

# Gender Influences Cerebral Vascular Responses to Angiotensin II Through Nox2-Derived Reactive Oxygen Species

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**Background and Purpose**—We tested whether gender influences cerebrovascular responses to angiotensin II (AngII) and the role(s) of Nox2.

**Methods and Results**—AngII-stimulated superoxide ( $O_2^-$ ) production by cerebral arteries from male and female wild-type (WT) and Nox2<sup>-/-</sup> mice was measured using lucigenin- or L-012-enhanced chemiluminescence. Hydrogen peroxide ( $H_2O_2$ ) production was measured using Amplex Red fluorescence. Western Blotting was used to measure expression of Nox2, endothelial nitric oxide synthase (eNOS), angiotensin receptors (AT<sub>1</sub> and AT<sub>2</sub>), and superoxide dismutases (SOD1–3). Immunofluorescence was used to localize Nox2 in middle cerebral arteries (MCA). Vascular responses to AngII were assessed in a perfusion myograph. AngII-stimulated  $O_2^-$  and  $H_2O_2$  production by cerebral arteries from female WT mice was ≈75% to 85% lower than in males ( $P<0.05$ ).  $O_2^-$  production was ≈60% lower in Nox2<sup>-/-</sup> versus WT males ( $P<0.05$ ), whereas Nox2 deletion did not affect  $O_2^-$  production in females. Expression of Nox2, eNOS, AT receptors, and SOD isoforms was similar between genders. Nox2 immunofluorescence was similarly localized in adventitial and endothelial cells of MCA from both genders. AngII elicited smaller contractions of MCA from females vs males ( $P<0.05$ ). Contractions were reduced in male, but not female, Nox2<sup>-/-</sup> mice ( $P<0.05$ ). The SOD mimetic, tempol, potentiated contractions to AngII in male WT mice ( $P<0.05$ ), whereas the SOD/catalase mimetic, EUK-134, virtually abolished contractions ( $P<0.05$ ).

**Conclusions**—AngII-stimulated  $O_2^-$  and  $H_2O_2$  production are greater in cerebral arteries from male versus female mice, and are associated with greater contractions to AngII mediated by  $H_2O_2$ . These gender differences are dependent on the expression of Nox2. (*Stroke*. 2009;40:00-00.)

**Key Words:** cerebral arteries ■ gender ■ NAD(P)H oxidase ■ reactive oxygen species ■ angiotensin II

Hypertension has profound effects on the cerebral circulation and is a major risk factor for stroke and vascular cognitive impairment.<sup>1</sup> Angiotensin II (AngII) is believed to be a critical mediator of the deleterious effects of hypertension on the cerebral circulation. For example, it has been reported to promote cerebrovascular remodeling<sup>2</sup> and inflammation,<sup>3</sup> as well as impairing functional hyperemia.<sup>4</sup> NADPH-oxidases are a major source of reactive oxygen species (ROS) such as superoxide ( $O_2^-$ ) and hydrogen peroxide ( $H_2O_2$ ) in the cerebral circulation.<sup>5</sup> These enzymes generate ROS by transferring electrons to molecular oxygen via a “Nox” catalytic subunit.<sup>6</sup> Nox1-, Nox2-, or Nox4-containing isoforms of NADPH-oxidase are thought to be important for the generation of ROS within the brain.<sup>6</sup> Evidence suggests that many of the deleterious effects of AngII on the cerebral circulation are mediated through the generation of ROS by Nox2-containing NADPH-oxidases.<sup>4,7,8</sup>

It is well documented that the incidence and severity of cerebrovascular diseases such as stroke is lower in premenopausal women than men of similar age.<sup>9</sup> The precise mechanisms responsible for this difference are not fully understood and are likely to be multifaceted. However, recent findings suggest that gender-dependent differences in vascular ROS production may play an important role.<sup>10</sup> Indeed, we recently reported that the activity and expression of NADPH-oxidase is lower in the cerebral circulation of female versus male rats because of the actions of estrogen.<sup>11</sup> Moreover, studies have reported a marked gender difference in the effects of AngII on mouse cerebral arteries.<sup>12,13</sup> However, the role of NADPH-oxidase in such gender differences has not been evaluated. Therefore, we first tested whether gender influences the levels of ROS generated in cerebral arteries in response to AngII, and then whether contractile responses to AngII were similarly gender-dependent. Secondly, using Nox2-deficient mice we evaluated the involvement of Nox2-containing

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NADPH-oxidases in these cerebrovascular responses to AngII.

## Materials and Methods

All procedures were approved by the institutional animal ethics committee. In total, 114 male C57B16/J wild-type (WT) (24.5±0.2g), 72 female WT (19.5±0.2g), 16 male Nox2<sup>-/-</sup> (23.2±0.5g), and 13 female Nox2<sup>-/-</sup> (20.1±0.7g) were studied. Nox2<sup>-/-</sup> mice were back-crossed to the C57B16/J strain for at least 10 generations. Mice were 8 to 12 weeks of age and were typically studied in pairs (ie, male and female) that were age-matched on the day of each experiment. Animals were killed by inhalation of isoflurane followed by decapitation.

