Experimental Pediatric Arterial Ischemic Stroke Model Reveals Sex-specific Estrogen Signaling

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Background and Purpose—Pediatric stroke, birth to 18 years, is a significant cause of long-term disability in the United States; however, there is currently little experimental data on the pathophysiology of childhood stroke owing to lack of animal models. We developed a novel mouse model of experimental childhood-onset arterial ischemic stroke to characterize the sex-specific response of the adolescent brain to cerebral ischemia and assess the neuroprotective effect of estrogen at this developmental stage.

Methods—Postnatal day 20 to 25 mice were subjected to 90 minutes experimental stroke via the intraluminal filament middle cerebral artery occlusion model and ischemic damage assessed 22 hours after reperfusion. Real-time quantitative real-time polymerase chain reaction was performed 22 hours after middle cerebral artery occlusion to determine the effects of ischemia and estrogen treatment on the proapoptotic gene Bax.

Results—Ischemic injury did not differ between male and female juvenile (postnatal day 20–25) mice after middle cerebral artery occlusion. However, estrogen reduced ischemic injury in female mice, whereas having no effect in juvenile males. No differences in estrogen receptor expression were observed on postnatal day between 20 males and females. In contrast, estrogen minimized the ischemia-induced increase in the proapoptotic gene Bax in female mice, whereas having no effect on Bax induction in the male brain.

Conclusions—Focal ischemia has fundamentally different effects in the juvenile brain compared with the adult, as evidenced by the lack of sex difference in ischemic injury in the murine postnatal day 20 to 25 middle cerebral artery occlusion model and the sexually dimorphic response to estrogen neuroprotection. (Stroke. 2013;44:759-763.)

Key Words: Bax, Bcl-2 ■ cerebral ischemia ■ childhood stroke ■ estrogen

Pediatric stroke, birth through age 18 years, is a significant cause of long-term disability, with an incidence of ≈1100000 children annually in the United States. A majority of pediatric strokes are arterial ischemic strokes (AIS) and include perinatal AIS (classically presenting with seizure in the first days of life) and childhood-onset AIS (occurring beyond the neonatal period). Permanent neurological deficits are observed in 50% to 90% of survivors. It is becoming increasingly appreciated clinically that early diagnosis and appropriate rehabilitation therapies can improve outcome in children after AIS, as we are seeing in the adult population. Children are not just small adults, as evidenced by dramatic differences in AIS risk factors. However, there are currently few viable experimental models aimed at determining the cause and modeling pathophysiology of childhood-onset AIS. There is extensive experimental literature modeling ischemia in perinatal animals and reports of pediatric stroke experiments in piglets, with no reports of the use of rodents to model pediatric ischemia. The current study describes a variation of the middle cerebral artery occlusion (MCAO) model modified for use in juvenile mice (modeling prepubertal children), providing a powerful new experimental tool to begin to study the effects of ischemia in the immature brain.

Incidence of childhood-onset AIS is sexually dimorphic, with boys at slightly greater risk; however, relative severity of outcome in boys and girls remains uncertain. In adult humans and animal models, females experience less damage after comparable ischemic insults compared with age-matched males. The relative advantage observed in adult females has been attributed to endogenous sex steroids, in particular, estrogen. Indeed, there is an extensive evidence that estrogen is neuroprotective in adult male and female animals after experimental AIS (MCAO). Estrogen is a pleiotropic steroid hormone that affects several signaling cascades to exert its neuroprotective effects. An important neuroprotective mechanism of estrogen is its ability to decrease ischemia-induced apoptotic cell death by increasing expression of the antiapoptotic protein Bcl-2 and decreasing the proapoptotic protein Bax, among others. Therefore, we used our novel model of experimental childhood-onset stroke to...
test 2 related hypotheses: (1) that juvenile male and female ischemic outcomes differ; and (2) that exogenous estrogen protects young brain by decreased expression of proapoptotic genes in a sex-specific manner.

Methods

Experimental Animals

All experimental protocols were approved by the Institutional Animal Care and Use Committee and conformed to the National Institutes of Health guidelines for the care and use of animals in research. To assess the effect of prolonged, controlled levels of hormones, 17β-estradiol (E2; 12.6 µg) was administered via subcutaneous silastic implants 2 days before experiments. We chose this dose based on previous studies in our laboratory.13 All MCAO experiments using vehicle or E2 were performed in a blinded, randomized manner using male and female C57Bl/6 mice at postnatal days 20 to 25.

