Sex-Specific Role of Thioredoxin in Neuroprotection Against Iron-Induced Brain Injury Conferred by Estradiol

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**Background and Purpose**—Accumulation of iron after intracerebral hemorrhage causes free radical formation and oxidative damage resulting in liquefaction. The aim of this study was the investigation of molecular mechanisms underlying estrogen-mediated neuroprotective effect against iron-induced brain injury in vivo.

**Methods**—Age-matched male and female Sprague-Dawley rats were stereotaxically infused with either ferrous citrate (FC) or saline (10 μL) into the right caudate nucleus. Beta-estradiol 3-benzoate (E2) capsule was implanted subcutaneously at 24 hours before infusion of FC. The severity of brain injury and neurological deficits were measured by histological quantification and forelimb asymmetry test, respectively. The role of thioredoxin (Trx) in E2-mediated neuroprotective effect was examined by intrastriatal administration of a Trx reductase inhibitor, 5,5-dithiobis-(2-nitrobenzoic acid), and small interfering RNA.

**Results**—FC induced greater brain injury in male rats than females. E2 treatment reduced FC-induced brain injury in both sexes. E2 significantly increased protein level and activity of Trx in the caudate nucleus of females but not males. Administration of female rats with 5,5-dithiobis-(2-nitrobenzoic acid) or Trx small interfering RNA to the caudate nucleus decreased the protective effect of E2 against FC-induced injury. The protein and mRNA levels of estrogen receptor β, but not estrogen receptor β, were more abundant in the caudate nucleus of female rats.

**Conclusions**—Increase of brain Trx activity might play an important role in the E2-mediated neuroprotective effect against FC-induced brain injury in female rats. Understanding of the sex differences in the Trx-mediated neuroprotective effect by E2 might help in improving treatment of brain dysfunction after hemorrhagic stroke and/or head trauma. (Stroke. 2010;41:160-165.)

**Key Words:** caudate nucleus ■ estrogen ■ ferrous citrate ■ hemorrhagic stroke ■ thioredoxin

Intracerebral hemorrhage (ICH) is caused by hypertension and related cerebral aneurysm and accounts for 10% to 30% of all stroke cases. ICH is associated with a high mortality rate, neurological deficits, and long-term disability. Reports indicate that females are less susceptible to stroke- and trauma-induced brain injury than age-matched males until reaching menopause. Animal studies of cerebral ischemia also indicate that female rats experience less neuronal damage than male rats, and estrogen treatment protects the brain from stroke in vivo. However, the underlying mechanisms remain to be elucidated. A recent in vitro study provided new evidence to support a possible role of thioredoxin (Trx) in mediating 17β-estradiol-mediated neuroprotection against brain injury.

Although the underlying mechanism of ICH-induced brain injury is not fully elucidated, one theory holds that the extensive lysis of extravascular red blood cells in the brain after ICH lead to overproduction of ferrous iron, which undergoes a redox cycle, including Haber-Weiss reaction and/or a Fenton reaction in the presence of oxygen, citric acid, and isocitric acids. Redox cycling of iron complexes (ie, bidentate and tridentate ferrous citrate and hemoglobin) causes persistent conversion of oxygen into reactive oxygen species that lead to axonal dystrophy and cell death. Because deferoxamine, an iron chelator, reduces acute ICH-induced brain edema and improves functional outcomes after bleeding, a detrimental role of iron in ICH has been indicated. Accordingly, we established the present rat model to mimic the ICH-induced iron accumulation and oxidative stress by intrastriatal infusion of the small molecular weight iron complexes, ferrous citrate (FC).

Cellular antioxidative activities include induction of anti-apoptotic or antioxidant molecules such as Trx and other survival proteins, which are crucial for reactive oxygen

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species scavenging and for preventing oxidative stress-induced apoptosis. Previous reports indicate that Trx induction mediates the preconditioning-induced neuroprotection and the β-estradiol 3-benzoate (E$_2$)-mediated neuroprotection against serum deprivation-induced cell death in vitro. Accordingly, we hypothesize that Trx might mediate the neuroprotection conferred by E$_2$ against iron-induced brain injury in vivo.

Materials and Methods

Animals
A total of 228 12-week-old male and female Sprague-Dawley rats (350 to 420 g; LASC; Charles River Technology, Taipei, Taiwan) were used. All procedures were approved by the Kaohsiung Medical University Committee for the Use of Experimental Animals.

Castration was performed under anesthesia with pentobarbital (35 mg/kg, intraperitoneally). A silastic tube (2-mm inner diameter, 30 mm in length; Shin-Etsu Polymer Co, Ltd) containing 1 mmol/L E$_2$ (Sigma) was implanted subcutaneously at 24 hours before infusion of FC. A microinfusion pump (CMA Microdialysis) was used to infuse 10 μL FC (2.55 μmol/L; Sigma) into the right caudate nucleus (CN; coordinates: 0.2 mm anterior, 3.5 mm lateral, and 5.5 mm ventral to bregma) at a rate of 10 μL/min. Sham-operated and saline-injected rats were used as normal and vehicle control groups, respectively.

