dependent in these vessels. On the other hand, in mesenteric arteries of intact mRen2.Lewis female rats, GPER is also located in both the intima and media, and vasodilation in response to G-1 was partially attenuated by both endothelial denudation and pretreatment with the NOS inhibitor L-NAME. This partial attenuation in G-1 response of small systemic artery is very similar to cerebral arterioles.

We did not examine GPER immunoreactivity in the rat cerebral penetrating arterioles. However, Broughton, et al. observed GPER expression in the aorta, carotid, and middle cerebral arteries of both male and female rats and found no gender differences in the GPER expression, indicating that differences in gene expression are not the cause for the observed gender difference. Further studies are required to clarify whether vasoreactivity and signaling mechanism in GPER activation differ in vascular beds and in health versus disease. Possible mechanisms to explain GPER-mediated vasodilation include calcium antagonistic or desensitizing effects because GPER activation by G-1 blocks serotonin-induced changes in intracellular calcium. Furthermore, G-1 induces a robust increase in extracellular signal-regulated kinase 1/2 phosphorylation, and thus GPER may antagonize change in intracellular calcium caused by vasoconstrictor agonists, possibly via extracellular signal-regulated kinase 1/2.

Our observation also contrasts the reported dilatory mechanisms of classical estrogen receptors. Signaling via the ER\( \alpha \) and ER\( \beta \) on vascular smooth muscle cells attenuates protein kinase C and activates potassium channels and results in a relaxation of smooth muscle cells. However, we found that potassium channel activation was not involved in the dilatory response to G-1, indicating that classical ERs and GPER may use different signaling pathways.

Selective GPER Antagonist G15

The selective GPER antagonist G15 attenuated the relaxations of either mesenteric arteries or endothelium-denuded coronary arteries in the response of G-1. In the present study, G15...
partially attenuated the G-1–induced vasodilation in the intact cerebral arterioles but not in the endothelial-impaired vessels. The likely cause is that both G-1 and G15 show similar affinities for GPER and that the G15 concentration used was at the lower competing concentration (1 μmol/L). It is also a possibility that G-1 stimulates other signaling pathways besides GPER, ERα, and ERβ in vascular smooth muscle cells, and further studies for elucidating of the signaling mechanism in G-1 are needed.

**Vasoprotection After H/RO Injury by GPER Activation**

Several mechanisms have been suggested to mediate I/R-induced vessel dysfunction. Especially, increased production of ROS inhibits vascular potassium channel function after I/R. Exogenous oxygen anion may impair ATP and calcium-sensitive potassium (K_{ATP} and K_{Ca}) channel functions via protein kinase activation. Estrogen is thought to protect vascular integrity under pathological conditions such as atherosclerosis, cerebral ischemia, and head injury. Some studies have indicated that endogenous and exogenous estrogens protect cerebral vasodilatory capacity during ischemia and reduce hyperemia after cerebral I/R injury. However, the specific mechanisms by which ERα and ERβ estrogen mediate vasoprotection after ischemia are not well known, though improving mitochondrial function and decreasing ROS activity were suggested. We previously reported that ATP-induced dilation is mediated by BKCa and intermediate-conductance calcium-sensitive potassium channels in the vascular endothelial cells. Additionally, we found that H/RO directly induces its cerebral microvessel dysfunction via oxygen radicals inhibiting K_{Ca} channel (Dietrich FASEB 2007). Our results demonstrated that G-1 as well as MnTBAP (Dietrich FASEB 2007 and the online-only Data Supplement) restored endothelial function after H/RO and G-1 thus may be a ROS scavenger. NADPH oxidases are believed to be the major source of vascular ROS; however, Broughton et al showed that G-1 directly scavenges ROS and activation of GPER does not suppress vascular NADPH oxidases. Lindsey et al reported that G-1 may reduce oxidative stress in kidney tubules, but the underlying mechanism is not known. Therefore, future studies are necessary to elucidate detailed mechanisms of G-1 against ROS with I/R. This GPER activation as a vasoprotectant after H/RO may play an important role in sex differences in the severity of ischemic stroke.

