Estrous Cycle–Dependent Neurovascular Dysfunction Induced by Angiotensin II in the Mouse Neocortex

Carmen Capone, Josef Anrather, Teresa A. Milner, Costantino Iadecola

Abstract—Female mice are protected from the cerebrovascular dysfunction induced by angiotensin II (Ang II), an effect attributed to estrogen. We examined whether such cerebrovascular protection from Ang II is related to the estrous cycle. Cerebral blood flow was monitored by laser-Doppler flowmetry in anesthetized (urethane-chloralose) C57BL/6 female mice equipped with a cranial window. The phase of the estrous cycle was determined by vaginal smear cytology and plasma estrogen measurement. Ang II (0.25 μg/kg per minute, IV, 30 to 45 minutes) elevated arterial pressure (15 to 20 mm Hg) equally across the estrous cycle. However, in proestrus and estrus, phases in which estrogen is relatively high, Ang II did not impair the increase in the cerebral blood flow induced by neural activity or by endothelium-dependent vasodilators (P>0.05 from vehicle). In contrast, in diestrus (lower estrogen), Ang II induced a marked cerebrovascular dysfunction comparable to that of male mice. For example, the cerebral blood flow responses to whisker stimulation and to the endothelium-dependent vasodilator acetylcholine were attenuated by 41±12% and 49±12%, respectively (P<0.05; n=6 per group). The protection from the cerebrovascular effects of Ang II in proestrus was abolished by the estrogen receptor inhibitor ICI182,780. Ang II also increased production of free radicals in cerebral blood vessels in diestrus (+116±13%; P<0.05) but not in proestrus and estrus (P>0.05 from control). Topical treatment with ICI182,780 reestablished Ang II–induced oxidative stress in proestrus (P>0.05 from diestrus). We conclude that the protection from the neurovascular dysfunction induced by acute administration of Ang II in females depends on the estrous cycle and may underlie the increased propensity to cerebrovascular damage associated with low estrogen states. (Hypertension. 2009;54:302-307.)

Key Words: functional hyperemia • endothelium-dependent relaxation • sex differences • reactive oxygen species • laser-Doppler flowmetry

Hypertension is a major risk factor for stroke and dementia, devastating diseases related to the cerebrovascular damage induced by elevated blood pressure and its mediators. Hypertension leads to hypertrophy and remodeling of cerebral blood vessels and disrupts critical regulatory mechanisms of the cerebral circulation. These alterations increase the susceptibility of the brain to vascular insufficiency and ischemic injury. Premenopausal women are relatively spared from hypertension and its deleterious effects, a protection that is lost at menopause. These observations have suggested that female reproductive hormones, estrogen in particular, protect women from cardiovascular diseases. The finding that the cardiovascular risk of premenopausal women may vary cyclically across the menstrual cycle also suggests a role for ovarian hormones in cardiovascular diseases in women.

Sex differences have also been observed in animal models of hypertension. In particular, the increase in blood pressure induced by chronic, but not acute, administration of the pressor peptide angiotensin II (Ang II) is markedly attenuated in female animals. The attenuation is eliminated by ovariotomy and re-established by exogenous estrogen. The deleterious effects of Ang II on the regulation of the cerebral circulation are also sexually dimorphic. Thus, systemic administration of Ang II to male mice disrupts the increase in cerebral blood flow (CBF) produced by neural activity or endothelium-dependent vasodilators. In contrast, young adult female mice (aged 2 to 3 months) are spared from the cerebrovascular dysfunction induced by Ang II, a protection abolished by ovariotomy and reinstated by estrogen. However, because these studies were performed in random cycling females, a precise relationship between cerebrovascular protection and changes in hormonal levels during the estrous cycle could not be established. Furthermore, it is not known whether the mechanism of the protection in females is related to reduced vascular oxidative stress, a major causative factor in Ang II-induced cerebrovascular dysfunction.

We used acute administration of Ang II in normally cycling female mice to determine whether the protection from the cerebrovascular effects of Ang II is related to the estrous cycle. We found that Ang II-induced cerebrovascular dys-
function and oxidative stress do not occur in female mice when plasma estrogens are high (proestrus and estrus) but occur only when they are low (diestrus). An estrogen-receptor inhibitor blocked the reduction in oxidative stress and protection from cerebrovascular dysfunction. Our data provide the first demonstration that the cerebrovascular effects of Ang II are estrous cycle dependent and raise the possibility that the susceptibility to cerebrovascular injury in females varies across the estrous cycle.