Forelimb Use Asymmetry Test

Individual rats were placed in a transparent cylinder (25 cm in diameter and 30 cm in height) in the dark, and the use of ipsilateral limbs (I), contralateral limbs (C), or simultaneous use of both forelimbs (B) was observed for a 5-minute period. The test was randomized, blind, and repeated twice in each rat. The forelimb use asymmetry ratio was calculated using the following equation: $\frac{[I+(I+C+B)]-[C+(I+C+B)]}{[I+(I+C+B)]+}[C+(I+C+B)]$.

Histological Examination

The paraffin-embedded tissue was serially sectioned into 10 μm thickness. After hematoxylin and eosin staining, the severity of brain lesions caused by the CN injury showed that the ratio of lesions caused by the CN injury in intact male rats (37.83%) was significantly higher than that in female rats (11.54%). Castration increased the FC-induced lesion ratio in female specimens, but there was no significant difference between castrated and intact male rats. To confirm the success of castration and intact male rats, the serum estradiol levels in female specimens using 2-way analysis of variance followed by Scheffe post hoc test using SPSS software (Statistical Package for the Social Sciences). The data for the effects of E$_2$, DTNB, or Trx on iron-induced brain injury and the Trx protein level and activity were analyzed by multiway analysis of variance using JMP Version 5.1.2 software (a business unit of SAS) to determine the effect of each factor and the interaction between 2 factors. Significance was accepted at $P<0.05$.

Results

Protective Effect of E$_2$ on FC-Induced Neurological Deficit and Brain Lesion

Neurological deficit was reflected by the higher forelimb use asymmetry ratio. Infusion of FC significantly increased the forelimb use asymmetry ratio in male and female rats (64%±11.47% and 34%±6.76%, respectively) and increased more in the ovariecctomized rats as compared with intact females, but there was no significant difference between castrated and intact male rats. To confirm the success of ovariecctomy or E$_2$ implantation, the serum estradiol levels were measured by using an enzyme immunoassay kit. E$_2$ implantation significantly reduced the forelimb use asymmetry ratio in male and female rats by 23%±11.7% and 26%±12.9%, respectively (Figure 1A).

Quantitative Real-Time Polymerase Chain Reaction

Total RNA (5 μg) was transcribed to cDNA using random primers (Promega Corporation). The sequences of primers for ER-α, ER-β, and glyceraldehyde-3-phosphate dehydrogenase were: ERα: 5’-GCAGCGAAGGGGAACATC-3’ (forward) and 5’-GCATAGTCGTACAACAGACAG-3’ (reverse); ERβ: 5’-TTCTGGGCACCTGTC-TCCITTTA-3’ (forward) and 5’-GGACTCTITTGAAGGTTGCA-TAGA-3’ (reverse); and glyceraldehyde-3-phosphate dehydrogenase: 5’-CTCTACCCACGGCAAGTGC-3’ (forward) and 5’-GGAAAGATGGTGATGGGTTC-3’ (reverse). The cDNA amplifications were carried out in a 25μL mixture containing 12.5 μL of 2X SYBR Green PCR Master Mix (Applied Biosystems), 200 nmol/L primers, and 20 ng cDNA. Thermal cycling was performed on the Applied Biosystems 7900HT real-time polymerase chain reaction system. Data were analyzed using SDS Version 2.1 software (Applied Biosystems).

Trx Small Interfering RNA

The Trx small interfering (si)RNA was synthesized by Ambion (Applied Biosystems Business). Two Trx siRNA sequences were used (Duplex 1 [5’→3’]: sense GCUUGAAGGCCACAUAAUCGt, antisense CGUAAUAGGGCUUCCAGCt; Duplex 2: sense GGUUAACCCUGU ACCUUUt, antisense AAAGGGUCACGUUUAACGgt). A 5μL (80 nmol/L) siRNA was mixed with 5 μL transfection reagent (Dharmacon), incubated for 20 minutes, and then infused into the right CN at 6 hours before infusion of FC. Dharmacon siCONTROL nontargeting siRNA (catalog number D-001206-13) was used as a negative control.

Statistical Analysis

FC-induced injury was compared between brains from male and female specimens using 2-way analysis of variance followed by Scheffé post hoc test using SPSS software (Statistical Package for the Social Sciences). The data for the effects of E$_2$, DTNB, or Trx siRNA on iron-induced brain injury and the Trx protein level and activity were analyzed by multiway analysis of variance using JMP Version 5.1.2 software (a business unit of SAS) to determine the effect of each factor and the interaction between 2 factors. Significance was accepted at $P<0.05$.

