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ESTROGEN-MEDIATED NEUROPROTECTION IN THE CORTEX MAY REQUIRE NMDA RECEPTOR ACTIVATION

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Abstract—Several studies have suggested that a potential mechanism for estrogen-mediated neuroprotection following experimental stroke is a result of modulating glutamate-mediated excitotoxicity. Our laboratory has shown that in male rats, estrogen injection (systemic or direct intracortical injection) resulted in an immediate depolarization of cortical neurons. Therefore, the present study was designed to investigate whether the estrogen-induced depolarization of cortical neurons was required in mediating the early events associated with this neuroprotection. We tested this hypothesis by co-injecting selective antagonists of the NMDA (MK-801) or AMPA (DNQX) glutamatergic receptors with estrogen. Systemic injection of estrogen significantly attenuated the MK-801-induced decrease in infarct volume following middle cerebral artery occlusion (MCAO). Similarly, when estrogen and MK-801 were co-injected directly into the cortex, no neuroprotection was observed. However, when estrogen or MK-801 was injected centrally 10 min prior to the injection of the other drug, significant neuroprotection was observed. This led us to hypothesize that estrogen-mediated neuroprotection required an initial activation of NMDA receptors. Furthermore, our results suggest that this estrogen-mediated neuroprotection was also associated with a significant increase in m-calpain and activation of an endoplasmic reticulum (ER) specific caspase-12. Finally, the results of current clamp experiments showed that estrogen significantly depolarized cortical neurons as well as enhanced NMDA-induced depolarization. Taken together, these results suggest that estrogen pretreatment may activate NMDA receptors resulting in modification of ER-associated molecular mechanisms involved in neuroprotection following MCAO. © 2007 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: caspase-12, endoplasmic reticulum stress, middle cerebral artery occlusion, m-calpain.

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Abbreviations: aCSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; DNQX, 6,7-dinitroquinoxaline-2,3-dione; ER, endoplasmic reticulum; MAP, mean arterial pressure; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; MK-801, 5S,10R-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; NMDA, N-methyl-D-aspartate; PBS, phosphate-buffered saline; RMP, resting membrane potential; TTC, 2,3,5-triphenol tetrazolium chloride.

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Stroke is a significant cause of morbidity and mortality in North America. Several researchers have consistently provided evidence to suggest that estrogen offers significant neuroprotection in both male and female animal models of cerebral ischemia (for review see [Hurn and MacRae, 2000](#); [Gibson et al., 2006](#)). In particular, studies have reported that female animals experience substantial protection from stroke compared with their male counterparts ([Gibson et al., 2006](#)) and this benefit disappears upon removal of plasma estrogen following ovariectomy ([Hurn and MacRae, 2000](#)). Our laboratory has previously shown that estrogen administered systemically or directly into the prefrontal cortex prior to middle cerebral artery occlusion (MCAO) significantly decreased the infarct size ([Saleh et al., 2001a,b, 2004](#)). This estrogen-mediated neuroprotection was observed as early as 2 h post-MCAO ([Saleh et al., 2004](#)). The exact mechanism(s) responsible by which estrogen acts within the ischemic cortex to limit the progression of cell death is not fully understood.

Evidence is available to suggest that estrogen-mediated neuroprotection involves a reduction of ischemia-induced excitotoxicity primarily through an interaction with the N-methyl-D-aspartate (NMDA) receptor ([Wong and Moss, 1992](#); [Weaver et al., 1997](#)). Within minutes of an ischemic insult, the anoxic depolarization leads to excitotoxic levels of glutamate in the extracellular space ([Storm-Mathisen et al., 1992](#)). This can lead to the initiation of several neurotoxic events including the over stimulation of both ionotropic and metabotropic glutamate receptors ([Szatkowski and Attwell, 1994](#)) and the subsequent influx of several ions, including calcium ([Lipton, 1999](#)). Several laboratories have demonstrated that large increases in neuronal intracellular calcium concentrations following ischemia contribute to both acute and delayed neuronal death ([Choi, 1995](#); [Lipton, 1999](#)). In support of the major role that NMDA receptors play in this excitotoxic cell death and continuing growth in infarct volume, studies have shown that administration of a highly potent and selective non-competitive NMDA receptor antagonist, MK-801, can provide significant neuroprotection against focal cerebral ischemia ([Buchan et al., 1992](#)). Estrogen has also been shown to interact with, and inhibit the activation of NMDA receptors to attenuate glutamate-mediated excitotoxicity using cultured cortical or hippocampal neurons *in vitro* ([Singer et al., 1996](#); [Weaver et al., 1997](#)). In contrast to evidence of estrogen-induced inhibition of NMDA activation, other electrophysiological studies have shown that, in general, estrogen increased the excitability of neurons within the CNS including those involved in cardiovascular regulation ([Woolley, 1999](#)). In our laboratory using male

rats, we have demonstrated that an i.v. injection of estrogen produced an increase in the spontaneous activity of neurons located within the insular cortex in conjunction with a decrease in endogenous GABA levels *in vivo* (Saleh and Connell, 2003; Saleh et al., 2004, 2005). In addition, in hippocampal slices estrogen has been shown to increase excitatory glutamatergic receptor currents mediated by NMDA receptors (Foy et al., 1999) and potentiate kainate-induced currents (Gu and Moss, 1998).

Glutamate receptor activation would lead to an increase in intracellular calcium. The endoplasmic reticulum (ER) has been linked to specific protective pathways following alterations in intracellular calcium levels such as those observed in ischemic animal models. Specifically, the activation of the ER resident caspase-12 has been observed following MCAO (Nakagawa et al., 2000). It is therefore possible that the neuroprotective effects of estrogen could be mediated via an interaction with glutamatergic receptors leading to a moderate influx of calcium and thus the activation of ER-specific, calcium-regulated protective pathways.