Methods

General Surgical Procedures
All of the procedures were approved by the Weill Cornell Medical College Institutional Animal Care and Use Committee and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Studies were conducted in young adult 2- to 3-month-old C57BL/6J female mice (weight: 20 to 25 g; Jackson Laboratory, Bar Harbor, Maine). Mice were anesthetized with isoflurane (maintenance: 2%) in oxygen, intubated, and artificially ventilated (SAR-830, CWE Inc).11,12 Femoral vessels were cannulated for recording mean arterial pressure (MAP), Ang II administration, and collection of blood samples at the conclusion of the experiment. Rectal temperature was maintained at 37°C. After surgery, anesthesia was maintained only with urethane (750 mg/kg IP) and chloralose (50 mg/kg IP).11,12

Monitoring of CBF
Portions of the parietal bone and underlying dura (2×2 mm) were removed, and a region of the cerebral cortex, including the whisker-barrel area, was superfused with a modified Ringer’s solution (37°C; pH 7.3 to 7.4).12 Relative CBF was monitored in the whisker barrel cortex with a laser-Doppler probe (Periflux System 5010, Perimed AB). The outputs of the flowmeter and blood pressure transducer were connected to a computerized data acquisition system (MacLab). CBF was expressed as percentage increases relative to the resting level. Zero values were obtained after the heart was stopped by an overdose of isoflurane.

Estrous Stage and Estrogen Assay
The estrous cycle stage (proestrus, estrus, and diestrus 2) was determined once a day by vaginal smear cytology.14 Diestrus 2 rather than diestrus 1 (ie, metestrus) was chosen to ensure that mice were completely out of the estrus phase. For simplicity, we will use the term “diestrus” when referring to diestrus 2 throughout the article. To avoid using pseudopregnant mice, only animals that showed 2 consecutive regular 4- to 5-day cycles were studied. Blood was collected from the femoral artery at the end of the experiment, usually at 12:00 to 1:00 PM. Plasma estradiol levels were measured with a commercially available ELISA (Bioquant), as described previously.5

Experimental Protocol
CBF experiments were performed without knowledge of the phase of the estrous cycle of the mice studied. CBF recordings were started after MAP and blood gases were in a steady state (Table S1, see the online data supplement at http://hyper.ahajournals.org). All of the pharmacological agents were dissolved in Ringer’s solution. The cranial window was first superfused with Ringer (vehicle). The whisker-barrel cortex was activated for 60 seconds by stroking the contralateral somatosensory cortex with a laser-Doppler probe (Periflux System 5010, Perimed AB). The outputs of the flowmeter and blood pressure transducer were connected to a computerized data acquisition system (MacLab). CBF was expressed as percentage increases relative to the resting level. Zero values were obtained after the heart was stopped by an overdose of isoflurane.

Detection of Reactive Oxygen Species by Hydroethidine
Hydroethidine (dihydroethidium; Molecular Probes; 2 μmol/L) in Ringer’s solution was superfused on the somatosensory cortex for 60 minutes, as described previously.23 Next, the brain was removed and frozen. Coronal brain sections (thickness: 20 μm) were cut on a cryostat and collected at 100-μm intervals throughout the region exposed by the cranial window. Fluorescence intensity was measured as described previously.12,23 Briefly, brain sections were examined under a fluorescence microscope (Nikon) equipped with an eudthiam filter set (Chroma Technology, No. 31014). Images were acquired with a digital camera (Coolsnap, Roper Scientific). Fluorescence intensity was assessed in the brain area superfused with hydroethidine. The analysis was performed in a blinded fashion using the IPLab software (Scanalytics). Fluorescence intensities of all of the sections (20 per animal) were averaged and expressed as relative fluorescence units.23 The contralateral somatosensory cortex not superfused with hydroethidine served as the control for background fluorescence. Reactive oxygen species (ROS) were assessed after intravenous infusion of saline or Ang II in proestrus, estrus, or diestrus. ROS were also assessed in proestrus mice treated with saline or Ang II, with or without topical application of vehicle or ICI182,780.

Data Analysis
Data are expressed as mean±SE. Multiple comparisons were evaluated by ANOVA and Tukey’s test. Differences were considered statistically significant for P<0.05.

