Estrogen-Mediated Neuroprotection After Experimental Stroke in Male Rats

Thomas J.K. Young, MD; Richard J. Traystman, PhD; Patricia D. Hurn, PhD

Background and Purpose—We have previously shown that 17β-estradiol reduces infarction volume in female rats. The present study determined whether single injection or chronic implantation of estrogen confers neuroprotection in male animals with middle cerebral artery occlusion (MCAO) and whether there is an interaction with endogenous testosterone.

Methods—Male Wistar rats were treated with 2 hours of reversible MCAO. In protocol 1, acute versus chronic estrogen administration was examined in groups receiving the following: Premarin (USP) 1 mg/kg IV, immediately before MCAO (Acute, n=13, plasma estradiol=171±51 pg/mL); 7 days of 25 µg (E25, n=10, 10±3 pg/mL) or 100 µg 17β-estradiol (E100, n=12, 69±20 pg/mL) by subcutaneous implant; or saline (SAL, n=21, 3±1 pg/mL). Laser-Doppler flowmetry was used to monitor the ipsilateral parietal cortex throughout the ischemic period and early reperfusion. At 22 hours of reperfusion, infarction volume was determined by 0 2,3,5-triphenyltetrazolium chloride staining and image analysis. In protocol 2, rats were castrated to deplete endogenous testosterone and then treated with estradiol implants: castration only (CAST, n=13, estradiol=5±2 pg/mL), sham-operated (SHAM, n=10, 4±2 pg/mL), estradiol implant 25 µg (CAST+E25, n=16, 7±2 pg/mL) or 100 µg (CAST+E100, n=14, 77±14 pg/mL).

Results—Cortical infarct volumes were reduced in all estrogen-treated groups: Acute (21±4% of ipsilateral cortex), E25 (12±5%), and E100 (12±3%) relative to SAL (38±5%). Caudate infarction was similarly decreased: Acute (39±7% of ipsilateral striatum), E25 (25±7%), and E100 (34±6%) relative to SAL (63±4%). Castration did not alter ischemic outcome; cortical and caudate infarction (percentage of respective ipsilateral regions) were 37±5% and 59±5% in CAST and 39±7% and 57±5% in SHAM, respectively. Estrogen replacement reduced infarction volume in castrated animals in cortex (19±4% in CAST+E25 and 12±4% in CAST+E100) and in caudate (42±6% in CAST+E25 and 20±7% in CAST+E100). Laser-Doppler flowmetry results during ischemia and reperfusion was not different among groups.

Conclusions—Both acute and chronic 17β-estradiol treatments protect male brain in experimental stroke. Testosterone availability does not alter estradiol-mediated tissue salvage after MCAO. (Stroke. 1998;29:1666-1670.)

Key Words: estrogen ■ cerebral ischemia ■ neuroprotection ■ stroke ■ testosterone ■ rats

Recent evidence emphasizes striking sex-linked differences in brain damage after experimental stroke. We have shown previously that female rats sustain approximately one third of the total tissue infarction observed in age-matched males during middle cerebral artery occlusion (MCAO).1 Furthermore, both endogenous and exogenous estrogens improve tissue damage after stroke and brain injury in female animals.3–4 Estrogen may act both by vascular mechanisms to enhance residual ischemic blood flow and by direct neuroprotection of neurons and glia in female brain,5–9 but effects in the male brain are unclear. The major male reproductive steroid, testosterone, has not been studied in experimental cerebral ischemia and remains an alternative to estrogen as the source of sex differences in stroke. Like estrogen, testosterone is a vasodilator of some vascular beds,10,11 possibly by a common mechanism involving vascular smooth muscle potassium channels.9–11 The purpose of the present study was to determine whether estrogen administered to adult male Wistar rats confers protection after MCAO and whether there is a potential interaction between exogenous estrogen and native testosterone.

