Effect of Gender on NADPH-Oxidase Activity, Expression, and Function in the Cerebral Circulation
Role of Estrogen
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Background and Purpose—This study tested whether NADPH-oxidase activity, expression, and functional effects on vascular tone are influenced by gender in the rat cerebral circulation and whether such differences are estrogen-dependent.

Methods—NADPH-stimulated superoxide production by cerebral (basilar [BA]; middle cerebral) arteries from male and female Sprague-Dawley rats was measured using lucigenin-enhanced chemiluminescence and dihydroethidium. Protein expression of Nox1, Nox2, Nox4, superoxide dismutase 1 (SOD1), SOD2, and SOD3 was measured using Western blotting. Vascular responses of BA to NADPH were assessed in a myograph. Some female rats were ovariectomized and treated with either vehicle (dimethyl sulfoxide) or 17β-estradiol.

Results—NADPH-stimulated superoxide production by BA and middle cerebral arteries from males was approximately 2-fold greater than vessels from females. Superoxide production was virtually abolished by the NADPH-oxidase inhibitor, diphenyleneiodonium. Protein expression of Nox1 and Nox4 in BA was also higher in males than in females (2.4- and 2.8-fold, respectively), whereas Nox2, SOD1, SOD2, and SOD3 expression did not differ between genders. NADPH induced greater vasorelaxant effects in BA from males versus females (P<0.05). The hydrogen peroxide scavenger, catalase, abolished these NADPH-induced relaxations. NADPH-stimulated superoxide production by BA from ovariectomized rats treated with vehicle was 3-fold greater than levels in intact females. Treatment of ovariectomized rats with 17β-estradiol decreased superoxide production (P<0.05). NADPH-induced relaxations of BA were smaller in 17β-estradiol-treated than in vehicle-treated ovariectomized rats (P<0.05).

Conclusions—NADPH-oxidase activity and function are lower in cerebral arteries of female rats. These gender differences are estrogen-dependent and are associated with lower Nox1 and Nox4 expression. (Stroke. 2007;38:2142-2149.)

Key Words: cerebral arteries ■ gender ■ NADPH oxidase ■ reactive oxygen species

NADPH-oxidases are an important source of reactive oxygen species (ROS) within the vasculature. These enzymes are membrane-associated and generate superoxide (O2−) by transferring electrons from NADPH to molecular oxygen through a flavin-containing “Nox” catalytic subunit. We have recently reported that the activity of vascular NADPH-oxidase is up to 100-fold greater in rat cerebral arteries versus systemic arteries, suggesting that this enzyme may be of particular importance within the cerebral circulation.

Epidemiological studies have revealed that the incidence of cerebrovascular diseases such as stroke is lower in premenopausal women than in men of similar age and postmenopausal women, perhaps consistent with the female sex hormone, estrogen, protecting the vasculature from disease. There is a growing body of evidence indicating that excessive generation of O2− by NADPH-oxidase contributes to oxidative stress associated with cardiovascular and cerebrovascular diseases. In vivo levels of biomarkers of oxidative stress are higher in healthy young males than in age-matched females. Moreover, in cultured cells, and in systemic arteries of ovariectomized mice, application of exogenous estrogen can inhibit NADPH-oxidase activity by downregulating the expression of several important subunits of the enzyme complex. It is unknown whether gender influences the generation of ROS by NADPH-oxidase in the cerebral circulation. The aim of the present study was to therefore test whether the activity, expression, and/or function of NADPH-oxidase in the cerebral circulation is gender-related and if any differences are attributable to the actions of endogenous estrogen.

Materials and Methods
All procedures were approved by the institutional animal ethics committee. In total, 98 male (weight, 295±5.3g) and 132 female...
quantification and localization of superoxide production by cerebral arteries
basilar and middle cerebral arteries were excised from male and female rats and cut into 5-mm-ring segments. NADPH (100 μmol/L)-stimulated O₂⁻ production was measured by 5 μmol/L lucigenin-enhanced chemiluminescence as previously described. 14 In some experiments, arteries were treated with the NADPH-oxidase inhibitor, diphenyleneiodonium (5 μmol/L) or the nitric oxide synthase inhibitor, N-nitro-l-arginine methyl ester (100 μmol/L). Background counts were subtracted and O₂⁻ production normalized for dry tissue weight.