Results

Estrous Cycle Determination and Estrogen Assay
On vaginal smear cytology, proestrus was characterized by a predominance of round nucleated epithelial cells, estrus by a large number of cornified squamous epithelial cells, and diestrus by a predominance of small leukocytes.14 The approximate length of the phases were as follows: proestrus 1.0 days, estrus 2.0 days, and diestrus 1.5 days.24 In agreement with reports by others,24 estradiol plasma levels (n=5 per group) were higher in proestrus (39.3±2.6 pg/mL; P<0.05 from estrus and diestrus, ANOVA), intermediate in estrus (23.7±3.0 pg/mL; P<0.05 from proestrus and diestrus), and lower in diestrus (11.6±3.5 pg/mL).

ICI182,780 was dissolved in DMSO (Sigma), or bradykinin (BK; 50 μmol/L; Sigma) or the endothelium-independent vasodilator adenosine (50 μmol/L; Sigma) was superfused topically on the neocortex for 5 minutes. Agents were applied at concentrations determined previously to induce 50% of maximal responses.14 After recording CBF responses, saline or Ang II was infused intravenously. The Ang II infusion was adjusted to elevate MAP by 15 to 20 mm Hg gradually over 10 to 15 minutes until a stable increase was obtained.11,12 At this time, the infusion rate was 0.25±0.02 μg/kg per min, which produces elevations in plasma Ang II within the upper range of those produced by endogenous activation of the renin-angiotensin system in rodents.16 To avoid confounding effects on resting vascular tone, the concentration of Ang II infused was chosen not to alter resting CBF and was identical to that used in previous studies.8,11,12,17,18 CBF responses elicited by whisker stimulation, A23187, ACh, BK, and adenosine were tested again after 30 to 45 minutes of Ang II infusion. In some experiments, the high-affinity estrogen receptor antagonist ICI182,780 (10 μmol) or vehicle was topically applied to the neocortex during infusion of Ang II or saline in proestrus females, and CBF responses were tested 60 minutes later. The effect of ICI182,780 on CBF responses was investigated only in proestrus, because plasma estrogen concentration is highest. The ICI182,780 concentration was selected based on published data.20,21 ICI182,780 was dissolved in dimethyl sulfoxide and then diluted with Ringer to the desired concentration. The final dimethyl sulfoxide concentration was <0.2%, which does not affect the cerebrovascular responses tested.22

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Data Analysis
Data are expressed as mean±SE. Multiple comparisons were evaluated by ANOVA and Tukey’s test. Differences were considered statistically significant for P<0.05.
Effect of Ang II on Cerebrovascular Responses During the Estrous Cycle

Relative CBF at baseline did not differ across the estrous cycle (proestrus: 22±3 perfusion units; estrus: 20±3 perfusion units; diestrus: 20±3 perfusion units; P>0.05, ANOVA; n=6 per group). Similarly, the CBF increase elicited by whisker stimulation, by the endothelium-dependent vasodilators A23187, ACh, and BK or by the smooth muscle relaxant adenosine, was comparable across the cycle (Figures 1 and S1; P>0.05; n=6 per group). Ang II produced comparable elevations in MAP during the different stages of the cycle (Figure 1A; P>0.05; n=6 to 7 per group). However, Ang II attenuated the CBF responses to whisker stimulation, A23187, ACh, and BK in diestrus (Figure 1B and 1C and Figure S1; P<0.05) but not in proestrus and estrus (Figure 1B and 1C and Figure S1; P>0.05). Ang II did not alter the CBF response to adenosine (Figure 1D; P>0.05). The attenuation in cerebrovascular responses in diestrus was comparable to that observed in male mice or in ovariectomized females.8

Effect of the Estrogen Receptor InhibitorICI182,780 on the Cerebrovascular Dysfunctions Induced by Ang II

To determine whether the lack of sensitivity to the cerebrovascular effects of Ang II in proestrus was attributable to estrogen, we used an estrogen receptor antagonist ICI182,780. We found that ICI182,780 abolished such attenuation and re-established the ROS increase to the levels comparable to those observed in proestrus (P>0.05 from diestrus; P<0.05 from vehicle; Figure 3B).

Discussion

We investigated whether the cerebrovascular dysfunction induced by Ang II is related to the phases of the estrous cycle. We found that Ang II elevates MAP equally across the estrous cycle but that its cerebrovascular effects are only observed in diestrus (low plasma estrogen) and not in proestrus and estrus (high and intermediate plasma estrogen). To determine whether the reduced susceptibility to the cerebrovascular effects of Ang II in proestrus was attributable to estrogen, we used an estrogen receptor inhibitor. We found that ICI182,780 eliminated the protection from the cerebrovascular dysfunction induced by Ang II observed in proestrus. Furthermore, to determine whether the estrous cycle dependence of the cerebrovascular effects of Ang II was related to changes in ROS, we investigated ROS production across the estrous cycle. Consistent with the cerebrovascular data, we found that Ang II-induced ROS production was attenuated in proestrus and estrus and was increased in diestrus. These observations unveil a previously unrecognized estrous cycle dependence of the cerebrovascular dysfunction.