Middle Cerebral Artery Occlusion

Methods are as previously published in mouse adult,14 with minor variations to accommodate the small size of postnatal day 20 to 25 mice (including 6-0 nylon suture that was heat-blunted and coated with silicone gel to obtain a smaller filament diameter of ≤0.18 mm). Cerebral ischemia was induced for 90 minutes of reversible MCAO via the intraluminal suture method under isoﬂurane anesthesia. Adequacy of MCAO was confirmed by laser Doppler flowmetry measured (≥70% drop required for inclusion) over the ipsilateral parietal cortex in all mice and by neurological deficit scoring at end of occlusion. Additionally, neurological deﬁcits were measured 22 hours after reperfusion. Neurological deﬁcit scored as follows: 0=no deficit, 1= failure to extend forelimb, 2=circling, 3=unilateral weakness, and 4=no spontaneous activity.15,16

Infarct Volume Analysis

After the period of reperfusion, the mice were anesthetized with isoﬂurane (2.0%–3.0%), and animals were then decapitated for brain removal. Each brain was sliced into five 2 mm thick coronal sections. The sections were placed in a 1.2% solution of 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, St Louis, MO) for 30 minutes at 37°C and fixed in 10% formalin for 24 hours. Both sides of each stained coronal slice were photographed using a digital camera, and infarction was measured with digital image analysis software (SigmaScan Pro; Jandel, San Rafael, CA) and integrated across all 5 slices. To account for the effect of edema, the infarcted volume was estimated and expressed as a percentage of the contralateral structure.

Quantitative Reverse Transcriptase-PCR

For quantitative polymerase chain reaction (PCR) measurement of estrogen receptor (ER) transcripts and apoptotic gene expression, cortical tissue was dissected from the penumbra of the ischemic hemisphere 22 hours after MCAO, and the corresponding cortical region was isolated from the nonischemic contralateral hemisphere. The penumbra was identified by cutting an adjacent 1 mm thick slice and staining with TTC to identify the infarcted region in each animal, as described previously. Total RNA was isolated using the RNAqueous-4 PCR kit (Ambion, Austin, TX) as per the manufacturer’s instructions. Briefly, ≈1 to 3 mg of tissue was lysed in lysis buffer and total RNA was isolated and eluted from a column with 50 µL RNase-free elution buffer and further treated with Turbo DNase (Ambion). First strand cDNA was reverse transcribed from 500 ng total RNA with high capacity cDNA archive Kit (Applied Biosystems, Foster City, CA). Real-time PCR reactions using 6-carboxyfluorescein (FAM) labeled primer/probe sets were performed on ABI Prism 7000 sequence detection system in triplicate using 50 ng cDNA. Probe/Primer sets used to detect Bax, Bcl-2, and the ERs were synthesized by Invitrogen. The housekeeping gene 18S was also assayed for each sample using 5 ng of cDNA. 18S RNA was chosen as the normalizer because of its abundance and stability, making it highly unlikely to be effected by ischemia. Cycle parameters used were 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Expression levels were calculated as the ratio of the target gene:18S.

Measurement of E2

Two hundred to 400 mg of brain tissue was homogenized using liquid nitrogen and kept at 4°C during the whole procedure. After homogenization, the samples were centrifuged at 13 000g and 4°C for 10 minutes. An agilent HPLC system consisting of 3 HPLC pumps, a column thermostat, and a 6-port switching valve coupled to an ABI-Sciex API5500 was used for the analysis of estrogen. A Zorbax XDB-C8 guard column (12x<4.6 mm) was used for an inline extraction and a Zorbax XDB-C8 analytic column (3.5 µm, 4.6x150 mm) was used for the separation of analytes (both columns from Agilent Technologies, Palo Alto, CA). Twenty percent of methanol and 80% of 0.1% formic acid were initially pumped at 500 µL/min, during the injection for loading the analytes onto the inline extraction column. Atmospheric pressure photo ionization was used for the ionization of steroids. Toluene at the flow rate of 200 µL/min was used as dopant. The API5500 mass spectrometer was operated in positive multiple reaction monitoring mode. The following parent-fragment ions were monitored: estrone and estriol 271.1>133.1, estradiol 255.1>159.1. Analyst Software version 1.5.2 was used for data acquisition and data processing. All calibration curves had a correlation coefficient R>0.99.