In order to reconcile these contradictory effects of estrogen action, it is hypothesized that estrogen may cause an immediate (within 30 min), mild increase in intracellular calcium levels, which in turn, triggers neuroprotective mechanisms to an ischemic stress (Bickler and Fahlman, 2004; Raval et al., 2006a,b). Then, as the infarct volume progresses, estrogen may cause inhibition of NMDA receptors either directly or via second messenger pathways. In this way, either inhibition of or mild activation of NMDA receptors could lead to neuroprotection. Thus, the main objective of this study was to determine if estrogen reduced infarct volume following MCAO via an early, initial activation of glutamatergic (NMDA or AMPA) receptors in the cortex of male rats. We then sought to determine if activation of NMDA receptors leads to activation of ER stress pathways by measuring changes in the levels of activated caspase-12. This would provide evidence of a potential neuroprotective mechanism for estrogen involving NMDA receptor activation and modulation of ER-specific neuroprotective pathways.

EXPERIMENTAL PROCEDURES

All experiments were carried out in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the University of Prince Edward Island Animal Care Committee (protocol # 04-032). All efforts were made to minimize the number of animals used and their suffering.

General surgical procedures

All experiments were conducted on male Sprague–Dawley rats (Charles Rivers; Montreal, PQ, Canada). The *in vivo* experiments were performed on 47 rats (250–300 g) and the *in vitro* electrophysiology experiments were conducted on 28 slices obtained from 22 rats (75–100 g). For all animals, food and tap water were available *ad libitum*. Rats were anesthetized with sodium thiobarbital (Inactin; RBI, Natick, MA, USA; 100 mg/kg; i.p.). Animals instrumented to record blood pressure and heart rate (HR) had a polyethylene catheter inserted into the right femoral artery (PE-50; Clay Adams, Parsippany,

NJ, USA), and rats receiving i.v. administration of drugs had a polyethylene catheter (PE-10) inserted into the right femoral vein. Arterial blood pressure was measured with a pressure transducer (Gould P23 ID; Cleveland, OH, USA) connected to a Gould Pressure Processor. HR was determined from the pulse pressure using a Gould ECG/Biotach tachograph. Blood pressure and HR data were displayed and quantified using the PolyView Pro/32 data acquisition and analysis system (Grass; Warwick, RI, USA). All animals had an endotracheal tube inserted within the trachea and body temperature was monitored with a digital rectal thermometer and maintained via a feedback warm water heating blanket at 37 ± 1 °C.

MCAOs

Animals receiving MCAO were placed in a David Kopf stereotaxic frame (Tujunga, CA, USA) and the right middle cerebral artery (MCA) was approached through a rostro-caudal incision of the skin and frontalis muscle at the approximate level of bregma. The frontalis and temporalis muscles were then reflected anteriorly and posteriorly to expose the squamosal bone to the point where the zygoma and the squamosal bone fuse. A hole was made in the rostro-dorsal part of the squamosal bone using a small handheld drill and a portion of the squamosal bone was then removed to expose the MCA. The bent tip of a 25-gauge hypodermic needle was used to cut and retract the dura mater above the MCA. The MCA was permanently occluded using three point bipolar electrical coagulation (Cameron-Miller; Chicago, IL, USA). The first occlusion was made just dorsal to the rhinal fissure, the second just ventral to the bifurcation of the MCA to the frontal and parietal cortices, and the third was made just proximal to the bifurcation of the MCA along the parietal cortex. This three-point occlusion protocol has been shown to result in very reproducible lesion volumes (Saleh et al., 2001a,b).

Effect of systemic drug administration on infarct volume, BP and HR

To examine the interaction between estrogen and the NMDA receptor following MCAO, *in vivo* experiments were conducted to measure infarct volumes following systemic pretreatment with estrogen (17 β -estradiol 3-sulfate; Sigma Aldrich, St. Louis; MO, USA) in combination with MK-801 (Tocris, Bristol, UK). Two groups of rats ($n=4$ /group) were administered MK-801 (0.5 mg/kg; 0.25 ml; i.p.) and one group ($n=4$) was administered physiological saline (0.09%; 0.25 ml; i.p.) 30 min prior to anesthesia (75 min prior to MCAO). The two groups administered MK-801 then received an i.v. injection of either estrogen (1×10^{-2} mg/kg; 0.2 ml; $n=4$) or physiological saline (0.9%; 0.2 ml; $n=4$) 45 min following the systemic administration of MK-801 (30 min prior to MCAO). The group of rats receiving the initial systemic administration of physiological saline was again administered an i.v. injection of physiological saline (0.9%; 0.2 ml; $n=4$) 30 min prior to MCAO. Two hours following MCAO, the animals were transcardially perfused with phosphate buffered saline (PBS; 0.1 M; 200 ml), the brains were removed and the area which included the insular cortex was sliced into 1 mm coronal sections using a rat brain matrix (Harvard Apparatus; Holliston, MA, USA). Sections were incubated in a 2% solution of 2,3,5-triphenol tetrazolium chloride (TTC; Sigma-Aldrich) for 3–4 min. Infarct volume at the level of the joining of the anterior commissure (Bregma 0 to –0.3 mm; Paxinos and Watson, 1986) was determined as previously described (Saleh et al., 2001a,b). Briefly, digital photographs of the appropriate section for each rat were taken to quantify infarct area using a computer-assisted imaging system (Scion Corporation; Frederick, MD, USA). Regions of infarct were outlined and the infarct volume was calculated as the percentage of the total ipsilateral hemisphere volume (to correct for MCAO-induced cerebral edema).