**Figure 1.** Cerebrovascular effect of acute intravenous administration of Ang II in cycling female mice. Effect of Ang II on MAP (A) and on the CBF increase produced by whisker stimulation (B), ACh (C), or adenosine (D). *P<0.05 from respective control before Ang II, ANOVA and Tukey’s test; n=6 per group.
induced by Ang II, a phenomenon attributable to variations in Ang II-induced ROS production throughout the estrous cycle.

Exclusion of Potential Sources of Artifacts
The differences in the cerebrovascular susceptibility to Ang II observed in the present study cannot be attributed to variations in body temperature or arterial blood gases, because these parameters were carefully controlled and did not differ among the groups of mice studied. Similarly, the differences in the cerebrovascular responses cannot be attributed to differences in baseline cerebrovascular reactivity across the estrous cycle, because the increases in CBF induced by whisker stimulation, ACh, BK, A23187, and adenosine were comparable in proestrus, estrus, and diestrus. This finding is consistent with studies in the rat tail artery or aorta in which vascular reactivity was not influenced by the estrous cycle.25,26 Finally, reversal of the protection from the cerebrovascular dysfunction induced by ICI182,780 in proestrus cannot be attributed to an indiscriminate worsening of vascular responses, because this agent did not affect the resting of cerebrovascular reactivity.

Estrous Cycle Dependence of the Cerebrovascular Effects of Ang II
We reported previously that the cerebrovascular effects of acute and chronic (7-day) Ang II administration are not observed in random cycling females.8 The vascular protection was abolished by ovariectomy and re-established by administration of estradiol,8 raising the possibility that estrogen was involved in the effect. Consistent with this hypothesis, we found that the protection from the cerebrovascular effects of Ang II is observed in proestrus and estrus, when plasma estrogen is higher, and not in diestrus, when estrogen levels are lowest. Because the neurovascular dysfunction is not observed in

Figure 2. Cerebrovascular effect of Ang II in proestrus mice treated with the estrogen receptor inhibitor ICI182,780 (10 μmol/L; neocortical application).
Effect of Ang II on MAP (A) and on the CBF increase produced by whisker stimulation (B), ACh (C), or adenosine (D).
*P<0.05 from vehicle, ANOVA and Tukey’s test; n=5 per group.

Figure 3. Ang II–induced ROS production in cycling female mice (A) and in proestrus mice topically treated with ICI182,780 (B). *P<0.05 from vehicle, ANOVA and Tukey’s test; n=5 per group.
proestrus and estrus, female mice are protected for most of the duration of the estrous cycle. This observation may explain why, in previous studies by us and others, random-cycling females were found to be protected from the cerebrovascular and hypertensive effects of Ang II.8–10 It remains to be established whether the cerebrovascular effects of chronic administration of Ang II or other pressor agents are also cycle dependent.

**Estrous Cycle Dependence of Ang II–Induced ROS Production**

ROS play a major role in the vascular effects of Ang II.27 In the model used in the present study, the increase in ROS induced by Ang II occurs only in cerebral blood vessels and, in males, is mediated by NADPH oxidase via activation of Ang II type 1 receptors.12 Therefore, we investigated whether the estrous cycle dependence of the cerebrovascular dysfunction elicited by Ang II is related to changes in vascular oxidative stress. We found that the increase in ROS production evoked by Ang II was present in diestrus, when cerebrovascular reactivity is impaired. However, Ang II–induced ROS production was attenuated in proestrus and estrus, when cerebral vessels are protected from the deleterious effects of Ang II. In proestrus, when estrogen levels are highest, ICI180,780 enhanced ROS production to levels comparable to those seen in diestrus, indicating that estrogen may have a role in the attenuation of ROS production. These findings indicate that the protection from the cerebrovascular effects of Ang II observed in proestrus and estrus is related to estrogen-induced attenuation of ROS production. However, because the estrogen receptor inhibitor ICI180,780 is not subtype specific, the estrogen receptor subtype(s) (α or β) involved in this effect remains to be determined. Although estrogen receptors are well known to fluctuate in neurons during the estrous cycle,28 it has not been established whether cycle-dependent fluctuations also occur in cerebrovascular estrogen receptors.29