Statistical Analysis

All data are presented as mean±SD. Histological damage was compared using 1-way and 2-way ANOVA followed by Newman–Keuls multiple comparison test to determine the source of variance. Student t test was used to compare Bax and Bcl-2 mRNA expression. Probability values ≤0.05 were considered statistically significant.

Results

Male and female mice at postnatal age 20 to 25 days were subjected to 90 minutes of MCAO, and infarct volume was analyzed after 22 hours reperfusion. Male and female mice were implanted with subcutaneous 17β-estradiol (E2) or vehicle 2 days before MCAO. A total of 85 mice were used for the study (69 for histological analysis and 16 used for brain E2 measurements); 5 were excluded for insufficient laser Doppler flow reduction; 5 were excluded because of premature mortality (2 male oil, 2 male E2, and 1 female oil mouse); additionally, 1 female E2 and 1 male E2 were excluded owing to the absence of behavioral deficit. No differences in intraischemic physiological parameters, laser Doppler flowmetry, or behavioral assessments were observed among the resulting experimental groups (Table 1). E2 implanted mice had significantly elevated levels of brain 17β-estradiol measured 2 days after implantation with either oil or E2 (Table 2).

To characterize possible sex differences in ischemic outcome in childhood experimental stroke, male and female mice of the same age (postnatal day 20 to 25) were exposed to 90 minutes MCAO. Male and female mice exhibited nearly identical ischemic damage after MCAO, with edema-corrected hemispheric infarct volumes of 41±12% (n=9) and 41±14% (n=8), respectively (Figure 1). Ischemic damage was not different between sexes in the cortex (52±12% in male and 66±8.2% in female) and striatum (65±17% in male and 73±12% in female).

In a separate cohort of mice, we examined the neuroprotective effectiveness of E2 in young male and female mice.
E2-treated female mice had significantly smaller infarct volumes than vehicle-treated female mice, exhibiting corrected hemispheric infarct volumes of 48±8.9% (n=7) in vehicle and 25±9.7% (n=7; P<0.05) in E2-treated females (Figure 2A). Estrogen was equally as protective in cortex (67±15% in vehicle and 39±25% in E2) and striatum (88±16% in vehicle and 38±23% in E2). In contrast, E2 had no effect on male infarct volume in any of the brain regions analyzed (Figure 2A). Importantly, infarct volume of E2-treated female pediatric brain remained reduced when analyzed 3 days after reperfusion, exhibiting 51±8.1% (n=6) in vehicle and 27±16% (n=6, P<0.05) in E2-treated female (Figure 2B).

To determine the relative expression of ERs α and β (ERα and ERβ) in the juvenile mouse brain, quantitative real-time PCR was performed from cortex of untreated males and females. Figure 3A demonstrates that a comparison of expression of each receptor in male and female cortex revealed a trend toward greater expression of each receptor in the male brain compared with the female brain (male ERα expression was 184±99% [n=5; P=0.13] to that of female ERα, and male ERβ was 165±44% [n=5; P=0.07] to that of female ERβ). However, relative expression of ERα to ERβ was similar between males and females (ERα/ERβ ratio was ≈20 in males and 18 in females).

Because estrogen inhibits cell death after ischemia in adult animals by decreasing apoptosis, we analyzed the expression of Bax and Bcl-2, known contributors to apoptosis signaling, in our juvenile mouse MCAO model. Expression of Bax and Bcl-2 was analyzed in ischemic (penumbra) and nonischemic cortex 22 hours after MCAO. We observed a significant increase in Bax mRNA expression in the penumbra relative to corresponding nonischemic region in both male and female brains (Bax increased to 160±56% [n=6; P<0.05 compared with vehicle; Figure 3B]. In contrast, E2 treatment did not significantly alter Bax expression in male animals (Figure 3B). Figure 3C demonstrates the minimal effects of ischemia and E2 treatment on Bcl-2 expression in the young brain.

**Discussion**

Our results indicate that ischemic injury is comparable between young male and female mice exposed to identical ischemic duration. This observation is in stark contrast to the adult experimental stroke literature, which provides overwhelming evidence that females are relatively resistant to ischemia compared with males. The simplest explanation for the lack of sexual dimorphism in our experimental pediatric model is the low level of endogenous estrogen during childhood. Estrogen levels are comparable in boys and girls before puberty, at which time girls begin producing large levels of estrogen and boys begin producing androgens. Female E2 significantly reduced the ischemia-induced increase in Bax expression, reducing Bax in the penumbra from 199±52% of nonischemic cortex to 130±23% of corresponding E2-treated nonischemic cortex (n=6; P<0.05 compared with vehicle; Figure 3B). In contrast, E2 treatment did not significantly alter Bax expression in male animals (Figure 3B). Figure 3C demonstrates the minimal effects of ischemia and E2 treatment on Bcl-2 expression in the young brain.