Materials and Methods
This study was conducted in accordance with the National Institutes of Health guidelines for the care and use of animals in research. All protocols were approved by the Animal Care and Use Committee of the Johns Hopkins University. All methods have been previously published.12 In brief, male Wistar rats (250 to 420 g) were anesthetized with 1% to 2% halothane delivered via face mask in oxygen-enriched air and instrumented with femoral artery catheters for physiological monitoring and blood gas measurement. Rectal and temporalis muscle temperatures were controlled at 37.5±0.5°C using heat lamps. Cortical perfusion as measured by laser-Doppler flowmetry (LDF, model MBF3D, Moor Instruments Ltd) was...
Physiological Data

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Values are mean±SE. MAP indicates arterial blood pressure; Acute E, intravenous estrogen 1 mg/kg; E25, chronic estrogen 25 µg implant; E100, chronic estrogen 100 µg implant; CAST, castrated; and SHAM, sham-operated.

determined as previously described, with probe placement at 2 mm posterior and 6 mm lateral to bregma." Focal cerebral ischemia was accomplished using the intraluminal filament model (4-0 nylon monofilament suture) of proximal MCAO. The right common carotid artery was exposed through a lateral incision, separated from the vagus, and ligated. The external carotid artery was ligated, the occipital branch cauterized, and pterygopalatine artery ligated. An occluding filament was advanced through the common carotid artery until the LDF signal displayed an abrupt and significant reduction, confirming ongoing ischemia, and then was secured in place. Ischemic LDF was determined over 5-minute periods throughout the 2-hour occlusion period, then the suture was withdrawn with prompt restoration of blood flow. Each animal was allowed to recover and supported with intravenous saline (0.6 mL/h) and supplemental oxygen as needed. After 22 hours of reperfusion, the animal was reanesthetized for blood sampling for plasma hormone levels and harvesting of brain. The tissue was sliced into seven 2-mm-thick coronal sections for 2,3,5-triphenyltetrazolium chloride (TTC) staining and quantification via standard photography and digital planimetry (SigmaScan Pro, Jandel). The infarcted area was numerically integrated across each section and over the entire ipsilateral hemisphere. Infarct volume was measured separately in the cortex and caudate putamen and expressed as a volume percentage of the ipsilateral structure. Plasma 17β-estradiol and testosterone levels were determined by radioimmunoassay (Diagnostic Products Corp) as previously described. All standards and samples were assayed in duplicate with an interassay and intraassay variability of 4% and 7%, respectively.

In protocol 1, acute versus chronic 17β-estradiol administration was examined in normal male rats. In animals randomized to chronic treatment, estradiol pellets (E25 [25 µg, n=10] or E100 [100 µg, n=12]) were implanted under halothane anesthesia in the skin at the dorsal neck at 7 to 10 days before MCAO. Premarin (USP) 1 mg/kg (Acute, n=13) or an equivalent volume of saline (SAL, n=21) was injected through the femoral venous catheter 30 minutes before MCAO.

In protocol 2, the effect of castration combined with estradiol treatment was determined. Castration (CAST, n=13) or sham operation (SHAM, n=10) was performed in 14- to 18-week-old male rats under halothane anesthesia. A longitudinal incision, approximately 1.5 cm in length, was made over the median septum, and each testicular capsule was incised. The spermatic cord was ligated above the head of the epididymis with 2-0 silk suture and cut with subsequent cauterization of all spermatic vessels. The surgical wounds were closed, and the animal was allowed to recover for 8 to 10 days before MCAO. Sham-operated males were treated with all surgical procedures except castration. Rats randomized to receive 17β-estradiol pellets were instrumented while still under anesthesia for castration (CAST+25 [25 µg, n=16] or CAST+100 [100 µg, n=14]).

All values are reported as mean±SE; all physiological variables were analyzed by 2-way ANOVA and post hoc Newman-Keuls test. Infarct volumes and mean residual laser-Doppler flow were analyzed by 1-way ANOVA and Newman-Keuls test. Statistical significance was confirmed at a value of P<0.05.

Results

Physiological data are summarized in the Table. Levels of arterial blood pressure, pH, blood gases, glucose, and hemoglobin were similar among groups during MCAO and early reperfusion. The ipsilateral LDF signal during MCAO decreased rapidly to ~25% of baseline values and remained at this level for the duration of occlusion (Figure 1). On reperfusion, the LDF signal returned toward baseline by 15 minutes. Averaged LDF over the ischemic period was equivalent in all groups: SAL, 23±2% of baseline; Acute, 24±3%; E25, 29±4%; and E100, 29±3%. Cortical infarction volume was reduced by acute estrogen treatment, and there was no

Figure 1. Ipsilateral parietal LDF during MCAO and reperfusion in normal estrogen-treated males. Reduction in ischemic LDF was not different among groups. Groups: ACUTE, n=13, 1 mg/kg IV injection; E25, n=10, 25 µg subcutaneous estradiol implant; E100, n=12, 100 µg estradiol implant; or SAL, n=21, equivalent volume of saline. Values are means for each group.
Further reduction with chronic estrogen treatment at either dose (Figure 2). Similarly, infarction was equally reduced in caudate putamen by both acute and chronic estrogen treatment.