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Sex Difference in Trx Protein Levels and Activities in the CN After Infusion of FC and E2 Implantation

The basal level of Trx protein in the CN was significantly higher in female rats than in males. Saline infusion did not change significantly the Trx protein level in the CN of either male or female rats, whereas infusion of FC significantly increased the protein level of Trx in male rats but not females. The Trx protein level in the CN did not differ between intact and castrated male rats but was significantly decreased in ovariectomized rats as compared with intact female rats. E2 implantation significantly increased the Trx level in FC-infused female but not male rats (Figure 2A). To evaluate the involvement of Trx in the neuroprotective effect of E2 on FC-induced brain injury, the reductive activity of Trx in the CN was examined. As shown in Figure 2B, infusion of FC significantly increased the reductive activity in the CN in male but not female rats. Castration did not change the reductive activity in the CN in male and female rats. Implantation of an E2 capsule significantly increased the reductive activity in the CN of FC-infused female rats but not males.

Involvement of Trx in the E2-Mediated Neuroprotection Against FC-Induced Brain Injury

Because E2-induced increase of Trx was observed only in FC-infused female rats, we further examined the role of Trx in E2-induced neuroprotection in female rats by intrastriatal administration of a Trx reductase inhibitor, DTNB. The DTNB per se did not significantly change the Trx protein level (data not shown). However, DTNB significantly de-

Figure 1. Sex difference in FC-induced brain injury and behavior deficit. FC induced more severe behavior deficit (A) and brain injury (B) in male rats. Ovariectomy increased the FC-induced behavior deficit and brain injury. E2 capsule implantation reduced the FC-induced behavior deficit and brain injury in both ovariectomized female and castrated male rats. The hemispheric area of the CN was quantified according to the intensity of the hematoxylin and eosin-stained tissue section by Image-pro plus software. The lesion ratio was estimated by dividing the hemispheric volume of the CN on the ipsilateral side by that on the contralateral side. Data are expressed as means±SE (n=6). *P<0.05.

Figure 2. Effect of E2 on the protein level and activity of Trx in the CN. FC treatment induced an increase of Trx protein level (A) and Trx activity (B) in the CN in male rats, but not females. Pretreatment with E2 increased the protein levels of Trx in the CN in FC-infused ovariectomized female rats, but not castrated male rats. Protein level of Trx was quantified by Western blot analysis. Beta-actin was used as a control for equal protein loading. The reductive activity of protein sampled from the CN was estimated by Ellman test. Five minutes after adding Ellman reagent, the absorbance was examined at a wavelength of 412 nm. Data are expressed as means±SE (n=6). *P<0.05.
creased the effects of E2 on Trx activity (Figure 3A), forelimb use asymmetry ratio (Figure 3B), and lesion ratio (Figure 3C). Moreover, Trx siRNA decreased the Trx protein level in the CN (Figure 3D) and significantly decreased the protective effect of E2 on FC-induced behavioral deficit (Figure 3E) and brain lesion (Figure 3F).

Expression of ERα and ERβ in the CN of Female and Male Rats
To test whether ER is involved in the sex dimorphism of Trx induction caused by E2, we examined the levels of mRNA and protein of both ERα and ERβ in the CN. As shown in Figure 4, the expression levels of ERα mRNA (Figure 4A) and protein (Figure 4B) and the number of ERα-immunoreactive cells (Figure 4C) in the CN were higher in female than male brains. However, the levels of brain ERβ mRNA and protein in the CN of female rats were similar to those in male rats.

Discussion
It has been suggested that Trx plays a critical role in E2-mediated neuroprotection against oxidative neuronal injury in vitro.17 This in vivo study demonstrated that Trx induction may participate in E2-mediated neuroprotection against iron-induced brain injury in female, but not male, rats. The present study demonstrated that the E2-mediated protection against the FC-induced brain injury in female rats was reduced by intrastriatal pretreatment with siRNA against Trx, which reduced the Trx protein levels (Figure 3D–F). Although the Trx reductive activity in the rats treated with siRNA Trx was not measured, the role of endogenous Trx in the brain was confirmed by using a Trx reductase inhibitor DTNB, which blocked the redox cycling of endogenous Trx and prevented the FC-induced brain injury in female rats (Figure 3A–C). These in vivo findings are consistent with the early in vitro findings using antisense oligonucleotide to decrease the Trx level and thus the Trx activity.17