### Quantification of O<sub>2</sub><sup>-</sup> Production by Cerebral Arteries

Experiments were carried out using pooled basilar and middle cerebral arteries (MCA). Basal O<sub>2</sub><sup>-</sup> production by cerebral arteries from WT mice was measured by 100 μmol/L L-012-enhanced chemiluminescence. AngII (0.1 μmol/L)-stimulated O<sub>2</sub><sup>-</sup> production by cerebral arteries from WT mice was measured by 5 μmol/L lucigenin-enhanced chemiluminescence in the presence of NADPH (100 μmol/L) and diethyldithiocarbamate (DETCA; 3 mmol/L) as previously described.<sup>5,14</sup> In some experiments, arteries were treated with the NADPH-oxidase inhibitor, diphenyleneiodonium (DPI; 5 μmol/L), or the cyclooxygenase inhibitor, indomethacin (10 μmol/L). O<sub>2</sub><sup>-</sup> production by cerebral arteries from WT and Nox2<sup>-/-</sup> mice in the absence and presence of AngII (0.1 μmol/L) was measured by L-012-enhanced chemiluminescence. In all experiments, background counts were subtracted and O<sub>2</sub><sup>-</sup> production normalized to dry tissue weight. In lucigenin experiments counts in vehicle-treated rings (ie, NADPH and DETCA treated) were subtracted from counts in vessel segments in the presence of AngII from the same animal. In L-012 experiments basal counts were subtracted from counts in the presence of AngII from the same animal.

### Quantification of H<sub>2</sub>O<sub>2</sub> Production by Cerebral Arteries

Experiments were carried out using pooled basilar arteries and MCA. The Amplex Red fluorescence bioassay was used to measure AngII (0.1 μmol/L)-induced H<sub>2</sub>O<sub>2</sub> production by cerebral arteries from male and female WT mice. H<sub>2</sub>O<sub>2</sub> standards (0 to 0.125 μmol/L) and cerebral arteries were transferred to a 96-well plate. Fluorescence was determined in 100 μL Krebs-HEPES solution containing 15 μmol/L Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) and 0.1 U/mL horseradish peroxidase in the absence and presence of AngII (1 nmol/L). In some experiments, arteries were treated with the O<sub>2</sub><sup>-</sup> dismutase (SOD) mimetic, 4-Hydroxy-TEMPO (tempol; 1 mmol/L) or the SOD/catalase mimetic, EUK-134 (100 μmol/L). Fluorescence was measured in a fluorimeter (Flexstation, Molecular Devices) using an excitation filter of 530 nm and an emission filter of 590 nm. Background fluorescence was subtracted and H<sub>2</sub>O<sub>2</sub> accumulation (pmoles/min) normalized for dry tissue weight. Fluorescence in the absence of AngII was subtracted from fluorescence in the presence of AngII in vessel segments from the same animal.

### Western Blotting

Experiments were carried out using pooled basilar and MCA arteries. Protein expression of Nox2, endothelial nitric oxide synthase (eNOS), AngII receptor subtypes 1 and 2 (AT<sub>1</sub> and AT<sub>2</sub>), and SOD isoforms (SOD1, SOD2, and SOD3) was measured in cerebral arteries using Western Blotting. Anti-Nox2 and anti-eNOS mouse monoclonal antibodies were purchased from BD Biosciences (North Ryde). Anti-AT<sub>1</sub> and anti-AT<sub>2</sub> rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology, anti-SOD1 rabbit polyclonal antibodies from Stressgen, and anti-SOD2 and anti-SOD3 rabbit polyclonal antibodies from Upstate (Chemicon).

Arteries were excised, snap frozen in liquid nitrogen, and homogenized in Laemmli buffer (25% Glycerin, 12.5% β-mercaptoethanol, 7.5% sodium dodecyl sulfate, 25% 1 mol/L TrisHCl pH 8.0, 0.25 mg/mL bromophenol blue) over liquid nitrogen. Protein concentration was determined using the RCD assay (BioRad). Equal amounts of protein were loaded onto a 7.5% (Nox2) or 10% (all other proteins) polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked in 5% skim milk for 1 hour and then incubated overnight (4°C) with the appropriate primary antibody (1:1000 for Nox2, eNOS, AT<sub>1</sub>, SOD2, and SOD3; 1:2000 for β-actin, 1:3000 for AT<sub>2</sub> and 1:4000 for SOD1) in 5% skim milk. Membranes were then incubated with a horseradish peroxidase-conjugated anti-mouse (Nox2 and eNOS) or anti-rabbit (all other antibodies) IgG for 1 hour. Immunoreactive bands were detected by enhanced chemiluminescence, quantified using a ChemiDoc XRS molecular imager (BioRad), and normalized to intensity of corresponding bands for β-actin.

### Localization of Nox2

MCA from male and female WT mice were mounted in an OCT Tissue-Tek (Bayer Diagnostics) mold and snap frozen in liquid nitrogen. Arteries were sectioned (10 μm) and thaw-mounted onto 0.1% gelatinised slides. Sections were fixed in acetone (15 minutes) and washed in 0.01 mol/L phosphate buffered saline (PBS; 3×10 minutes). Sections were then incubated with an anti-Nox2 mouse monoclonal antibody (1:1000) overnight at 4°C. The following day, the tissues were washed in 0.01 mol/L PBS (3×10 minutes) and incubated in a fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (1:500; Zymed Laboratories) for 3 to 4 hour at 4°C. The tissues were then washed in 0.01 mol/L PBS (3×10 minutes) and mounted in buffered glycerol (0.5 mol/L Na<sub>2</sub>CO<sub>3</sub> added dropwise to 0.5 mol/L NaHCO<sub>3</sub> to pH 8.6, combined 1:1 with glycerol). Tissue mounted slides were viewed and photographed on a Leica confocal scanning laser system.