Dihydroethidium (DHE; 2 μmol/L) was used to localize and measure O₂⁻ production in frozen sections (16 μm) of basilar arteries from male and female rats as previously described. 15,16 In the presence of O₂⁻, DHE is oxidized to ethidium and hydroxyethidium, which intercalate into DNA producing a nuclear staining pattern. Arterial sections were treated with NADPH (100 μmol/L) for 30 minutes before treatment with DHE. Some sections were treated with polyethylene glycol-superoxide dismutase (250 U/mL) for 30 minutes before incubation with DHE. Fluorescent images were acquired as 8-bit (256-intensity levels) and were analyzed with Image-Pro Plus software (version 5.0.1.11, Media Cybernetics Inc.). For each arterial section, pixels in the upper 50% intensity level were counted, background fluorescence was subtracted, and data were presented as fluorescent units per millimeter squared of cross-sectional artery wall area. Incubations and fluorescent intensity measurements were carried out on a basilar artery from a male and female rat in paired fashion on the same day.

protein expression of nox subunits and superoxide dismutase isoforms
protein expression of NADPH-oxidase catalytic subunits, Nox1, Nox2, and Nox4, and SOD isoforms, SOD1, SOD2, and SOD3, was measured in basilar arteries from male and female rats using Western blotting. Anti-Nox1 and anti-Nox4 rabbit polyclonal antibodies were raised against the Nox1 and Nox4 peptides, respectively. We have previously demonstrated that preincubation of Nox1 or Nox4 antibody sera with Nox1 or Nox4 peptide (10 μg/mL), respectively, results in undetectable immunoreactive bands, implying specificity of the antibodies. 17 Anti-Nox2 goat polyclonal antibodies were obtained from Santa Cruz Biotechnology. Anti-SOD1, anti-SOD2, and anti-SOD3 rabbit polyclonal antibodies were obtained from Upstate (Chemicon; Boronia, Australia). Basilar arteries were homogenized in ice-cold lysis buffer (composition in mmol/L: sucrose 250; HEPES 10) with protease inhibitors (Roche Complete Mini, #1836153) using 0.2-mL glass homogenizers. Homogenates were cleared by centrifugation (10,000 relative centrifugal force, 5 minutes, 4°C) and protein concentration was determined using the Bradford protein assay (BioRad). For Western blotting, gel sample buffer was added to supernates and boiled for 5 minutes. Equal amounts of protein were loaded onto a 10% polyacrylamide gel and buffer was added to supernates and boiled for 5 minutes. Equal amounts of protein were loaded onto a 10% polyacrylamide gel and buffer was added to supernates and boiled for 5 minutes. Equal amounts of protein were loaded onto a 10% polyacrylamide gel and buffer was added to supernates and boiled for 5 minutes. Equal amounts of protein were loaded onto a 10% polyacrylamide gel and buffer was added to supernates and boiled for 5 minutes. Equal amounts of protein were loaded onto a 10% polyacrylamide gel and buffer was added to supernates and boiled for 5 minutes.
icant effect on NADPH-stimulated O$_2^\cdot$ production by basilar arteries from either gender (male, control, 5.1±0.5; N-nitro-$L$-arginine methyl ester, 7±1×10$^3$ counts/mg of dry tissue, n=6; female, control 2.5±0.1; N-nitro-$L$-arginine methyl ester, 2.9±0.3×10$^3$ counts/mg of dry tissue, n=7).

**Localization and Quantification of Superoxide Production by NADPH-Oxidase**

O$_2^\cdot$ production by NADPH-oxidase was localized and measured in arterial sections of basilar arteries from male and female rats using DHE. In the presence of NADPH, ethidium/hydroxyethidium fluorescence in basilar arteries from female rats was more confined to the adventitial side of the artery, whereas in males, fluorescence was found throughout the wall. Nevertheless, the important finding was that fluorescence was found to be approximately 50% lower than in males (P<0.01; Figure 2). Treatment of sections with polyethylene glycol-superoxide dismutase abolished fluorescence in arteries from both genders (data not shown), confirming that O$_2^\cdot$ was the ROS detected by DHE. By contrast, treatment with native Cu$^{2+}$/Zn$^{2+}$-SOD did not attenuate the signal (data not shown), indicating that the DHE fluorescence reflected intracellular O$_2^\cdot$.  

**Nox and Superoxide Dismutase Expression**

Protein expression of Nox1 and Nox4 was 2.4- and 2.8-fold higher, respectively, in basilar arteries from male versus female rats (P<0.05; Figure 3A, C). By contrast, Nox2 expression did not differ between genders (Figure 3B). Protein expression of SOD1, SOD2, and SOD3 in basilar arteries also did not differ between genders (Figure 3D–F).