Numerous clinical studies indicate that premenopausal women have lower risk in stroke than age-matched men, and estrogen improves stroke outcome after vascular occlusion in animal4 and human18 studies. However, the precise mechanisms underlying sex differences in functional outcomes after stroke are still unclear. A previous report showed that male rats with cortex lesions exhibited persistent water maze
deficit throughout 10 days of test, whereas brain-damaged females did not, suggesting that females are less susceptible to injury after brain lesion than males. During the past decade, knowledge of the mechanisms underlying brain injury induced by ICH has rapidly accumulated. The coagulation cascade, inflammation, and breakdown of hemoglobin products (iron in particular) all contribute to ICH-induced injury. Because deferoxamine, an iron chelator, improved the functional outcome by reducing ICH-induced brain edema and atrophy, we applied the FC-infused rat model to examine the neuroprotective mechanism of E2. After the intrastriatal infusion of FC, both the forelimb use asymmetry ratio and lesion ratio showed significant differences between male and female rats (Figure 1). The FC-induced increases in forelimb use asymmetry ratio and lesion ratio showed significant differences between male and female rats (Figure 1). The FC-induced increases in forelimb use asymmetry ratio and lesion ratio in male rats (64.86% and 35.32%, respectively) were significantly higher than those observed in females (30.96% and 8.9%, respectively). Additionally, the ratios of forelimb use asymmetry ratio and lesion caused by intrastriatal infusion of FC were decreased in castrated male rats but were increased in ovariectomized rats. These results imply that the endogenous sex hormonal milieu might affect the severity of brain injury. The E2 implantation significantly protected the CN of both male and female rats against the FC-induced behavioral deficit and brain lesion. Accordingly, the rat model of brain injury induced by intrastriatal infusion of FC in this study provides a good model for studying sex-specific brain injury caused by iron overload and the protective mechanism conferred by E2.

Excessive ferrous iron enters a redox cycle and causes persistent reactive oxygen species production as well as oxidative stress, which induce survival proteins such as Trx to quench the stress. Many in vitro and in vivo models have confirmed the antioxidant effects of estrogen. Physiological concentrations of E2 (<10 nM) have been reported to upregulate Trx expression in vitro. This estrogen-mediated induction of Trx plays a pivotal role in the ER-mediated neuroprotection in human neuroblastoma cells. Estrogen can also provide neuroprotection through an ER-independent pathway. However, the exact neuroprotective mechanism of E2 under neuronal iron overload is still unclear. Surprisingly, the results from our present study showed that Trx induction by FC in the CN was significantly greater in male than female rats. A possible explanation is that a higher concentration of endogenous E2 in female rats can reduce the FC-induced increase of oxidant stress, which is a stimulant for Trx expression, through an ER-independent mechanism. Notably, the FC-induced increase of Trx was not significantly changed in E2-implanted castrated males but significantly increased in E2-implanted ovariectomized female rats. Because activation of ER can increase the Trx expression and more abundance of ERα was observed in the CN of female rats (Figure 4), a more prominent ER-dependent protective effect of estradiol may exist in female brains and sex differences in Trx induction in response to E2 implantation might be explained by the sex difference in the levels of ER in the CN. The involvement of ER isoforms in the estrogen-mediated Trx induction during basal physiological condition or under iron-induced stress has to be confirmed by further studies. Antagonist or siRNA of either ERα or ERβ may be applied to address this issue.

Figure 4. Sexual dimorphism of ERα expression in the CN. Female rats expressed more ERα, but not ERβ, mRNA (A) and protein (B) in the CN. A 3-mm-thick sample was taken from the CN. The levels of mRNA expression were quantified by quantitative real-time polymerase chain reaction. Protein levels of both ERα and ERβ were quantified by Western blot analysis. Glyceraldehyde-3-phosphate dehydrogenase mRNA and β-actin protein were used as a control for equal mRNA and protein loading, respectively. (C) Immunocytochemical staining showed more ERα-positive cells (white arrow) in the CN of female rats than males. Data are expressed as means ± SE (n = 6). *p < 0.05 different from male. Po, positive control.
Stroke is recognized as a sexually dimorphic disease. Reports indicate that females have better free radical homeostasis and stronger defense capacity against oxidative brain damage as compared with males. Moreover, stress-induced HSP72 expression in the brain also exhibits sexual dimorphism. The present study revealed that the protein levels of Trx in the CN of female brains were greater than in males. Furthermore, Trx was found to be involved in the neuroprotective effect of E2 in a sex-dependent manner, which implies a sexual dimorphism in the molecular pathogenesis of iron-induced brain injury. The underlying mechanism of sex-specific neuroprotection requires further investigation to develop sex-specific therapeutic strategies for preventing brain dysfunction after hemorrhage.

Conclusions
E2 increased the Trx expression, which in turn protected the CN against FC-induced injury in female but not male rats. This evidence of sexual dimorphism in the Trx-mediated neuroprotective effect of E2 may be useful for developing sex-specific therapeutic strategies for preventing brain dysfunction caused by hemorrhage and/or by neurodegenerative disease associated with iron overload.

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Disclosures
None.

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