### Preparation of Isolated Cannulated MCA Segments

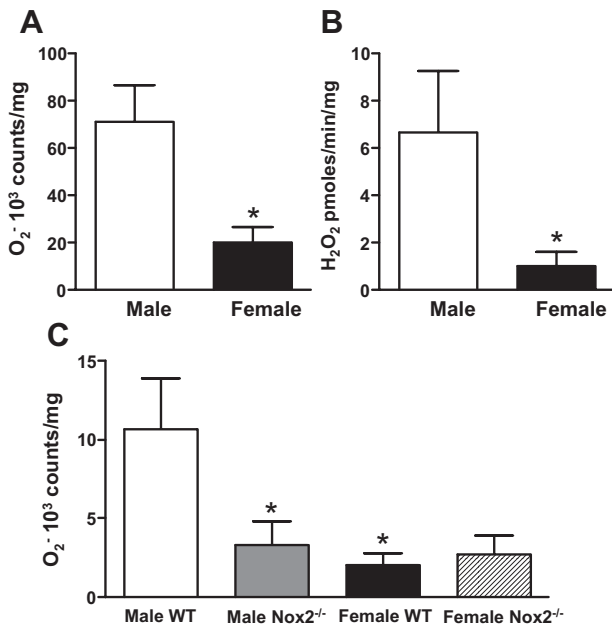
MCA from male and female WT and Nox2<sup>-/-</sup> mice were mounted between two microcannulae in a pressure myograph (Living Systems Instrumentation Inc). Arteries were constantly superfused with warm (37°C), carbogen-bubbled (95% O<sub>2</sub>, 5% CO<sub>2</sub>) Krebs-bicarbonate solution (composition in mmol/L; NaCl 118, KCl 4.5, MgSO<sub>4</sub> 0.45, KH<sub>2</sub>PO<sub>4</sub> 1.03, NaHCO<sub>3</sub> 25, glucose 11.1, CaCl<sub>2</sub> 2.5). Intraluminal pressure was gradually increased to 60 mm Hg and maintained at this level with a pressure servo unit without further intraluminal perfusion. After equilibration and on completion of the experimental protocol, arteries were exposed to a high potassium physiological salt solution (KPSS) containing 122.7 mmol/L KCl (equimolar replacement of NaCl with KCl).

### Functional Study

Cumulative doses of AngII (0.1 nmol/L to 1 μmol/L) were added extraluminally to MCA from male and female WT and Nox2<sup>-/-</sup> mice. In some MCA from male WT mice, AngII (0.1 nmol/L to 1 μmol/L) concentration-response curves were performed in the presence or absence of tempol (1 mmol/L) or EUK-134 (100 μmol/L). The effect of exogenous H<sub>2</sub>O<sub>2</sub> (10 to 300 μmol/L), in the presence or absence of EUK-134 (100 μmol/L), was also tested in MCA from male WT mice.

### Drugs

AngII was purchased from Auspep, Amplex Red fluorescence bioassay kit from Molecular Probes (Invitrogen), EUK-134 from Sapphire Biosciences and all other drugs from Sigma. AngII was dissolved in 0.05 mol/L acetic acid and then diluted in either Krebs-HEPES (lucigenin/L-012 experiments) or Krebs bicarbonate (myograph experiments). DPI and EUK-134 were prepared at 10 mmol/L and 100 mmol/L in dimethyl sulfoxide (DMSO), respectively, and diluted in Krebs-HEPES or Krebs-bicarbonate solution, respectively, such that the final concentra-



**Figure 1.** Vascular ROS production by cerebral arteries from male and female mice. **A**, The effect of AngII ( $0.1 \mu\text{mol/L}$ ) on  $\text{O}_2^-$  production by cerebral arteries from wild-type (WT) mice as measured by  $5 \mu\text{mol/L}$  lucigenin-enhanced chemiluminescence. Experiments were performed in the presence of NADPH ( $100 \mu\text{mol/L}$ ) and DETCA ( $3 \text{mmol/L}$ ). The data represent counts in AngII-treated rings minus counts in vehicle treated rings from the same animal. **B**, The effect of AngII ( $0.1 \mu\text{mol/L}$ ) on  $\text{H}_2\text{O}_2$  production by cerebral arteries from WT mice as measured by Amplex Red fluorescence. **C**, The effect of AngII ( $0.1 \mu\text{mol/L}$ ) on  $\text{O}_2^-$  production by cerebral arteries from WT and  $\text{Nox2}^{-/-}$  mice as measured by  $100 \mu\text{mol/L}$  L-012-enhanced chemiluminescence. Results are given as mean  $\pm$  SEM ( $n=7$  to  $10$ ).  $*P<0.05$  vs WT males (1-way ANOVA with a Bonferroni multiple comparison posthoc test).

tion of DMSO was  $<0.1\%$ . All other drugs were dissolved and diluted in either Krebs-HEPES (lucigenin/L-012 experiments) or Krebs-bicarbonate (myograph experiments).