Our data suggest that the cerebrovascular effects of Ang II depend on the stage of the estrous cycle. Other biological actions of Ang II are also estrous cycle dependent. For example, the dipsonic effect of centrally administered Ang II is attenuated in proestrus and estrus and more marked in diestrus.30 Considering that the dipsonic effect of Ang II requires ROS production in the subfornical organ,31 this observation is consistent with our finding that the ROS production induced by Ang II also depends on the estrous cycle. Therefore, the estrous cycle may modulate ROS production not only in cerebral blood vessels but also in the subfornical organ, suggesting a broader role for reproductive hormones in the biological effects of Ang II.

**Mechanisms of the Neurovascular Protection**

The mechanisms of estrogen-dependent variation in ROS production induced by Ang II in females remain to be established. Cerebral blood vessels of female rats have lower ROS production than those of males, a reduction abolished by ovariectomy and reinstated by estradiol administration.29 Expression of vascular Ang II type 1 receptors, which are coupled to ROS production, as well as the balance between Ang II type 1 and Ang II type 2 receptors, could also be modulated by sex hormones.32 In addition, estrogen increases the production of vasoprotective agents, eg, prostacyclin and NO, which could counteract the deleterious effects of Ang II.29 Direct antioxidant effects of estrogen and/or modulation of antioxidant defenses could also play a role.33 For example, the brain activity of the superoxide-scavenging enzyme Mn-superoxide dismutase is higher in proestrus than in diestrus.34

**Perspectives**

Although the incidence of cardiovascular and cerebrovascular diseases in premenopausal women is relatively low, the risk for myocardial ischemia in women with coronary artery diseases fluctuates with the phases of the estrous cycle and is highest when estrogen is low.5 Furthermore, the cardiovascular responses to acute stress in black women are enhanced when estrogen is low.6 To our knowledge, no evidence of estrous cycle–dependent variations in the susceptibility to acute stroke has been thus far provided. Nevertheless, the present data raise the possibility that the susceptibility to cerebrovascular events in women may depend on the phase of the estrous cycle. This hypothesis is supported by the observations that focal cerebral ischemia in rats produces larger infarcts in diestrus than in proestrus35 and that cerebral microcirculatory flow and thrombotic tendency are highest in diestrus.36 Therefore, our data suggest that the vulnerability of the female brain to vascular injury may vary according to the phases of the estrous cycle, as it is well established to occur in other neurological diseases, including epilepsy, migraine, and multiple sclerosis.37–39

**Conclusions**

We have demonstrated that the cerebrovascular dysfunction induced by Ang II varies across the estrous cycle. Thus, the dysfunction is not observed in proestrus and estrus but is present in diestrus. The protection from the cerebrovascular actions of Ang II in proestrus is abolished by the nonselective estrogen receptor antagonist ICI182,780, suggesting the involvement of estrogen in the mechanisms of the protection. Like the cerebrovascular effects, Ang II–induced ROS production is also estrous cycle dependent and is maximal in diestrus and attenuated in proestrus and estrus. ICI182,780 enhances the ROS increase in proestrus, also attesting to a role of estrogen receptors in the Ang II–induced ROS generation. These findings, collectively, unveil a previously unrecognized dependence of the cerebrovascular effects of Ang II on the estrous cycle and raise the possibility that the vulnerability of the female brain to cerebrovascular injury varies cyclically across the estrous cycle.

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**Disclosures**

None.
References

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Estrous cycle dependent neurovascular dysfunction induced by angiotensin II in the mouse neocortex

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Running head: Estrus cycle and cerebrovascular effects of AngII

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### Supplemental table S1

Arterial blood gases and pH in the mice in which CBF was studied

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Figure S1: Cerebrovascular effect of acute i.v. administration of AngII in cycling female mice. Effect of AngII on CBF increase produced by A23187 (A) or BK (B). *p<0.05 from respective control before AngII; analysis of variance and Tukey’s test; n=6/group.

Figure S2: Cerebrovascular effect of AngII in proestrus mice treated with the estrogen receptor inhibitor ICI182,780 (10µM; neocortical application). Effect of AngII on the CBF increase produced by A23187 (A) or BK (B) *p<0.05 from vehicle; analysis of variance and Tukey’s test; n=5/group.