**Effect of Gender on NADPH-Oxidase-Dependent Vasorelaxation in Cerebral Arteries**

Acetylcholine (10 μmol/L) elicited similar relaxation responses of basilar arteries from both genders (male, 84±3%; female, 81±4%), indicating a functional endothelium. NADPH (10 to 100 μmol/L) elicited concentration-dependent relaxations of basilar arteries from both genders (Figure 4A). However, relaxations were smaller in arteries from female rats (P<0.01). By contrast, relaxations to SNP were similar in both genders (Figure 4B).

**Effect of Gender on Vascular Responsiveness to Endogenous and Exogenous H$_2$O$_2$ in Cerebral Arteries**

In both genders, relaxation of basilar arteries to NADPH (10 to 100 μmol/L) was abolished by catalase (1000 U/mL; P<0.01; Figure 5A; data only shown for 100 μmol/L NADPH). Catalase had no effect on responses to SNP in vessels from either gender (1 μmol/L; Figure 5B). These experiments suggest that cerebrovascular relaxation to NADPH is mediated by endogenous H$_2$O$_2$ presumably derived from NADPH-oxidase activity.
We next tested whether basilar arteries from female rats were less sensitive to exogenous H$_2$O$_2$. Exogenous H$_2$O$_2$ (10 to 1000 μmol/L) induced a biphasic response: a small transient contraction followed by a large, sustained relaxation. There were no differences between genders with respect to contractions (data not shown) or relaxations (Figure 5C). However, catalase abolished relaxations to H$_2$O$_2$ in all arteries ($P<0.01$, data not shown).

**Effect of Ovariectomy and Estradiol Therapy**

Body weights of OVX rats treated with vehicle (OVX+Veh; 234±8 g, n=19) were significantly greater ($P<0.05$) than those treated with 17β-estradiol (OVX+E2; 212±7 g, n=20). OVX reduced uterine weight (control females, 1.9±0.2 g/kg of body weight, n=8; OVX+Veh, 0.8±0.04 g/kg of body weight, n=20, $P<0.001$). Uterine weight was also lower than normal in OVX+E2 rats (1.4±0.3 g/kg of body weight, n=20, $P<0.001$).
body weight, n=19, P<0.001), but was greater than in OVX/Veh rats (P<0.001).

NADPH-stimulated O₂⁻ production by basilar arteries from OVX/Veh rats was significantly greater (3-fold) than levels generated by arteries from intact females (P<0.05, Figure 6A). However, 17β-estradiol treatment in OVX rats decreased NADPH-stimulated O₂⁻ production as compared with vehicle-treated OVX rats (P<0.05, Figure 6A).

Acetylcholine elicited similar relaxation responses in basilar arteries from vehicle- or 17β-estradiol-treated OVX groups (OVX/Veh, 85±4%; OVX+E2, 83±5%). Relaxation of the basilar artery to 100 μmol/L NADPH was greater in OVX+Veh versus OVX+E2 rats (P<0.01; Figure 6B). Vasodilator responses to SNP did not differ between vehicle- and 17β-estradiol-treated OVX groups (Figure 6C). There were also no differences between OVX groups with respect to

Figure 5. Effect of catalase (1000 U/mL) on relaxation of basilar arteries from male and female rats to (A) NADPH (100 μmol/L) and (B) SNP (0.1 μmol/L). The effect of H₂O₂ (10 to 1000 μmol/L) on the vascular tone of basilar arteries from male and female rats (C). Values are expressed as percent relaxation of serotonin-induced tone and are given as mean±SEM (n=5 to 6). *P<0.05 versus control (unpaired t test).

Figure 6. The effect of NADPH (100 μmol/L) on O₂⁻ production of basilar arteries from intact female controls and OVX female rats treated with either vehicle or E2 for 14 days post-OVX (A, n=5 to 6). Vascular O₂⁻ production was measured using lucigenin-enhanced chemiluminescence. Also shown is the effect of NADPH (B, 10 to 100 μmol/L; n=5 to 7), SNP (C, 0.01 to 1 μmol/L; n=5 to 7), and H₂O₂ (D, 10 to 1000 μmol/L; n=5 to 7) on the tone of basilar arteries from OVX+Veh and OVX+E2 rats. Arteries were precontracted (50% of contraction to KPSS) with serotonin before addition of NADPH, SNP, or H₂O₂. Values are given as mean±SEM. *P<0.05 versus O₂⁻ production in arteries from intact female controls. **P<0.05 versus OVX+Veh rats (one-way analysis of variance). ***P<0.05 versus relaxations to 100 μmol/L NADPH in basilar arteries from OVX+E2 rats (unpaired t test).
relaxations to exogenous H_2O_2 (Figure 6D). Catalase (1000 U/mL) abolished NADPH-induced relaxations of basilar arteries from both OVX groups (data not shown).