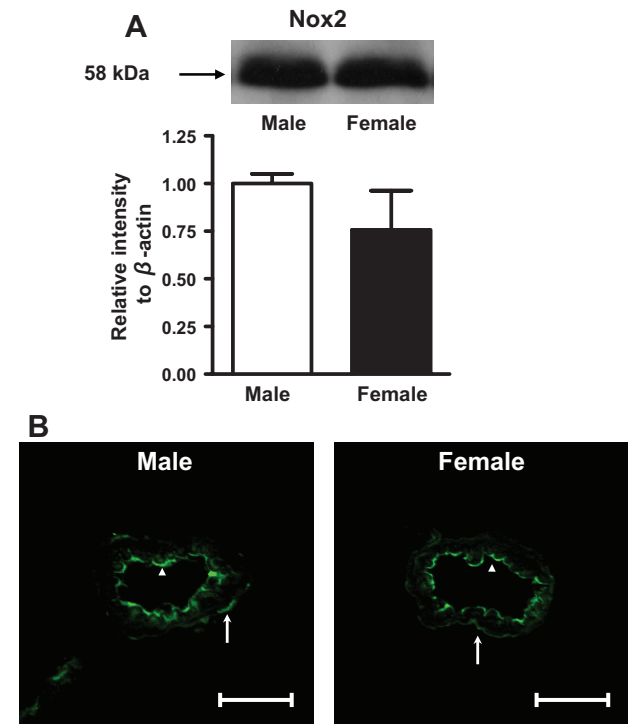
### Data Analysis

All results are presented as mean  $\pm$  SEM. Statistical comparisons were performed using either 2-way ANOVA, 1-way ANOVA with a Bonferroni multiple comparison posthoc test, or using student paired or unpaired  $t$  test, as appropriate.  $P<0.05$  was considered statistically significant.

## Results

### Gender Influences AngII-Stimulated ROS Production: Role of Nox2

Basal  $\text{O}_2^-$  production by cerebral arteries was similar between genders (male,  $12.98 \pm 0.94$ ; female,  $14.28 \pm 3.82 \times 10^3$  counts/mg dry tissue,  $n=7$ ). Using either lucigenin or L-012, AngII-stimulated  $\text{O}_2^-$  production by cerebral arteries from female WT mice was  $\approx 70\%$  and  $\approx 80\%$  lower, respectively, than levels generated by arteries from male mice ( $P<0.05$ , Figure 1A and 1C). The NADPH-oxidase inhibitor, DPI virtually abolished AngII-stimulated  $\text{O}_2^-$  production by cerebral arteries (male,  $8.9 \pm 3.9\%$  of control; female  $6.8 \pm 4.7\%$  of control  $P<0.05$ ,  $n=3$  to  $4$ ). The COX inhibitor, indomethacin had no significant effect on AngII-stimulated  $\text{O}_2^-$  production by cerebral arteries from male mice (control,



**Figure 2.** **A**, Representative Western blot showing protein expression of the NADPH-oxidase catalytic subunit Nox2 in cerebral arteries from male and female WT mice (top). Also shown is a summary of immunoreactive band intensity (bottom). Values are expressed as relative intensity normalized to  $\beta$ -actin intensity and are given as mean  $\pm$  SEM ( $n=6$ ). **B**, Representative photomicrographs showing sections of the middle cerebral artery from male and female mice after processing for Nox2 immunohistochemistry. In arterial sections from both males and females, Nox2-staining appeared to be similar in the endothelium (arrowheads) and adventitia (arrows). Magnification,  $\times 400$ , scale bar= $40 \mu\text{m}$ .