Effect of central drug injections on infarct volume, BP and HR

To determine if estrogen receptor activation or ionotropic-glutamate receptor blockade in the cortex altered infarct volume or cardiovascular parameters, BP and HR were measured before and after estrogen, MK-801 or DNQX (6,7-dinitroquinoxaline-2,3-dione; Tocris; a competitive non-NMDA (kainate and quisqualate) receptor antagonist) was centrally injected. Holes were drilled through the right temporal bone to permit the stereotaxic insertion of a 30-gauge stainless steel 1 μ l Hamilton micro-syringe (Hamilton Co., Reno, NV, USA). Injection coordinates were obtained from a stereotaxic atlas of the rat brain (Paxinos and Watson, 1986). A unilateral injection of estrogen (0.5 μ M in 200 nl; $n=4$), MK-801 (0.025 μ g/ μ l in 200 nl; $n=5$), DNQX (100 μ M in 200 nl; $n=4$) or physiological saline (0.9%; 200 nl; $n=4$) was made into the right cortex 30 min prior to MCAO. For all rats, BP and HR were measured immediately prior to each central drug administration, immediately prior to MCAO, and then at 5, 10, 15, 20, 30, 60, 90 and 120 min following MCAO.

To determine if there was an interaction between estrogen receptor activation and ionotropic-glutamate receptor blockade, an injection of a mixture of either estrogen and MK-801 (0.5 μ M and 0.025 μ g/ μ l in 200 nl respectively; $n=4$) or estrogen and DNQX (0.5 μ M and 100 μ M in 200 nl respectively; $n=4$) was made into the right cortex 30 min prior to MCAO. To determine if the estrogen-mediated neuroprotection was dependent upon activation of the NMDA receptor, MK-801 was injected into the right cortex either 10 min prior to ($n=4$; 200 nl), or 10 min following ($n=4$; 200 nl) the injection of estrogen into the right cortex. The first injection was made 30 min prior to MCAO and the second injection 20 min prior to MCAO. For all rats, BP and HR were measured immediately prior to each central drug administration and immediately prior to MCAO, and then at 5, 10, 15, 20, 30, 60, 90 and 120 min following MCAO. In all groups, infarct volumes were determined 120 min following MCAO at the level of the joining of the anterior commissure as described above.

Tissue harvesting for Western blot analysis

The following experiments were carried out to determine if micro-injection of estrogen, alone or in combination with the prior systemic administration of MK-801, alters an ER-stress pathway following MCAO. Two groups of rats ($n=6$ /group) were administered MK-801 (0.5 mg/kg; 0.25 ml; i.p.) or physiological saline (0.25 ml; i.p.) 30 min prior to anesthesia. Three rats in each of these groups received an i.v. injection of estrogen (1×10^{-2} mg/kg; 0.2 ml) and the remaining three rats in each group received an i.v. injection of physiological saline (0.9%; 0.2 ml) 45 min following the i.p. administration of MK-801 or saline (30 min prior to MCAO). These four groups of rats were not instrumented to record BP or HR. Two hours following MCAO the experiment was terminated. Animals were transcardially perfused with PBS (0.1 M; 200 ml) and the brains removed. Ipsilateral and contralateral cortices were dissected using biopsy needles with an internal diameter of 4 mm to collect tissue from the region directly affected by the stroke (infarct core). The corresponding tissue regions on the contralateral side were also collected to be used as internal controls. The tissue samples were added to cryovials containing 300 μ l of PBS (0.1 M) and 3 μ l protease inhibitor (Sigma-Aldrich), flash frozen in liquid nitrogen and stored at -80°C until used. The samples were then thawed on ice, homogenized (2×10 s) and centrifuged at $9000 \times g$ for 10 min. The resulting supernatant was collected and protein concentrations were determined using a protein assay (Bio-Rad Laboratories, Mississauga, ON, Canada).

Western blot analysis of caspase-12 and m-calpain levels

Aliquots of each sample (50 μ g of protein) were added to 4 $\times$ Laemmli buffer and PBS (0.1 M). The mixture was boiled for 3 min and loaded onto a 15% polyacrylamide gel. Following electrophoresis, the separated proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories) using a wet transfer method (Bio-Rad Laboratories). The membranes were blocked for 1 h in 5% non-fat milk, and incubated overnight at room temperature with a 1:500 dilution of rat monoclonal anti-caspase-12 antibody (Sigma-Aldrich) or a 1:1000 dilution of anti-m-calpain antibody (Triple Point Biologics; Forest Grove, OR, USA). After washing with PBS (0.1 M), the membranes were incubated with a 1:10,000 dilution of horseradish peroxidase-conjugated anti-rat IgG (Sigma-Aldrich) for caspase-12 or 1:10,000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (Sigma-Aldrich) for m-calpain. The membranes were again washed, treated with ECL Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ, USA), and immediately exposed on an image station (Kodak Image Station 440CF; New Haven, CT, USA). To ensure correct protein loading, membranes were also immunostained with a 1:2000 dilution of mouse monoclonal anti- β -actin (Sigma-Aldrich) followed by 1:10,000 dilution of horseradish peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich). The protein band densities were normalized to the density of the β -actin band from the same sample.