In protocol 2, plasma testosterone ranged from 0.05 to 1.62 ng/mL in noncastrated rats and below detection in all castrated animals. Ischemic LDF remained stable throughout occlusion and returned toward baseline in all animals. Averaged LDF over the ischemic period was equivalent in all groups: CAST, 23±2% of baseline; SHAM, 24±4%; CAST+25, 26±2%; and CAST+100, 27±2%. There was no difference in cortical or caudate infarction volume in CAST relative to SHAM animals (Figure 3). Furthermore, estrogen treatment consistently decreased tissue injury after MCAO in castrated males at both implant doses.

**Figure 3.** Infarction volume as a percentage of ipsilateral structure. Groups: CAST, n=13, castrated 8 to 10 days before MCAO; SHAM, n=10, sham-operated; CAST+E25, n=16, castrated and 25 μg estradiol implant; or CAST+E100, n=14, castrated and 100 μg estradiol implant. *P*≤0.05 from CAST. Values are mean±SE.

**Discussion**

This study demonstrates 4 important findings. First, exogenous estrogen reduces injury in the male brain after MCAO, but this was not associated with preservation of the LDF signal during vascular occlusion. Second, intravenous estrogen injection immediately before ischemia provides protection that is equivalent to that of chronic hormone treatment over time. Therefore, castration and loss of testosterone does not alter tissue outcome in acute experimental stroke. Finally, testosterone availability does not diminish or enhance estradiol-mediated tissue salvage after MCAO in male brain. These findings clearly demonstrate that exogenous estrogen provides rapid neuroprotection in male brain subsequently insulted by vascular occlusion and cerebral ischemia.

Numerous studies now indicate that the magnitude of injury after experimental stroke is gender linked.1–4 We have previously found that females “primed” with estrogen sustain strikingly smaller brain infarcts in both cortex and striatum than age-matched males or estrogen-deficient females.1 The capacity of estrogen to alter ischemic pathology has been reported across animal species1–4 and intra-species strains.1,3 The present findings strengthen the hypothesis that estrogen is a major mediator of sex differences in stroke and clearly demonstrate that the benefits of estrogen can be extended to the male brain, reducing tissue injury consequent to cerebral ischemia. The large differences in infarction volume are not explained by differences in intraschismic LDF signal; the percent reduction in LDF was not different among estrogen-treated or deficient groups. This observation suggests, but does not prove, that the ability of exogenous estrogen to salvage tissue after MCAO in the male brain is not dependent on preservation of ischemic blood flow. Because LDF measures relative changes in cortical perfusion, differences in absolute blood flow at end-ischemia cannot be excluded. Endogenous estrogen is associated with a striking preservation of LDF and of striatal, but not cortical, ischemic blood flow as measured by [14C]Jodoantipyrine in the estrous rat. In addition, estrogen augments cognition-activated regional cerebral blood flow in young women11 and improves perfusion in older women with vascular pathology.14 The lack of effect on the ischemic LDF signal in the present study may be due to a sex difference or to differing activity by pharmacological estrogens versus the endogenous steroid on the cerebral vasculature during experimental stroke.

Estrogen exhibits an array of actions in parenchymal cells that potentially accounts for the hormone’s ability to salvage ischemic tissue, including antioxidant actions,15,16 amelioration of glutamate-induced excitotoxicity,17,18 and amplification of trophic mechanisms through cross talk with growth factors.19 Autoradiographic studies using 1H-estradiol injections demonstrate a wide range of sites of nuclear accumulation and retention of radioactivity in brain and spinal cord of both sexes.20 Estrogen receptor mRNA–containing cells have been identified in numerous brain areas not associated with reproductive function, including cortex and hippocampus.21 Consequently, there are numerous potential target sites.
for direct neuroprotective actions in estrogen-treated male brain.

