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.

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...
(weight, 245±3.5g) Sprague-Dawley rats were studied. Rats were killed by inhalation of 80% CO2:20% O2.

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 transferred to a nitrocellulose membrane. Membranes were blocked in 5% skim milk for 1 hour at room temperature and then incubated overnight (4°C) with the appropriate primary antibody (1:300 for Nox2 and SOD1, 1:1000 for all other antibodies in 5% skim milk). Membranes were then incubated with a horseradish peroxidase–conjugated anti-goat (Nox2) or anti-rabbit (for all other antibodies) immunoglobulin for 1 hour at room temperature. Equal protein loading was verified by β-actin immunostaining (rabbit polyclonal β-actin antibody). Immunoreactive bands were detected by enhanced chemiluminescence (Progen Biosciences) and quantitated densitometrically with the use of a Kodak Image Station 440CF (Perkin-Elmer Life Sciences). Relative densities of immunoreactive bands were normalized to intensity of corresponding bands for β-actin.

In Vitro Preparation
Basilar arteries were excised from male and female rats and placed in cold carbogen (95% O2, 5% CO2)-bubbled Krebs-bicarbonate solution (composition in mmol/L: NaCl 118, KCl 4.5, MgSO4 0.45, KH2PO4 1.03, NaHCO3 25, glucose 11.1, CaCl2 2.5). Arteries were cut into approximately 3-mm rings, threaded onto two wires, and mounted in a Mulvany-Halpern myograph (Danish Myo Technology A/S). Resting tension was slowly increased to 5 mN. Arterial segments were continuously bathed in warm (37°C) carbogen-bubbled Krebs-bicarbonate solution. After an equilibration period of 60 minutes, rings were exposed to a high potassium physiological salt solution (KPSS) containing 122.7 mM NaCl to KCl (equimolar replacement of NaCl with KCl) to induce vascular contraction defined as “100% of KPSS.” In all rings, endothelial integrity was assessed by measuring acetylcholine (10 μmol/L)-induced relaxation after precontraction with serotonin (0.1 to 1 μmol/L; 50% of contraction to KPSS).

In Vitro Protocol
Ring segments of basilar arteries were again contracted submaximally (50% to 60% of maximal contraction to KPSS) with serotonin (0.1 to 1 μmol/L). Once contractions were stable, the effect of the following relaxing agents on vascular tone was investigated: NADPH (10 and 100 μmol/L), sodium nitroprusside (SNP; 0.01 to 1 μmol/L), and hydrogen peroxide (H2O2; 10 to 1000 μmol/L). Responses were examined in the presence and absence of the H2O2 scavenger, catalase (1000 U/mL). No more than 2 relaxing agents were tested in each ring.

Ovariectomy of Female Rats
Ovariectomy (OVX) was performed in anesthetized (60 mg/kg sodium pentobarbital) female Sprague-Dawley rats (weight, 164±5g) as previously described.18 OVX rats were then injected daily with either vehicle (0.01% dimethyl sulfoxide subcutaneously; weight, 233±7g, n=19) or 17β-estradiol (10 μg/kg subcutaneously; weight, 212±5g, n=20) for 14 days post-OVX. Rats were then killed and the uterus was harvested and weighed. Basilar and middle cerebral arteries were excised for measurement of NADPH-stimulated O2- production (lucigenin assay) and in vitro functional experiments.

Drugs
H2O2 was purchased from Merck, DHE from Molecular Probes, and all other drugs from Sigma. Diphenyleneiodonium and DHE were prepared at 10 mmol/L in dimethyl sulfoxide and diluted in Krebs-HEPES solution such that the final concentration of dimethyl sulfoxide was <0.05% or <0.001%, respectively. All other drugs were dissolved and diluted in either Krebs-HEPES (lucigenin/DHE experiments) or Krebs-bicarbonate (myograph experiments).

Data Analysis
All results are expressed as mean±SEM. Statistical comparisons were performed using either one-way analysis of variance with Bonferroni multiple comparisons post hoc test or using Student’s unpaired t test as appropriate. P<0.05 was considered statistically significant.

Results
Effect of Gender on NADPH-Oxidase Activity in Intracranial Cerebral Arteries
In the presence of NADPH, O2- production by basilar and middle cerebral arteries from female rats was approximately 50% lower than levels generated by arteries from male rats (P<0.05; Figure 1A, B). The NADPH-oxidase inhibitor, diphenyleneiodonium, virtually abolished O2- production (P<0.05; Figure 1A, B). The nitric oxide synthase inhibitor, N-nitro-L-arginine methyl ester (100 μmol/L), had no signifi-
Significant effect on NADPH-stimulated $O_2^-$ production by basilar arteries from either gender (male, control, $5.1 \pm 0.5$; $N$-nitro-L-arginine methyl ester, $2.5 \pm 0.1$; $N$-nitro-L-arginine methyl ester, $2.9 \pm 0.3 \times 10^3$ counts/mg of dry tissue, $n = 6$; female, control $2.5 \pm 0.1$; $N$-nitro-L-arginine methyl ester, $2.9 \pm 0.3 \times 10^3$ counts/mg of dry tissue, $n = 7$).

Localization and Quantification of Superoxide Production by NADPH-Oxidase
$O_2^-$ 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 $O_2^-$ 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 $O_2^-$.

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, $84 \pm 3$%; female, $81 \pm 4$%), 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 $H_2O_2$ 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 $H_2O_2$ 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=10, $P<0.05$ versus males).

**Figure 3.** Representative Western blot showing protein expression of the NADPH oxidase catalytic subunits: (A) Nox1, (B) Nox2, (C) Nox4, and SOD isoforms: (D) SOD1, (E) SOD2, and (F) SOD3 in basilar arteries from male and female rats (top). Also shown is a summary of densitometric data (bottom). Values are expressed as relative intensity/μg of protein normalized to β-actin expression and are given as mean±SEM (n=5 to 13). *$P<0.05$ versus males.

**Figure 4.** Effect of (A, 10 to 100 μmol/L) NADPH and (B, 0.01 to 1 μmol/L) SNP on the tone of basilar arteries from male and female rats. Arteries were precontracted (50% of contraction to KPSS) with serotonin (0.1 to 1 μmol/L) before addition of NADPH or SNP. Values are expressed as percent relaxation of serotonin-induced tone and are given as mean±SEM (n=6 to 8). *$P<0.05$ versus males (unpaired t test).
body weight, n=19, P<0.001), but was greater than in OVX/Veh rats (P<0.001).

NADPH-stimulated O$_2^-$ 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 O$_2^-$ 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 H$_2$O$_2$ (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 O$_2^-$ 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 O$_2^-$ 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 H$_2$O$_2$ (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 H$_2$O$_2$. Values are given as mean±SEM. *P<0.05 versus O$_2^-$ 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 H₂O₂ (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 O₂⁻ production by NADPH-oxidase is 50% lower in basilar and middle cerebral arteries from female than male rats. This lower level of O₂⁻ 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 F2α, are greater in healthy males than age-matched females. Furthermore, measurements of O₂⁻ 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-
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 are related to vascular dysfunction in premenopausal women. Theoretically, estrogen deficiency results in higher NADPH-oxidase activity in cerebral arteries of female rats. Indeed, hormone replacement therapy with 17β-estradiol reduces superoxide production in aorta of ovariectomized rats. 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, Radical 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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