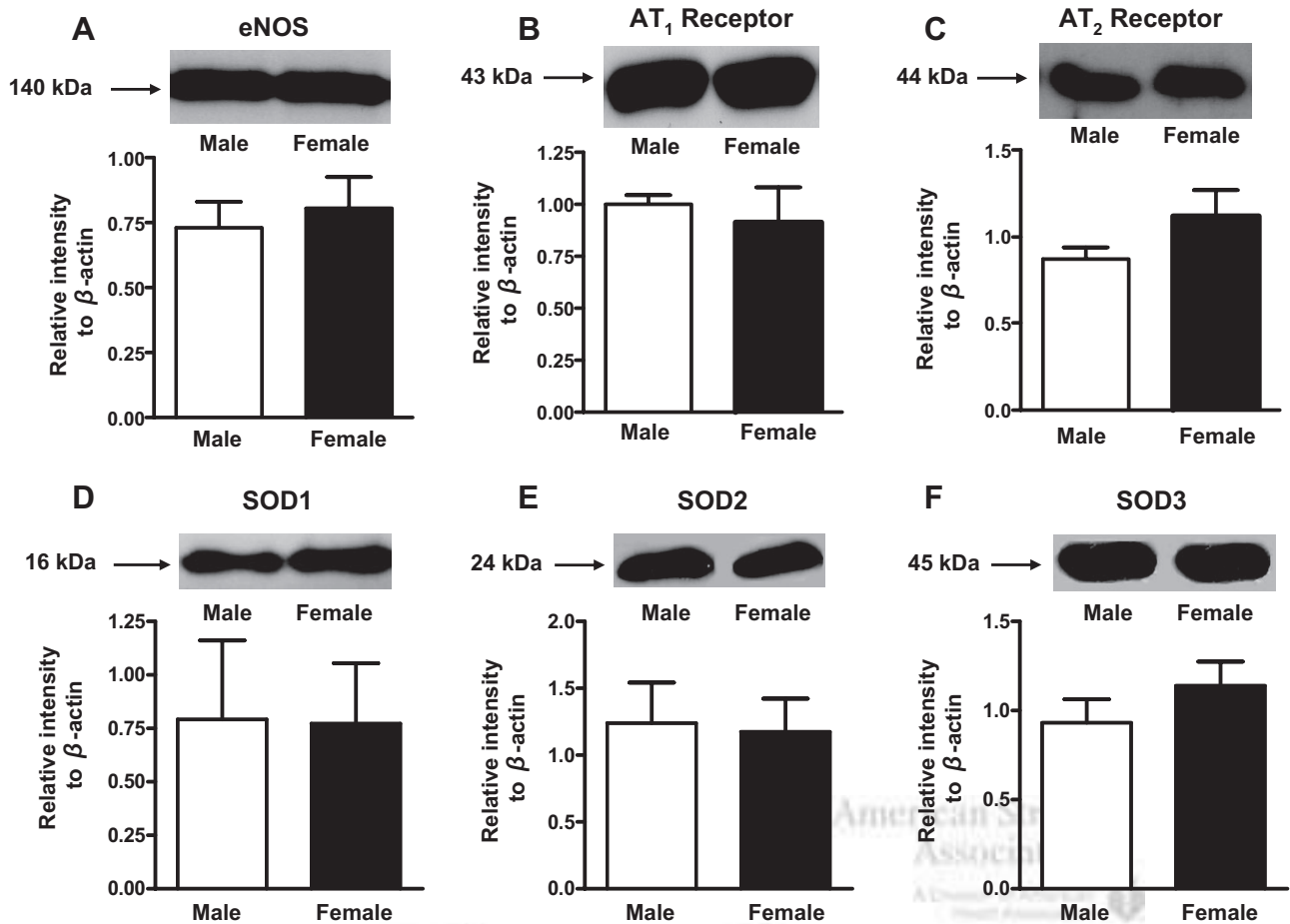
$122.5 \pm 23.3$ ; indomethacin,  $175.4 \pm 44.8 \times 10^3$  counts/mg dry tissue,  $n=6$ ). AngII-stimulated  $\text{H}_2\text{O}_2$  production by cerebral arteries from female mice was  $\approx 85\%$  lower than levels generated by arteries from male mice ( $P<0.05$ , Figure 1B). AngII-stimulated  $\text{O}_2^-$  production by cerebral arteries from male  $\text{Nox2}^{-/-}$  mice was  $\approx 60\%$  lower than levels generated by arteries from male WT mice ( $P<0.05$ , Figure 1C), such that levels were comparable to those in WT females ( $P>0.05$ ). There was no significant difference between  $\text{O}_2^-$  production by female WT and  $\text{Nox2}^{-/-}$  mice (Figure 1C).

### Nox2, eNOS, AT Receptors, and SOD Expression

Protein expression of Nox2, eNOS,  $\text{AT}_1$  and  $\text{AT}_2$  receptors, and SOD isoforms<sup>1-3</sup> in cerebral arteries did not differ between genders (Figures 2A and 3).

### Localization of Nox2

Nox2 immunoreactivity was predominantly observed in the endothelium and to a lesser extent in the adventitia of arterial sections from both males and females (Figure 2B). No specific Nox2 immunoreactivity was located in the vascular smooth muscle. Nox2 immunofluorescence intensity and distribution appeared similar in MCA from both genders.



**Figure 3.** Representative Western blot showing protein expression of (A) eNOS, (B) AT<sub>1</sub> receptor, (C) AT<sub>2</sub> receptor, (D) SOD1, (E) SOD2, and (F) SOD3 in cerebral arteries from male and female mice. Also shown is a summary of immunoreactive band intensities (bottom). Values are expressed as relative intensity normalized to  $\beta$ -actin intensity, and are given as mean  $\pm$  SEM ( $n=4$  to 14).

### Gender Influences Contractions to AngII: Role of Nox2

Baseline diameters of MCA were not significantly different between genders or genotypes (male WT,  $110 \pm 2 \mu\text{m}$ ; male Nox2<sup>-/-</sup>,  $122 \pm 4 \mu\text{m}$ ; female WT,  $98 \pm 8 \mu\text{m}$ ; female Nox2<sup>-/-</sup>,  $120 \pm 4 \mu\text{m}$ ). AngII (0.1 nmol/L to 1  $\mu\text{mol/L}$ ) elicited contractions of MCA from both male and female WT mice, however contractions were significantly smaller in MCA from female mice ( $P < 0.05$ , Figure 4A). AngII-induced contractions of MCA from male Nox2<sup>-/-</sup> mice were significantly smaller than responses in male WT mice ( $P < 0.05$ , Figure 4A), such that responses were comparable to those found in female WT mice ( $P > 0.05$ , Figure 4A). In contrast, responses to AngII did not differ between female Nox2<sup>-/-</sup> and WT mice (Figure 4A). Contractile responses to KPSS (also measured from baseline) were similar between all four groups (Figure 4B).