Reduction of stroke volume was achieved at both physiological and pharmacological plasma estradiol levels. In the E25 and CAST+25 groups, plasma levels were 10±3 and 7±2 pg/mL, respectively; these are normal physiological values for cycling females compared with the Acute, E100, and CAST+100 groups, which received pharmacological doses. Steroid hormones are typically thought to produce major effects on target cell structure or function via intracellular receptors that translocate to the nucleus and alter gene transcription. Although classic estrogen receptors are present in numerous male brain regions,

21 the neuroprotection afforded in this study by a single injection immediately before MCAO prompts a consideration of nongenomic, as well as genomic, mechanisms at work. Rapid membrane-associated actions unrelated to transcription also have been reported for estrogen in brain, but these remain controversial at present.22,23 Specifically, high concentrations of estrogen are required to elicit cell surface events, which would be consistent with the pharmacological injection doses used in the present study.

The basal testosterone levels in our male Wistar rats (1 ng/mL) are the same as those previously reported in young male rodents.24 These data are important in confirming that testosterone is not the cause of enhanced stroke injury in male versus female animals. Although the incidence of stroke is well known to be higher in men than in age-matched premenopausal women, it is not clear whether testosterone plays a role in stroke risk or outcome once an ischemic event has occurred. Testosterone has been implicated as a risk factor for acute myocardial infarction,25 increased thrombogenicity,26 and altered lipid metabolism in young men27 and in cardiovascular collapse after trauma and hemorrhagic shock in animals.28 Our data suggest that endogenous testosterone may have little effect in acute stroke. Furthermore, testosterone is vasoactive in some regional vascular beds, although its effects on the cerebral vascular factors should be considered of importance in the male brain. Elucidating the reproductive steroid mechanisms in stroke may yield new treatment insights and strategies for both sexes.

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References

The article by Toung and colleagues concisely defines a protective effect of both acute and chronic administration of 17β-estradiol to limit ischemic damage following occlusion of the middle cerebral artery in male rats. This study is a logical extension of previous studies that have identified a gender-associated difference in infarct size after cerebral ischemia. The results extend previous observations and demonstrate that the female sex hormone when administered before cerebral ischemia limits the size of cerebral infarct in male animals. This “protective” effect of estrogen was not limited by endogenous testosterone, as the size of infarcts was reduced comparably in gonadally intact and castrated male rats. These results are exciting in that they suggest that the neural protective effects of estrogen are not gender specific.

The concept that therapeutic benefit of estrogens may be uncoupled from gender is consistent with vascular effects of estrogen. Indeed, estrogen treatment limits transplant-associated atherosclerosis in male animals by mechanisms that may require receptor activation and transcriptional regulation of growth factors and expression of major histocompatibility complex class II antigens. Whether activation of specific estrogen receptors (α and/or β) is required for vascular effects of estrogen to be mediated is unclear. Estrogen receptor α may not be required, as estrogen reduces proliferation after arterial injury and increases endothelium-mediated vasodilatation in male mice and humans deficient in this receptor. Whether neural protective effects of estrogens require integrity of estrogen receptors remains to be determined, as do the mechanisms (genomic or nongenomic) of the effects. After ischemia, estrogen may provide protection against cellular injury related to antioxidant properties of the molecule.

The results of the study of Toung et al also point to potential therapeutic uses of estrogen to limit cerebral damage in acute clinical situations, for example, in patients with crescendo transient ischemic attacks or with stroke in evolution. Estrogen treatment also may have the potential to prolong safe occlusion time during carotid endarterectomy in high risk patients with poor collateral blood supply to the ipsilateral hemisphere and reduce the risk of ischemic stroke. As the authors point out, the “therapeutic window” for application of estrogen to extend to postischemic times to reduce ischemic penumbra needs to be investigated. “Designer estrogens” that could mimic the vascular and neural protective actions of the native hormone in the postischemic period may provide new therapeutic options in the treatment of cerebral ischemia in both men and women.

Virginia M. Miller, PhD, Guest Editor
Department of Surgery and Physiology
Mayo Foundation
Rochester, Minnesota

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