Slice preparation for *in vitro* experiments

To further investigate whether estrogen modulates glutamatergic receptor activation, *in vitro* electrophysiological recordings were obtained from the cells within the cortex of freshly prepared brain slices. Methods for preparing the brain slices were similar to those previously published (Fatehi et al., 2006a,b). Briefly, male Sprague-Dawley rats (75–100 g; Charles River) were anesthetized with isoflurane vapor (Isoflo; Abbott Laboratories, Saint-Laurent, PQ) and decapitated. Brains were removed and immersed in ice-cold ($2-3^\circ\text{C}$) artificial cerebrospinal fluid (aCSF) of the following composition (in mM): 145 NaCl, 2.5 KCl, 10 D-glucose, 26 NaHCO₃, 1.2 NaH₂PO₄, 1.3 MgCl₂, 2.5 CaCl₂ (pH 7.4, osmolarity of 295–305 mOsmol/L, continuously bubbled with 95% O₂–5% CO₂). The brain was then mounted in a vibratome (Model 1000 plus, Ted Pella Inc., Redding, CA, USA) and cut coronally into 350 μ m thick slices while submerged in ice-cold aCSF. Prior to the initiation of recording, slices containing the cortex were incubated for 30 min in warmed aCSF ($30^\circ\text{C} \pm 1^\circ\text{C}$), then for 1 h in aCSF at room temperature. For experimentation, slices were transferred to an experimental chamber on an upright microscope and superfused at 3 ml/min with aCSF ($30^\circ\text{C} \pm 1^\circ\text{C}$). Stock solutions of drugs were made in Milli-Q water then aliquots of these stock solutions were dissolved in aCSF to the final drug concentration. All drugs were applied to the slices by bath perfusion. Evidence provided from a dose-response relationship conducted in our laboratory in a previous study (Fatehi et al., 2006b) showed that a dose of estrogen of 50 μ M via bath application was required to significantly alter excitatory neurotransmission *in vitro*.

In vitro electrophysiological recordings

"Blind" whole-cell patch-clamp recordings (Blanton et al., 1989) were performed on healthy neurons from the cortex using a MultiClamp 700B amplifier with CV-7B head stage (Axon Instruments, Union City, CA, USA). Patch pipettes (KG-33; Garner Glass Co., Claremont, CA, USA) were pulled with a Flaming-Brown micropipette puller (Model P-87; Sutter Instruments Co., Novato, CA, USA) and fire polished to between 5 and 8 M Ω resistance. Patch pipettes contained the following solution (in mM): 130 K-gluconate, 6 NaCl, 10 Hepes, 2.5 Na-ATP, 0.1 Na-

GTP; pH 7.3 with KOH, osmolality of 285–295 mOsmol/L). Before breaking into the cell with suction fast electrode capacitance was compensated. Access resistance routinely ranged between 15 and 35 M Ω . Electrophysiological criteria for accepting cells were based on their excitability, resting membrane potential (RMP) more negative than -55 mV, and the access resistance from the beginning to the end of the experiment did not change by more than 15%. Data acquisition and analysis were performed using Clampex and Clampfit 9.2 software, respectively (Axon Instruments). All data were acquired at a sampling rate of 10 kHz.

Data analysis

All data are presented as mean $\pm$ standard error of the mean (S.E.M). Changes from baseline in mean arterial blood pressure, HR and sympathetic nerve activities were analyzed with one-way analysis of variance (ANOVA) for repeated measures, and when necessary, followed by a Student-Newman-Keuls post hoc analysis. Differences between groups at identical time points were determined using unpaired Student's *t*-tests with a Bonferroni correction for multiple comparisons. For the analysis of the Western blots, an unpaired *t*-test was used to determine if significant differences existed between treatment groups. A paired *t*-test was used to determine difference between ipsilateral (infarct side) and contralateral protein levels. In the *in vitro* electrophysiological experiments, changes in RMP from baseline to peak response were measured. In most cases, cells were first treated with drug, and following washout, treated with drug plus estrogen. Due to the long-lasting (genomic) effects, estrogen was only applied once per slice. Therefore, only one cell per slice was recorded from and hence sample size (*n*) represents the number of slices used. For these experiments, differences between treatment groups were determined using paired Student's *t*-test. For comparison of treatment groups from different slices, means were analyzed by one-way ANOVA followed by a Dunnett multiple comparison test. In all cases, differences were considered significant if $P \leq 0.05$.

RESULTS

For all animals, rectal temperature was maintained throughout the experiment at 36.0 ± 1.0 °C. Prior to drug injection into the cortex, mean arterial pressure (MAP) and HR were 98 ± 12 mm Hg and 307 ± 23 beats/min, respectively ($n=33$). The values for MAP and HR were not significantly altered throughout the 2 h of the experiment when MCAO was combined with the injection of any individual drug, when combined with two separate drug injections, or when combined with any drug mixture into the cortex ($P > 0.05$ at all time points). Also, baseline (pre-MCAO) cardiovascular parameters in animals pretreated with MK801 (i.p.) were not significantly different from those pretreated with saline ($P > 0.05$).

Effect of systemic administration of MK-801 on infarct volume

The group of rats receiving both a systemic and an i.v. injection of physiological saline had an infarct volume of $30.6 \pm 7\%$ of the right hemisphere 2 h following MCAO (Fig. 1A and B). When MK-801 was injected i.v. 75 min prior to MCAO followed 45 min later by i.v. saline, the infarct area was significantly smaller compared with the saline/saline control group ($12 \pm 2\%$; $P < 0.05$; Fig. 1A and B). However, when MK-801 was followed 45 min later by i.v. injection of estrogen, there was no significant difference in infarct vol-

ume compared with the saline/saline control group 2 h post-MCAO ($20.0 \pm 5\%$; $P > 0.05$; Fig. 1A and B).