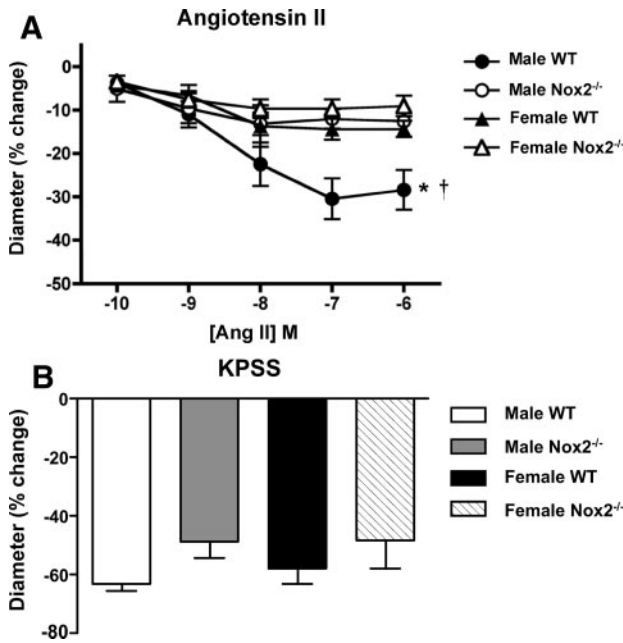
### Contractions to AngII: Role of ROS

Tempol (1 mmol/L) increased AngII-stimulated H<sub>2</sub>O<sub>2</sub> production (measured using Amplex Red) by cerebral arteries from male WT mice by  $\approx 80\%$  as compared with control arteries ( $P < 0.05$ , Figure 5A), whereas EUK-134 (100  $\mu\text{mol/L}$ ) decreased H<sub>2</sub>O<sub>2</sub> levels by  $\approx 45\%$  ( $P < 0.05$ , Figure 5B). In MCA from male WT mice, contractions to

AngII were potentiated by tempol ( $P < 0.05$ , Figure 6A). In the absence or presence of tempol, EUK-134 virtually abolished contractions to AngII ( $P < 0.05$ , Figure 6A). EUK-134 had no significant effect on contractile responses to KPSS (Figure 6B). Exogenous H<sub>2</sub>O<sub>2</sub> (10 to 300  $\mu\text{mol/L}$ ) elicited contractions of the MCA from male mice (Figure 6C; data shown only for 100  $\mu\text{mol/L}$ ). Contractions to exogenous H<sub>2</sub>O<sub>2</sub> were virtually abolished by EUK-134 (Figure 6C).

### Discussion

One major novel finding of this study is that gender influences cerebrovascular responses to AngII by modulating Nox2-dependent ROS generation. Specifically, we found that the production of O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> in response to AngII is lower in cerebral arteries from female WT mice compared with males. Furthermore, this lower level of ROS production was associated with selectively smaller constrictor responses to AngII. In Nox2-deficient males, O<sub>2</sub><sup>-</sup> production and contractile responses to AngII were reduced, whereas deletion of Nox2 had no effect on either O<sub>2</sub><sup>-</sup> production or contractile responses in female mice. A second novel finding is that the SOD mimetic, tempol, potentiated contractions of the MCA to AngII, whereas the SOD/catalase mimetic, EUK-134, virtually abolished contractions to AngII. Thus, these results suggest that contractile responses to AngII in the male mouse



**Figure 4.** A, Cumulative concentration-response curves showing contractile responses to AngII (0.1 nmol/L to 1  $\mu$ mol/L) of isolated middle cerebral arteries from male and female wild-type (WT) and Nox2<sup>-/-</sup> mice. B, Contractile responses to high potassium physiological salt solution (KPSS). Results are expressed as % change in intraluminal diameter and are given as mean  $\pm$  SEM (n=5 to 6). \**P*<0.05 vs female WT, †*P*<0.05 vs male Nox2<sup>-/-</sup> (2-way ANOVA with a Bonferroni multiple comparison posthoc test).

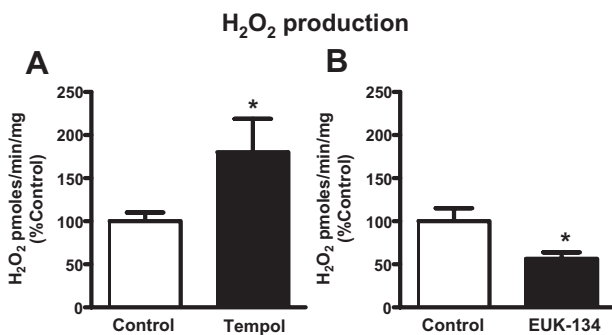
cerebral circulation are mediated by Nox2-dependent H<sub>2</sub>O<sub>2</sub> generation.

AngII is a critical mediator of the deleterious effects of hypertension on the cerebral circulation.<sup>2-4</sup> Investigators have reported that many of the effects of AngII on the cerebral circulation are mediated by ROS, generated by Nox2-containing NADPH-oxidases.<sup>4,7,8</sup> Epidemiological studies have revealed that the incidence of cerebrovascular disease is lower in premenopausal women than males.<sup>9</sup> Moreover, evidence suggests that gender-dependent differences in the generation of vascular ROS may play an important role.<sup>10</sup> However, no study has specifically addressed whether gender influences AngII-induced ROS production in the cerebral