**Perspectives**

The observed endothelial NO–dependent and smooth muscle cell–dependent intracerebral arteriolar dilatory response as well as restored endothelial function following H/RO after selective GPER activation in the present study may have therapeutic implications for human ischemic stroke. This hypothesis is supported by a recent study that GPER agonist may be neuroprotective after cerebral ischemia. Our findings may make G-1 an attractive candidate for therapeutic intervention against I/R injury, especially in the light of the low vasoactive concentration needed. However, further characterizations of G-1 mechanisms are needed.

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**Disclosures**

None.

**References**

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Method for vessels isolation and cannulation

83 Male and 33 female Sprague-Dawley rats weighing between 240 and 450 g (14 to 20 weeks olds, Harlan, Indianapolis, IN) were anesthetized with ketamine (250 mg/kg intraperitoneally) and decapitated. A penetrating arteriole was isolated from the distribution of middle cerebral artery and transferred to a vessel chamber (2.5-ml organ bath) mounted on the stage of an inverted video microscope (Zeiss 200), and was cannulated with glass micropipettes. No intraluminal flow was applied, and the transmural pressure was set at 60 mmHg and monitored continuously. We observed the internal diameter of the arterioles using a computerized diameter tracking system (Diamtrak, T.O. Neild, Flinders University, Adelaide, Australia) with a spatial resolution of 0.5 μm/pixel and a data acquisition rate of 10Hz. The maximal passive internal diameter of the arteriole was determined at 60 mmHg intraluminal pressure at room temperature. The organ bath was continuously circulated with a physiological saline solution (37.5°C; pH7.3) of the following composition (in mmol/L): 144 NaCl, 3.0 KCl, 2.5 CaCl₂, 1.4 MgSO₄, 2.0 pyruvate, 5.0 glucose, 0.02 ethylenediaminetetraacetic acid (EDTA), 1.21 NaH₂PO₄, and 2.0 3-(N-morpholino)propanesulfonic acid (MOPS). After an equilibration period, the arterioles developed spontaneous tone and we confirmed their viability by changing the extraluminal pH from 7.3 to 6.8 and from 7.3 to 7.65. Arterioles with poor tone (less than 20% decrease from the maximal passive diameter) or poor response to pH (less than 15% change in diameter after pH change) were excluded.

Hypoxia and reoxygenation injury (H/RO)
To simulate I/R we applied a method of H/RO. The isolated pial sheath with the attached penetrating arterioles was placed into small organ tubes which had the ends covered by nylon mesh to allow for free buffer access. We placed the tubes into a temperature-controlled (37°C) organ bath with two chambers (Diamond General). One chamber was bubbled with air (normoxia, \(PO_2\) of 21%), while the adjacent chamber was bubbled with nitrogen gas (hypoxia, \(PO_2 < 2\%\)). Oxygen tension was measured in both chambers using an oxygen electrode (Diamond General). To induce H/RO, pial sheaths where incubated for one hour in the hypoxic bath and then transferred to the normoxic bath to induce reoxygenation. Subsequently, we dissected penetrating arterioles from the pial sheaths and cannulated them as described above earlier. Vessels from pial sheaths incubated for one hour in the normoxic bath served as time controls.

*Results for superoxide dismutase mimetic Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP)*

In control study, we found that MnTBAP 1 and 10 \(\mu\)mol/L diluted neither normoxic nor hypoxic vessel from male, but MnTBAP 100 \(\mu\)mol/L caused dilation equally in both vessels (Supplemental Fig.S1). After H/RO injury, the dilation to 100 \(\mu\)mol/L ATP was significantly reduced compared to normoxic control (n=5, respectively). Then, the H/RO injured vessels were incubated 1 \(\mu\)mol/L MnTBAP, and the dilation to 100 \(\mu\)mol/L ATP were fully restored (Supplemental Fig.S2).

Supplemental Figure Legends
S1: Vasodilatory response to increasing concentration of the superoxide scavenger MnTBAP in both cerebral penetrating arterioles at normoxic control or after H/RO.

S2: Vasodilation response to 100 µmol/L concentration of ATP after H/RO and treatment of MnTBAP (subthreshold concentration 1 µmol/L) in cerebral penetrating arterioles from male rats.

*P<0.05 vs. control (normoxia).