### Table 1. LDF, Temporals Muscle Temperature, and Behavioral Assessment in 90 Minutes MCAO

<table>
<thead>
<tr>
<th>Group</th>
<th>LDF, % MCAO (85 mins)</th>
<th>Temporal Muscle Temperature, °C</th>
<th>Behavior Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male V (n=6)</td>
<td>20±6</td>
<td>36.5±0.2</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td>Male E2 (n=8)</td>
<td>22±4</td>
<td>36.8±0.3</td>
<td>2.1±0.2</td>
</tr>
<tr>
<td>Female V (n=7)</td>
<td>25±5</td>
<td>36.8±0.1</td>
<td>2.3±0.3</td>
</tr>
<tr>
<td>Female E2 (n=7)</td>
<td>18±4</td>
<td>36.4±0.4</td>
<td>2.3±0.2</td>
</tr>
</tbody>
</table>

Data are presented as mean±SEM.

E2 indicates 17β estradiol, LDF, laser Doppler flowmetry, MCAO, middle cerebral artery occlusion; and V, vehicle.

No differences were found among groups.

### Table 2. Brain E2

<table>
<thead>
<tr>
<th>Group</th>
<th>% Infarct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male V (n=3)</td>
<td>2.1±1.3</td>
</tr>
<tr>
<td>Male E2 (n=5)</td>
<td>336.2±41.7*</td>
</tr>
<tr>
<td>Female V (n=4)</td>
<td>8.8±3.3</td>
</tr>
<tr>
<td>Female E2 (n=4)</td>
<td>454.5±25.3*$</td>
</tr>
</tbody>
</table>

Data are presented as mean±SEM.

E2 indicates 17β estradiol; and V, vehicle.

* P<0.05 compared with V; and $P<0.05 compared with male E2.
mice experience a sharp increase in estrogen production, that is puberty, at ≈5 weeks of age (postnatal day 28–49).17,18 We used mice before postnatal day 25 and observed a lack of benefit in females, consistent with the presumed similar levels of estrogen between male and female mice at this developmental stage. To directly test this hypothesis, we administered estrogen to female mice, hypothesizing that we would produce a sex difference in ischemic outcome reminiscent of that observed in the adult. Indeed, female mice in the presence of exogenous estrogen have significantly smaller infarcts compared with their male counterparts.

We made the notable observation that estrogen failed to protect the juvenile male brain from transient focal ischemia. This is in contrast to the large body of literature that reports potent estrogen neuroprotection in adult male and female animals after experimental ischemia.8,9,19 Although the molecular mechanism of this novel observation warrants further study, our findings suggest that the failure of estrogen to have a neuroprotective effect on the juvenile male brain after AIS is not because of a lack of receptor expression, as we found robust expression of ERs in the male brain at this age. This suggested that a downstream mediator of estrogen signaling was implicated, which differed fundamentally between the juvenile male and female brain. There is strong evidence in the adult stroke literature that estrogen neuroprotection in both males and females is in part mediated by its potent antiapoptotic actions.10–12 However, we observe female-specific regulation of apoptosis in our novel model of childhood-onset AIS. E2 regulation of Bax in female brain and not male brain indicates a female-specific neuroprotective signaling that may underlie the reduced ischemia observed histologically in this study. Interestingly,
we did not observe an effect of E2 on Bcl-2 expression in either male or female mice after MCAO. This finding may provide insight in E2 neuroprotection in the young brain, implicating cell-extrinsic apoptosis signaling in the cell death and neuroprotection pathways engaged in childhood-onset AIS. Further research is necessary to fully elucidate this intriguing observation.

Conclusions

We have developed a new experimental model of childhood-onset AIS in mice that will provide an important and clinically relevant platform for the study of ischemic signaling in the young brain. Our data demonstrates that the overall injury after transient focal ischemia is not different in young male and females; however, we revealed fundamental sex differences in signaling. Female-specific estrogen neuroprotection in our juvenile mouse MCAO model is an important observation because it provides the first experimental evidence that protective strategies developed in the adult may not translate directly to children, making it extremely important to obtain experimental preclinical data in this age group.

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Disclosures

None.

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