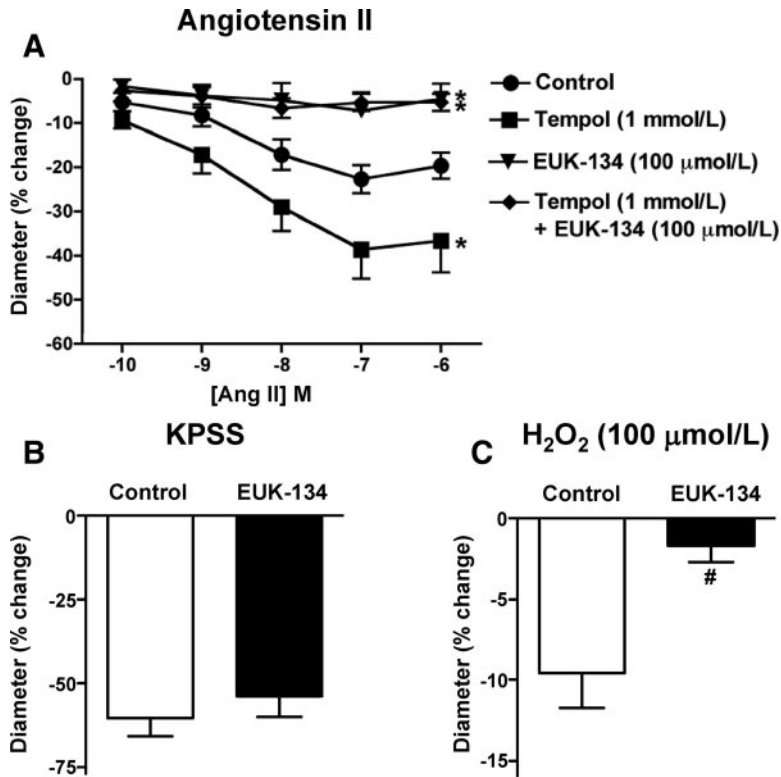
circulation. Using the chemiluminescence probe, lucigenin, we found that AngII increases O<sub>2</sub><sup>-</sup> production by cerebral arteries from mice of both genders, an effect that was virtually abolished by the NADPH-oxidase inhibitor, DPI. The major novel finding of these experiments was that O<sub>2</sub><sup>-</sup> production in response to AngII was approximately 70% lower in female WT mice than in males. Importantly, these findings were made in the presence of the SOD1 and SOD3 inhibitor, DETCA, thus it seems unlikely that differing rates of O<sub>2</sub><sup>-</sup> inactivation by SODs account for these gender differences in detected O<sub>2</sub><sup>-</sup> levels. Consistent with this interpretation was our finding that protein expression of all three SOD isoforms did not differ between genders. Similarly, expression levels of eNOS and angiotensin receptors (AT<sub>1</sub> and AT<sub>2</sub>) were not influenced by gender. Thus, it is unlikely that the gender-related effects on O<sub>2</sub><sup>-</sup> levels are attributable to differences in the production of and inactivation by NO, or to differential signaling through AT<sub>1</sub> and AT<sub>2</sub> receptors. It has been previously suggested that AngII may increase levels of O<sub>2</sub><sup>-</sup> in the cerebral circulation via activation of cyclooxygenase (COX).<sup>15</sup> However, in the present study we found that the COX inhibitor, indomethacin, did not reduce AngII-stimulated O<sub>2</sub><sup>-</sup> production by cerebral arteries. Under physiological conditions, O<sub>2</sub><sup>-</sup> is rapidly metabolized by SODs to form H<sub>2</sub>O<sub>2</sub>. In contrast to O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub> is highly diffusible and relatively stable. Consequently, H<sub>2</sub>O<sub>2</sub> is now thought to be an important ROS molecule for modulating vascular function.<sup>16</sup> Using Amplex Red fluorescence, we found that AngII increases H<sub>2</sub>O<sub>2</sub> production by cerebral arteries from both genders. However, H<sub>2</sub>O<sub>2</sub> production was approximately 85% lower in arteries from female mice than males. Thus, overall these experiments reveal that the production of both O<sub>2</sub><sup>-</sup> and its downstream metabolite H<sub>2</sub>O<sub>2</sub> in response to AngII is lower in the cerebral circulation of female versus male WT mice.

We next tested for any gender difference in the regulation of cerebral vascular tone associated with the differing levels of ROS production in response to AngII. Numerous studies of cerebral arteries, including those in humans, have found that AngII elicits constriction.<sup>5,12,17,18</sup> Consistent with these findings we found here that application of AngII to pressurized isolated mouse MCA results in vasoconstriction in both genders. However, the contractile response to AngII was approximately 50% smaller in female versus male mice. Furthermore, responses to KPSS were similar between genders, consistent with the gender-dependent difference being somewhat selective for AngII. Moreover, our results in MCA are consistent with findings in basilar arteries where constrictor responses to AngII, but not U-46619, were smaller in female versus male mice.<sup>12</sup> Furthermore, our findings are conceptually consistent with a recent study reporting that AngII administration attenuates the increase in cerebral blood flow induced by the endothelium-dependent vasodilator, acetylcholine, or whisker stimulation in male, but not female mice.<sup>13</sup>

To test for a role for Nox2-containing NADPH-oxidases in the gender differences in cerebrovascular responses to AngII we studied Nox2-deficient mice. Firstly, we measured AngII-stimulated O<sub>2</sub><sup>-</sup> production by cerebral arteries from WT and



**Figure 5.** Effect of (A) tempol (1 mmol/L) and (B) EUK-134 (100  $\mu$ mol/L) on AngII (0.1  $\mu$ mol/L)-stimulated H<sub>2</sub>O<sub>2</sub> production by cerebral arteries from male WT mice as measured by the Amplex Red Fluorescence Bioassay. Results are given as mean  $\pm$  SEM (n=6). \**P*<0.05 vs control (paired *t* test).