Effect of MCAO and central drug treatments on infarct volume

Two hours following the injection of physiological saline directly into the cortex, infarct volume was measured to be $34 \pm 4\%$ of the right hemisphere volume (Fig. 2). Consistent with previous results from our laboratory (Saleh et al., 2001b), estrogen injected into the cortex 30 min prior to MCAO resulted in a significant reduction in infarct volume by approximately 55% compared with saline-injected rats to $15 \pm 3\%$ of the right hemisphere ($P < 0.05$; Fig. 2). Similarly, injection of MK-801 into the right cortex 30 min prior to MCAO significantly decreased infarct volume by approximately 60% compared with saline-injected rats to $13 \pm 6\%$ ($P < 0.05$; Fig. 2). However, when estrogen was combined with MK-801 and co-injected into the right cortex 30 min prior to MCAO, the infarct volume was not significantly different compared with saline ($25 \pm 8\%$ of right hemisphere; $P > 0.05$; Fig. 2). When there was a 10 min interval between the injection of MK-801 and estrogen, or, between estrogen and MK-801, the infarct volumes remained significantly decreased compared with saline-injected animals ($19 \pm 4\%$ and $13 \pm 5\%$ of right hemisphere respectively; $P < 0.05$; Fig. 2). Injection of DNQX into the right cortex 30 min prior to MCAO did not significantly decrease infarct volume compared with saline-injected animals ($27 \pm 6\%$ of right hemisphere; $P > 0.05$; Fig. 2). However, the infarct volume was significantly decreased compared with saline when estrogen was co-injected with DNQX into the right cortex 30 min prior to MCAO ($14 \pm 3\%$ of right hemisphere; $P < 0.05$; Fig. 2).

Effect of MCAO and drug treatments on caspase-12 and m-calpain levels in the cortex

To determine if the estrogen-induced neuroprotection was mediated through ER stress pathways, immunoblots for the expression of procaspase-12 (observed at a molecular weight of ~ 57 kDa), activated caspase-12 (molecular weight of ~ 30 kDa) and m-calpain (molecular weight of ~ 108 kDa) were measured 2 h following MCAO. Fig. 3A contains a representative immunoblot demonstrating the changes in the expression of each protein in the MCAO-induced infarct region at 2 h post MCAO.

The level of procaspase-12 in tissue from the infarct region was significantly decreased following estrogen treatment compared with similar tissue from rats administered saline while the levels of activated caspase-12 and m-calpain were significantly elevated compared with tissue from rats treated with saline ($P < 0.05$; Fig. 3B). MK-801 treatment alone did not alter any of the protein levels compared with saline treatment ($P > 0.05$; Fig. 3A and B). However, the prior systemic treatment with MK-801 did result in a significant alteration in the estrogen-induced changes in the levels of each of these proteins ($P > 0.05$; Fig. 3A and B).

Fig. 1. Effect of systemic drug injections on infarct volume. (A) Representative digital photomicrographs of TTC-stained coronal brain sections (at bregma -0.25 mm) illustrating the extent of the infarct 2 h post-MCAO and saline, MK-801+saline, or MK-801+estrogen pretreatment. Infarct size is outlined in hash marks on each photomicrograph. (B) Graphic representation of the change in infarct volume (measured as percentage (%) ipsilateral hemisphere) 2 h following MCAO with either saline ($n=4$), MK-801+saline ($n=4$), or MK-801+estrogen ($n=4$) pretreatment. Each data point represents the mean $\pm$ S.E.M. and (*) indicates significant difference ($P<0.05$) of the infarct volumes between each drug group and the saline group.

Effect of acute estrogen application on glutamatergic agonist-induced depolarization of cells in the cortex

In order to determine if an interaction exists between estrogen and glutamate receptors, *in vitro* electrophysiological experiments were performed in cortical cells from male rats. In these experiments, cells were recorded in current clamp mode and their RMP was monitored. The average RMP for all cells recorded was -66 ± 11 mV ($n=39$). Bath application (30 s) of $50 \mu\text{M}$ estrogen caused a significant increase (depolarization) in the RMP of cells (1.9 ± 0.3 mV; $n=5$; $P<0.05$) compared with control (aCSF; Fig. 4A and B). However, when the same amount of estrogen was co-applied in combination with $10 \mu\text{M}$ MK-801, the membrane depolarization was significantly less (0.8 ± 0.3 mV; $n=6$; $P<0.05$) than that caused by estrogen alone and was

not different from control ($P>0.05$). Co-application of $20 \mu\text{M}$ NMDA and $50 \mu\text{M}$ estrogen also caused a significantly greater depolarization (3.6 ± 0.9 mV) than that caused by application of $20 \mu\text{M}$ NMDA alone (1.5 ± 1.0 mV; $n=6$; $P<0.05$; Fig. 4C and D). Similarly, the depolarization caused by co-application of $2.5 \mu\text{M}$ AMPA and $50 \mu\text{M}$ estrogen was significantly greater (10.2 ± 2.8 mV) than that caused by application of $2.5 \mu\text{M}$ AMPA alone (6.9 ± 1.8 mV; $n=5$; $P<0.05$; Fig. 4E and F).

DISCUSSION

The results of these studies suggest a novel interaction between estrogen and the NMDA receptor following MCAO that may contribute to estrogen's neuroprotective properties in the cortex. Previous studies from several

Fig. 2. Effect of intra-cortical drug injections on infarct volume. Graphic representation of the change in infarct volume (measured as percentage (%) ipsilateral hemisphere) 2 h following MCAO with either saline ($n=4$), estrogen ($n=4$), MK-801 ($n=5$), co-injection of MK-801+estrogen ($n=4$), MK-801 injected 10 min prior to estrogen (MK-801 (10)+Estrogen; $n=4$), estrogen injected 10 min prior to MK-801 (Estrogen (10)+MK-801; $n=4$), DNQX ($n=4$), or co-injection of DNQX and estrogen ($n=4$). Each data point represents the mean $\pm$ S.E.M. and (*) indicates significant difference ($P<0.05$) of the infarct volumes between each drug group and the saline group.