Discussion
This study reveals that gender influences the activity, expression, and function of NADPH-oxidase within the rat cerebral circulation. Specifically, we found that O_2^- production by NADPH-oxidase is 50% lower in basilar and middle cerebral arteries from female than male rats. This lower level of O_2^- production by NADPH-oxidase in the basilar artery was associated with lower protein expression of the Nox catalytic subunits, Nox1 and Nox4. Activation of NADPH-oxidase in vitro elicited relaxation of the basilar artery from both genders; however, this response was smaller in females. Ovariectomy of female rats increased NADPH-oxidase activity and vasodilator function, and this was restored to normal levels by 17β-estradiol therapy. Thus, we report for the first time that the female gender is associated with lower cerebrovascular NADPH-oxidase activity, expression, and function than males. Furthermore, we provide strong evidence that this gender difference is dependent on estrogen and that it is possibly attributable to lower Nox1 and/or Nox4 activity.

The incidence of cerebrovascular disease is lower in premenopausal women than their male counterparts and postmenopausal women. Recently, NADPH-oxidase has attracted considerable attention as a contributor to the pathogenesis of several cerebrovascular diseases, including stroke. Indeed, there is substantial evidence supporting a key role for NADPH-oxidase in vascular dysfunction associated with cerebral ischemic reperfusion. Clinical studies have found that two commonly used markers for oxidative stress, malondialdehyde and 8-isoprostaglandin F_2α, are greater in healthy males than age-matched females. Furthermore, measurements of O_2^- production in the aorta have shown greater amounts in male than in female rats. However, no previous study has specifically addressed whether gender influences the activity of NADPH-oxidase in the cerebral circulation. Using the chemiluminescent probe, lucigenin, we found that the substrate for NADPH-oxidase, NADPH, in vitro elicited relaxation of the basilar artery from both genders without affecting basal O_2^- production. The primary source of O_2^- generated by these arteries is likely that the smaller functional response to NADPH in cerebral arteries may differ between genders. Nevertheless, in accordance with our chemiluminescence data, overall NADPH-stimulated O_2^- production as measured using dihydroethidium was approximately 50% lower in females. Thus, using two separate approaches, we provide strong evidence that the activity of NADPH-oxidase is lower in cerebral arteries from female versus male rats.

Three isoforms of the Nox catalytic subunit are thought to be important for the generation of cerebrovascular ROS: Nox1, Nox2, and Nox4. In this study, we report for the first time that gender influences the expression of some of these Nox isoforms in cerebral arteries. Specifically, we found that Nox1 and Nox4 protein are both expressed at lower levels in the basilar artery of female rats. In contrast, protein expression of the Nox2 catalytic subunit was similar between genders. Furthermore, protein expression of all three SOD isoforms in the basilar artery did not differ between genders, suggesting that the gender difference in O_2^- levels was not attributable to different rates of metabolism by SOD. Thus, it is likely that the lower expression of Nox1 and/or Nox4 protein contributes to the lower production of ROS by cerebrovascular NADPH-oxidase in females.