**Figure 6.** A, Effect of tempol (1 mmol/L) and EUK-134 (100 μmol/L) on contractile responses to AngII of isolated cannulated middle cerebral arteries (0.1 nmol/L to 1 μmol/L) from male WT mice. B, Effect of H<sub>2</sub>O<sub>2</sub>, in the absence or presence of EUK-134 (100 μmol/L), on the diameter of middle cerebral arteries. C, Effect of EUK-134 (100 μmol/L) on contractile responses to high potassium physiological salt solution (KPSS). Results are expressed as % change in intraluminal diameter and are given as mean ± SEM (n=5 to 10). \**P*<0.05 vs control (2-way ANOVA with a Bonferroni multiple comparison posthoc test).

Nox2<sup>-/-</sup> mice using the chemiluminescent probe, L-012. Consistent with our lucigenin data, O<sub>2</sub><sup>-</sup> production was markedly lower in female versus male WT mice. Levels of O<sub>2</sub><sup>-</sup> were not altered in female Nox2<sup>-/-</sup> versus WT mice, whereas levels were markedly reduced in male Nox2<sup>-/-</sup> versus WT. Indeed, O<sub>2</sub><sup>-</sup> levels in male Nox2<sup>-/-</sup> mice were comparable to levels found in female WT mice. We next examined contractile responses to AngII in Nox2<sup>-/-</sup> mice. Again, in females, responses of the MCA to AngII were similar between WT and Nox2<sup>-/-</sup> mice, whereas contractions were markedly reduced in male Nox2<sup>-/-</sup> mice such that responses were comparable to those found in female WT. Taken together, these experiments provide the first direct evidence that Nox2-containing NADPH-oxidases play a major role in mediating the ROS generated in response AngII in the cerebral circulation of males but not females.

These data also provide a mechanistic basis for the marked gender difference in cerebrovascular responses to AngII. We have previously reported that Nox2 protein is expressed at similar levels in basilar arteries from male and female rats.<sup>11</sup> Similarly, we found here that levels of Nox2 protein in the mouse cerebral circulation do not differ between genders. Consistent with previous studies in mouse cerebral arterioles,<sup>4</sup> Nox2 immunoreactivity was predominantly localized to adventitial and endothelial cells of MCA in male and female mice. Thus, although AngII-stimulated Nox2 activity appears to be higher in males than females, differing expression levels of Nox2 or its cellular localization apparently do not account for the Nox2-dependent gender differences in responses to AngII. These differences presumably reflect higher Nox2 catalytic activity in males than females. Numerous factors may regulate the activity of vascular Nox2,

including its association with p47phox, Rac, and NADPH.<sup>6</sup> Greater levels or association of these factors with Nox2 in males could conceivably result in higher Nox2 activity.

The mechanism(s) that mediate the constrictor effects of AngII in the cerebral circulation are not fully understood. Previous studies have reported that AngII elicits constriction of cerebral arteries via activation of AT<sub>1</sub> receptors<sup>12,19</sup> and the RhoA/Rho-Kinase pathway.<sup>12</sup> Furthermore, in the present study we provide the first direct evidence that Nox2-derived ROS is a critical component in this signaling cascade. It is well documented that ROS can act as vasodilators or vasoconstrictors to modulate cerebral vascular tone. Indeed, we have reported that NADPH-oxidase-derived H<sub>2</sub>O<sub>2</sub> offsets contractions of the rat basilar artery to AngII.<sup>5</sup> Furthermore, we found that exogenous application of H<sub>2</sub>O<sub>2</sub> to the rat basilar artery induced a small constriction followed by sustained relaxation.<sup>5</sup> In the present study, the SOD mimetic, tempol, increased AngII-stimulated H<sub>2</sub>O<sub>2</sub> levels (measured using Amplex Red fluorescence), and potentiated contractions of the MCA to AngII. By contrast, the SOD/catalase mimetic, EUK-134, decreased H<sub>2</sub>O<sub>2</sub> levels and virtually abolished contractions without affecting responses to KPSS. Moreover, we found here that exogenous application of H<sub>2</sub>O<sub>2</sub> elicited a sustained contraction of the mouse MCA. Thus, these data seem to indicate that, in contrast to the rat cerebral circulation, H<sub>2</sub>O<sub>2</sub> (or a downstream ROS thereof) actually mediates contractions to AngII in the mouse cerebral circulation. Species-dependent differences in the pathways mediating the effects of AngII on the cerebral circulation have been previously noted. For example, Haberl et al provided evidence that responses to AngII in the rat are COX-dependent,<sup>15</sup> whereas the same authors reported no role for

COX in the rabbit.<sup>20</sup> Clearly, the reason(s) for the species-dependent difference in cerebral vascular responses to H<sub>2</sub>O<sub>2</sub>, and especially what role H<sub>2</sub>O<sub>2</sub> plays in cerebral responses to AngII in humans, requires further investigation.

In summary, the findings of this study indicate that levels of ROS generated in response to AngII are lower in the cerebral circulation of female versus male mice, and are associated with selectively weaker contractions of the MCA to AngII. Deletion of Nox2 significantly attenuated ROS generation and vasoconstrictor responses to AngII in males but not females. Moreover, we found strong pharmacological evidence that contractions to AngII in cerebral arteries of males are predominately mediated by H<sub>2</sub>O<sub>2</sub>. Thus, these findings indicate a key role for Nox2-containing NADPH-oxidase-derived ROS in gender-dependent differences in cerebrovascular responses to AngII. If similar processes apply at these concentrations of AngII in humans, such an effect could conceivably contribute to the lower clinical incidence of cerebrovascular diseases in females.

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### Disclosures

C.G.S. and G.R.D. are consultants for, and have significant ownership interests in, Radical Biotechnology Pty Ltd of Australia.

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