laboratories including our own have demonstrated that estrogen mediated a powerful neuroprotection in male rats when administered prior to permanent MCAO (Hurn and MacRae, 2000; Saleh et al., 2001a,b; Gibson et al., 2006). Weaver and colleagues (1997) suggested that this estrogen-mediated neuroprotection may have been due to a direct inhibition of the NMDA receptor, similar to the neuroprotection afforded by NMDA receptor antagonists, such as MK-801 (Buchan et al., 1992). Our results demonstrated that i.v. estrogen treatment either prevented or decreased the potency of systemic MK-801-induced neuroprotection following MCAO indicating that a negative interaction occurred between estrogen and MK-801. To investigate this further, both estrogen and MK-801 were directly injected into the cortex prior to MCAO and once again, the neuroprotective effect of either drug was significantly attenuated following co-administration. This study suggests that an interaction between these two drugs was occurring within the brain and possibly directly at the level of the NMDA receptor. Results from our *in vitro* electrophysiological study demonstrated that estrogen depolarized cortical neurons and enhanced NMDA-mediated depolarization and this effect was significantly attenuated with MK-801. This supports our suggestion that estrogen may activate or enhance the activation of NMDA receptors in cortical neurons. However, we should mention that although significant changes were observed, based on the low sample size and the pharmacological dose of estrogen required to observe an effect, results from the *in vitro* study may not be correlated with those observed *in vivo*.

The form of the negative interaction observed between estrogen and MK-801 could be one where estrogen binds to a membrane bound estrogen receptor in close proximity to the NMDA receptor providing steric interference and inhibiting MK-801 from binding within the open NMDA receptor channel. Membrane bound estrogen receptors have been characterized electrophysiologically (Woolley, 1999), and the use of the membrane impermeable forms of estrogen (i.e. BSA-estradiol) has been shown to produce equivalent electrophysiological effects as unconjugated forms of estrogen (Fatehi et al., 2006b) indicating that estrogen acts on a receptor located on the cell surface. Alternatively, estrogen could bind directly to a binding site on the NMDA receptor whereby estrogen would compete or interfere with the MK-801 binding site on the NMDA receptor. It is also possible that the interaction between estrogen and MK-801 is chemical in nature where estrogen and MK-801 physically interact with each other within the pre-synaptic cleft as well. Although no data exist in the literature to support a negative interaction between estrogen and MK-801, similar estrogen-mediated negative interactions have been reported where estrogen replacement therapy in postmenopausal women has been shown to prevent the beneficial effects of caffeine in reducing the risk of developing Parkinson's disease (Ascherio et al., 2004). Further, Xu et al. (2006) demonstrated that estrogen treatment prevented the neuroprotective effects of caffeine in a mouse model of Parkinson's disease. Neither of these studies investigated or suggested the nature of the negative interaction.

Fig. 3. Effect of intra-cortical drug injections on ER stress proteins. (A) Representative immunoblots showing m-calpain (108 kDa), pro-caspase-12 (57 kDa), activated caspase-12 (30 kDa), and the corresponding β -actin levels (43 kDa) from the infarct region (ipsilateral) 2 h following MCAO in rats receiving co-injections of saline+saline (lane 1), saline+estrogen (lane 2), MK-801+saline (lane 3), or MK-801+estrogen (lane 4). (B) Graphical representation of the mean density of bands corresponding to procaspase-12, activated caspase-12, and m-calpain in rats co-injected with saline+saline, saline+estrogen, MK-801+saline, or MK-801+estrogen ($n=3$ per group). Each data point represents the mean $\pm$ S.E.M. and (*) indicates significant difference ($P<0.05$) from control (saline+saline group; lane 1).

If the drugs were interfering with one another, then the presence of either estrogen or MK-801 in the infarct area

may alter the neuroprotection afforded by the subsequent injection of the other drug. This hypothesis was tested by

Fig. 4. Effect of bath application of drugs on the RMP recorded from cortical neurons *in vitro*. (A) Whole cell patch clamp recordings made from cortical neurons. RMP was measured for 10 min (1 min baseline followed by 30 s during drug application and 8.5 min washout). (A) Representative current clamp recordings demonstrating the effect of aCSF (top), 50 μ M estrogen (middle), and 50 μ M estrogen+10 μ M MK-801 (bottom) on RMP. (B) Graphical representation of the magnitude of depolarization caused by application of either aCSF ($n=6$), estrogen ($n=5$), or estrogen+MK-801 ($n=6$). Each data point represents mean $\pm$ S.E.M. and (*) indicates significant difference ($P<0.05$) from aCSF control and (†) indicates significant difference from estrogen treatment. (C) Representative current clamp recordings demonstrating the effect of 20 μ M NMDA (top) and 20 μ M NMDA+50 μ M estrogen (bottom) on RMP. (D) Graphical representation of the magnitude of depolarization caused by application of NMDA or NMDA+estrogen. Each data point represents mean $\pm$ S.E.M. and (*) indicates significant difference ($P<0.05$) between the groups ($n=6$). (E) Representative current clamp recordings demonstrating the effect of 2.5 μ M AMPA (top) and 2.5 μ M AMPA+50 μ M estrogen (bottom) on RMP. (F) Graphical representation of the magnitude of depolarization caused by application of AMPA or AMPA+estrogen. Each data point represents mean $\pm$ S.E.M. and (*) indicates significant difference ($P<0.05$) between the groups ($n=5$).