We next tested for any gender difference in the regulation of cerebral vascular tone associated with the differing levels of NADPH-oxidase activity. Several studies have reported that NADPH elicits ROS-mediated cerebral vasodilatation by activating NADPH-oxidase in vitro and in vivo. Catalase abolished NADPH-stimulated O_2^- production by rat cerebral arteries can also be attenuated by the NADPH-oxidase inhibitors, apocynin and gp91ds-tat, suggesting that NADPH-oxidase is the primary source of O_2^- generated by these arteries. The important new finding from these experiments was that O_2^- production by NADPH-oxidase in cerebral arteries was approximately 50% lower in females than in males. O_2^- is known to react rapidly with nitric oxide in solution with a rate constant of 6.7×10^9 M^-1s^-1, which is considerably faster than its reaction with lucigenin. Several authors have reported greater vascular nitric oxide bioactivity in females than in males. Nevertheless, in this assay, we found that the nitric oxide synthase inhibitor, N-nitro-L-arginine methyl ester, had no significant effect on NADPH-stimulated O_2^- production by the basilar artery from either gender. Thus, in agreement with previous studies, endogenous nitric oxide does not appear to regulate the concentration of O_2^- detected by this assay. In addition to using quantitative chemiluminescence, we localized and semiquantified NADPH-stimulated O_2^- production using the oxidative fluorescent dye dihydroethidium. In the basilar artery from male rats, ethidium/hydroxyethidium fluorescence was visible in endothelial, vascular smooth muscle, and adventitial cells, indicating that NADPH-oxidase is expressed and active throughout the male cerebral vasculature. In contrast, fluorescence was mainly visible in adventitial cells, and to some extent in vascular smooth muscle cells, in the basilar artery from female rats, suggesting that the localization profile of NADPH-oxidase in cerebral arteries may differ between genders. Nevertheless, in accordance with our chemiluminescence data, overall NADPH-stimulated O_2^- production as measured using dihydroethidium was approximately 50% lower in females. Thus, using two separate approaches, we provide strong evidence that the activity of NADPH-oxidase is lower in cerebral arteries from female versus male rats.
cerebral arteries from female rats is a consequence of lower ROS generation by NADPH-oxidase and not attributable to lower vascular sensitivity to H₂O₂.

We tested whether estrogen modulates the activity of NADPH-oxidase in the rat cerebral circulation. After ovariectomy, NADPH-stimulated O₂⁻ production by the basilar artery was almost 3-fold higher than levels generated by arteries from intact females, suggesting that estrogen deficiency results in higher NADPH-oxidase activity in cerebral arteries. Indeed, hormone replacement therapy with 17β-estradiol prevented this increase in NADPH-stimulated O₂⁻ production with O₂⁻ levels being comparable to those found in intact females. Basilar arteries from estrogen-deficient rats were more responsive than arteries from 17β-estradiol-treated rats in terms of NADPH-oxidase-mediated relaxation to NADPH, whereas responses to acetylcholine, SNP, and exogenous H₂O₂ were each similar between the two groups. Thus, the data suggest that estrogen suppresses the activity and function of NADPH-oxidase in the cerebral circulation. Our data are conceptually consistent with previous data from systemic vessels and cultured cells. For example, Wassmann et al recently reported that in female mice, ovariectomy resulted in increased NADPH-stimulated O₂⁻ production by the aorta and that hormone replacement with 17β-estradiol decreased O₂⁻ production. Furthermore, application of exogenous estrogen decreases the generation of ROS by NADPH-oxidase in human and bovine endothelial cells and rat aortic vascular smooth muscle cells.

It is widely believed that excessive generation of ROS by NADPH-oxidase contributes to vascular dysfunction associated with several cerebrovascular disease. Thus, a higher level of ROS production by cerebral arteries of males would be consistent with their observed higher risk of cerebrovascular diseases versus premenopausal women. Interestingly, recent findings have raised the possibility that low (ie, subpathological) levels of ROS derived from NADPH-oxidase activity may serve as a cerebral vasodilator mechanism under physiological conditions, thus possibly playing a beneficial role in the regulation of the cerebral circulation. Indeed, studies in rats, rabbits, and mice have shown that activation of NADPH-oxidase using NADPH elicits cerebral vasodilation in vitro and in vivo. Furthermore, NADPH-oxidase-derived H₂O₂ appears to offset contractions of the basilar artery to angiotensin II in vivo and partially mediate flow-dependent cerebral vasodilatation. However, we postulate that in the presence of multiple cardiovascular risk factors that are known to increase NADPH-oxidase activity, males may have a lower threshold than females to reach pathological levels of ROS, thus making them more susceptible to develop cerebrovascular disease. Although this may be one plausible explanation to reconcile the various findings to date, it is clear that further studies are needed to clarify whether this gender difference in NADPH-oxidase is relevant for the development of cerebrovascular disease.

In summary, the findings of this study indicate that the activity and function of NADPH-oxidase are lower in the cerebral circulation of female versus male rats. Furthermore, we provide strong evidence that these differences are dependent on estrogen and are likely attributable to suppression of Nox1- and/or Nox4-dependent NADPH-oxidase activity.

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Disclosures
C.G.S. and G.R.D. have potential conflicts of interest in that they are consultants for, and have significant ownership interest in, Radikal Biotechnology Pty. Ltd. of Australia. H.H.H.W.S. has a potential conflict of interest in that he is a consultant for, and has significant ownership interest in, Vasopharm GmbH, Germany.

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