introducing a 10 min delay between the injection of the drugs. Our results demonstrated that whether estrogen injection preceded MK-801 or MK-801 injection preceded estrogen, significant neuroprotection was observed. Initial blockade of NMDA receptors with MK-801 would prevent any increase in intracellular calcium and thus result in neuroprotection. However, we are suggesting that initial injection of estrogen resulted in a moderate activation of the NMDA receptor to increase intracellular calcium to a level required to activate ER-stress-induced neuroprotective pathways. Estrogen has been shown to increase intracellular calcium levels in neurons, perhaps through receptor-operated calcium channels such as the NMDA receptor, or through the activation of voltage-gated calcium channels (Zhao et al., 2005). The results of the *in vitro* study also support the hypothesis that estrogen caused a significant depolarization of cortical neurons. In addition, estrogen enhanced both NMDA- and AMPA-induced cellular depolarization, although the effect of estrogen could simply have been additive to the depolarizing effects of either NMDA or AMPA. Interestingly, the co-application of estrogen and MK-801 to cortical slices significantly decreased the estrogen-induced depolarization. This would seem to suggest that either estrogen acts directly on NMDA receptors resulting in mild activation, or estrogen may act presynaptically to release a moderate amount of glutamate which would in turn activate NMDA receptors. This latter hypothesis is currently under investigation using patch-clamp recordings in TTX and quantifying the frequency of spontaneous mini-EPSCs in slices before and following bath application of estrogen. Also, convention-

ally, whole cell patch clamp recordings are done in younger animals to increase the probability of success in forming high resistance seals, gaining access and holding cells with a stable RMP for a prolonged period of time (Blanton et al., 1989). Therefore, as mentioned above, due to this difference in the age of the animals used in the *in vivo* and *in vitro* study, caution should be taken when correlating the results of the two studies.

Recently, estrogen has been shown to modulate apoptotic cell death through manipulation of the ER stress response (Ejima et al., 1999). Also, the prior upregulation of various ER stress proteins following disruptions in intracellular calcium has been shown to provide protection against a variety of chemical and biological insults in both *in vitro* and *in vivo* systems (Cribb et al., 2005). A possible physical connection between glutamate receptor activation and the ER has been described whereby a traumatic stimulation activated transcription of the homer-1a gene leading to an upregulation of the homer-1 protein as quickly as 10 min following traumatic brain injury in cultured cortical neurons (Huang et al., 2005). Homer proteins belong to a family of post-synaptic density proteins (Brakeman et al., 1997), and contain complexes creating a physical link between glutamate receptors and the ER. As these homer proteins seem to physically link the intracellular domain of glutamate receptors with the ER (Fagni et al., 2004), it is possible that these homer proteins may mediate signaling between the NMDA receptor and intracellular calcium regulation by the ER. It may also be possible that in our *in vivo* model of MCAO, a small influx of calcium ions following estrogen-induced activation of NMDA re-

ceptors is sufficient to activate ER stress pathways and upregulate protective ER stress proteins. It has been demonstrated *in vitro* that moderate sub-lethal increases in calcium levels may induce immediate or long-term tolerance of ischemia or other stresses (Bickler and Fahlman, 2004).

Procaspase-12 is an ER resident caspase and activated caspase-12 is a well-known mediator of ER stress-induced pathogenesis (Nakagawa et al., 2000). Procaspase-12 can be converted into the active enzyme following cleavage by m-calpain, a calcium dependent cytosolic protease that is activated by an increase in intracellular calcium (Nakagawa and Yuan, 2000; Goll et al., 2003). Calpains have mainly been implicated in excitotoxic neuronal injury and necrosis (Goll et al., 2003). An increase in intracellular calcium (via NMDA receptor activation) would increase m-calpain levels and lead to the cleavage of procaspase-12 into activated caspase-12 (Wang, 2000). This is consistent with the results of the immunoblot analysis in that the levels of procaspase-12 decreased, while the levels of m-calpain and activated caspase-12 were significantly elevated (Fig. 3) in estrogen-treated animals following MCAO. It has been demonstrated that m-calpain may be responsible for the activation of procaspase-12, indicating a strong link between calcium dysregulation and ER stress (Nakagawa and Yuan, 2000). In this way, low level activation of NMDA receptors by estrogen may initiate neuroprotective processes which protect neurons from the excitotoxic injury following MCAO.

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REFERENCES

- Ascherio A, Weisskopf MG, O'Reilly EJ, McCullough MI, Calle EE, Rodriguez C, Thun MJ (2004) Coffee consumption, gender, and Parkinson's disease mortality in the cancer prevention study II cohort: the modifying effects of estrogen. *Am J Epidemiol* 160:977–984.
- Bickler PE, Fahlman CS (2004) Moderate increases in intracellular calcium activate neuroprotective signals in hippocampal neurons. *Neuroscience* 127:673–683.
- Blanton M, LoTurco J, Kriegstein A (1989) Whole cell recording from neurons in slices of reptilian and mammalian cerebral cortex. *J Neurosci Methods* 30:203–210.
- Brakeman PR, Lanahan AA, O'Brien R, Roche K, Barnes CA, Huganir RL, Worley PF (1997) Homer: a protein that selectively binds metabotropic glutamate receptors. *Nature* 386:284–288.
- Buchan AM, Slivka A, Xue D (1992) The effect of the NMDA receptor antagonist MK-801 on cerebral blood flow and infarct volume in experimental focal stroke. *Brain Res* 574:171–177.
- Choi DW (1995) Calcium: still center stage in hypoxic-ischemic neuronal death. *Trends Neurosci* 18:58–60.
- Cribb AE, Peyrou M, Muruganandan S (2005) The endoplasmic reticulum in xenobiotic toxicity. *Drug Rev Metab* 37:405–422.
- Ejima K, Nanri H, Araki M, Uchida K, Kashimura M, Ikeda M (1999) 17 β -Estradiol induces protein thiol/disulfide oxidoreductases and protects cultured bovine aortic endothelial cells from oxidative stress. *Eur J Endocrinol* 140:608–613.
- Fagni L, Ango F, Perroy J, Bockaert J (2004) Identification and functional roles of metabotropic glutamate receptor-interacting proteins. *Semin Cell Dev Biol* 15:289–298.
- Fatehi M, Kombian SB, Saleh TM (2006a) 17 β -Estradiol inhibits outward potassium currents recorded in rat parabrachial nucleus cells *in vitro*. *Neuroscience* 135:1075–1086.
- Fatehi M, Zidichouski JA, Kombian SB, Saleh TM (2006b) 17 β -Estradiol attenuates excitatory neurotransmission and enhances the excitability of rat parabrachial neurons *in vitro*. *J Neurosci Res* 84:666–674.
- Foy MR, Xu J, Xie X, Brinton RD, Thompson RF, Berger TW (1999) 17 β -Estradiol enhances NMDA receptor-mediated EPSPs and long term potentiation. *J Neurophysiol* 81:925–929.
- Gibson CL, Gray LJ, Murphy SP, Bath PMW (2006) Estrogens and experimental ischemic stroke: a systematic review. *J Cereb Blood Flow Metab* 26:1103–1113.
- Goll DE, Thompson VF, Li H, Wei W, Cong J (2003) The calpain system. *Physiol Rev* 83:731–801.
- Gu Q, Moss RL (1998) Novel mechanism of non-genomic action of 17 β -estradiol on kainate-induced currents in isolated rat CA1 hippocampal neurons. *J Physiol* 506:745–754.
- Huang WD, Fei Z, Zhang X (2005) Traumatic injury induced homer-1a gene expression in cultured cortical neurons of rat. *Neurosci Lett* 389:46–50.
- Hurn PD, MacRae IM (2000) Estrogen as a neuroprotectant in stroke. *J Cereb Blood Flow Metab* 20:631–652.
- Lipton P (1999) Ischemic cell death in brain neurons. *Physiol Rev* 79:1431–1568.
- Nakagawa T, Yuan J (2000) Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *J Cell Biol* 150:887–892.
- Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, Yuan J (2000) Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid- β . *Nature* 403:98–103.
- Paxinos G, Watson C (1986) The rat brain in stereotaxic coordinates. New York: Academic Press.
- Raval AP, Bramlett H, Perez-Pinzon MA (2006a) Estrogen preconditioning protects the hippocampal CA1 against ischemia. *Neuroscience* 141:1721–1730.
- Raval AP, Dave KR, Perez-Pinzon MA (2006b) Resveratrol mimics ischemic preconditioning in the brain. *J Cereb Blood Flow Metab* 26:1141–1147.
- Saleh TM, Connell BJ (2003) Central nuclei mediating estrogen-induced changes in autonomic tone and baroreceptor reflex in male rats. *Brain Res* 961:190–200.
- Saleh TM, Connell BJ, Cribb AE (2005) Sympathoexcitatory effects of estrogen in the insular cortex are mediated by GABA. *Brain Res* 1037:114–122.
- Saleh TM, Cribb AE, Connell BJ (2001a) Estrogen-induced recovery of autonomic function after middle cerebral artery occlusion in male rats. *Am J Physiol* 281:R1531–R1539.
- Saleh TM, Cribb AE, Connell BJ (2001b) Reduction in infarct size by local estrogen does not prevent autonomic dysfunction after stroke. *Am J Physiol* 281:R2088–R2095.
- Saleh TM, Connell BJ, Legge C, Cribb AE (2004) Estrogen attenuates neuronal excitability in the insular cortex following middle cerebral artery occlusion. *Brain Res* 1018:119–129.
- Singer CA, Rogers KL, Strickland TM, Dorsa DM (1996) Estrogen protects primary cortical neurons from glutamate neurotoxicity. *Neurosci Lett* 212:13–16.
- Storm-Mathisen J, Danbolt NC, Rothe F, Torp R, Zhang N, Aas JE, Kanner BI, Langmoen I, Ottersen OP (1992) Ultrastructural immunocytochemical observations on the localization, metabolism and transport of glutamate in normal and ischemic brain tissue. *Prog Brain Res* 94:225–241.
- Szatkowski M, Attwell D (1994) Triggering and execution of neuronal death in brain ischemia: two phases of glutamate release by different mechanisms. *Trends Neurosci* 17:359–365.

- Wang KK (2000) Calpain and caspase: can you tell the difference? Trends Neurosci 23:20–26.
- Weaver CE Jr, Park-Chung M, Gibbs TT, Farb DH (1997) 17 β -Estradiol protects against NMDA-induced excitotoxicity by direct inhibition of NMDA receptors. Brain Res 4761:338–341.
- Wong M, Moss RL (1992) Long-term and short-term electrophysiological effects of estrogen on the synaptic properties of hippocampal CA1 neurons. J Neurosci 12:3217–3225.
- Woolley CS (1999) Electrophysiological and cellular effects of estrogen on neuronal function. Crit Rev Neurobiol 13:1–20.
- Xu K, Xu Y, Brown-Jermyn D, Chen JF, Ascherio A, Dluzen DE, Schwarzschild MA (2006) Estrogen prevents neuroprotection by caffeine in the mouse 1-methyl-4-phenyl-1,2,3,6-tetrahydropyriding model of Parkinson's disease. J Neurosci 26:535–541.
- Zhao L, Chen S, Ming J, Brinton RD (2005) 17 β -Estradiol induces Ca^{2+} influx, dendritic and nuclear Ca^{2+} rise and subsequent cyclic AMP response element-binding protein activation in hippocampal neurons: a potential initiation mechanism for estrogen neurotrophism. Neuroscience 132:299–311.

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