Development/Plasticity/Repair

Estradiol Attenuates Programmed Cell Death after Stroke-Like Injury

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Estradiol is a known neurotrophic and neuroprotective factor. Our previous work demonstrated that replacement with physiological concentrations of estradiol protects the cortex against middle cerebral artery occlusion (MCAO)-induced cell death. The cerebral cortex exhibits caspase-dependent programmed cell death (PCD) in many models of focal cerebral ischemia. We hypothesized that estradiol attenuates PCD during stroke injury. The current study explored the temporospatial pattern of markers of PCD, their relationship to the evolution of injury, and their modulation by estradiol. Rats were ovariectomized and treated with either estradiol or vehicle. One week later, rats underwent MCAO, and brains were collected at 1, 4, 8, 16, and 24 hr. We assessed the temporospatial evolution of infarction volume, DNA fragmentation, and levels of spectrin cleavage products in ischemic cortex. Estradiol led to a delay and attenuation of injury-mediated DNA fragmentation as early as 8 hr after MCAO. Estradiol also dramatically reduced the level of the 120 kDa caspase-mediated spectrin breakdown product (SBDP120) at 4 hr but not at 8 or 16 hr. The SBDP150, produced by caspase and calpain, showed peak levels at 16 hr but was not altered by estradiol. These results strongly suggest that estradiol protects the ischemic cortex by attenuating PCD, thereby reducing caspase activity, DNA fragmentation, and subsequently, overall cell death. These studies deepen our understanding of the mechanisms underlying estrogen-mediated neuroprotection.

Key words: **apoptosis; ischemia; stroke; middle cerebral artery occlusion; TUNEL; estrogen (estradiol); caspase**

Introduction

Middle cerebral artery occlusion (MCAO) leads to a characteristic pattern of expanding tissue damage. The permanent occlusion of the MCA primarily affects two regions of the brain: the striatum and the overlying cortex. Occlusion at the base of the MCA leads to severe metabolic impairment and necrotic cell death in the striatum. However, the ischemic cortex is less severely damaged as a result of the presence of collateral perfusion from branches of other major cerebral arteries. Because of this difference in perfusion, the cortex contains cells undergoing programmed cell death (PCD) (Li et al., 1995, 1997; Guegan and Sola, 2000), involving the activation of protease cascades (Asahi et al., 1997; Namura et al., 1998; Guegan and Sola, 2000; Luo et al., 2002). Studies have demonstrated that an attenuation of PCD reduces the amount of tissue damage after ischemic injury (Friedlander et al., 1997; Endres et al., 1998; Kitagawa et al., 1998a; Jover et al., 2002). Apoptosis is a name for at least one specific pathway of PCD. Because the precise definition of this term is still a topic of active debate, we will use PCD to refer to the caspase-mediated cell death pathway known to characterize the ischemic cortex after MCAO.

It is accepted that estradiol influences the hypothalamic–pituitary–ovarian axis and thereby plays a critical role in reproduction. In recent years, it has become increasingly clear that estradiol also plays important nonreproductive roles in the brain, such as enhancing synaptic plasticity and exerting neurotrophic and neuroprotective actions (Woolley, 1999; Hurn and Macrae, 2000; Roof and Hall, 2000; Garcia-Segura et al., 2001; Wise et al., 2001). Both pharmacological and physiological concentrations of estradiol protect against brain injury (Hall et al., 1991; Simpkins et al., 1997; Dubal et al., 1998; Toung et al., 1998; Zhang et al., 1998; Culmsee et al., 1999; Rusa et al., 1999; Shughrue and Merchenthaler, 2003).

Our laboratory has shown that pretreatment with a low, physiological dose of estradiol protects the ischemic cortex against the later stages of cell death induced by MCAO, independent of alterations in cerebral blood flow (Dubal et al., 1998). We hypothesized that estradiol attenuates injury by blocking PCD in the ischemic cortex.

We tested our hypothesis by exploring the temporospatial patterns of PCD markers in ischemic cortex. PCD can be explored immunohistochemically via terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) staining, an indicator of DNA fragmentation (Willingham, 1999), and by observing caspase-3 activation (Namura et al., 1998; Guegan and Sola, 2000; Davoli et al., 2002; Wang et al.,

Received May 29, 2003; revised Oct. 8, 2003; accepted Oct. 16, 2003.

This work was supported by National Institutes of Health Grants AG17164, AG02224, and AG00242 (P.M.W). We give special thanks to Dr. Rodney P. Guttman of the Sanders Brown Center on Aging at the University of Kentucky for excellent technical support and advice.

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2003). We evaluated the relative levels of caspase-3 activation by measuring levels of 150 kDa and 120 kDa spectrin breakdown products (SBDP150 and SBDP120, respectively), which reflect activity of enzymes associated with necrosis and programmed cell death, respectively (for review, see Wang, 2000). TUNEL staining and spectrin cleavage profiles of both oil- and estradiol-treated rats were compared with hematoxylin and eosin (H&E) staining to observe the temporal relationships of caspase activation, DNA fragmentation, and infarction after MCAO in oil- and estradiol-treated animals.

Materials and Methods

Cerebral ischemia

Female Sprague Dawley rats (225–275 gm) were maintained in a 14/10 hr light/dark cycle with *ad libitum* access to food and water. All procedures were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and have been approved by the University of Kentucky Medical Center Institutional Animal Care and Use Committee. Under methoxyflurane anesthesia, rats were bilaterally ovariectomized to eliminate endogenous estradiol production and then implanted with a SILASTIC capsule (Konigsberg Instruments, Pasadena, CA) containing oil or 17β-estradiol (180 μg/ml). This paradigm of low-dose estradiol treatment produces ~20 pg/ml in rats (Dubal and Wise, 2001). These levels are equivalent to basal circulating levels found during the estrous cycle of rats (Smith et al., 1975).

Two separate groups of animals were collected for these studies. The first group was used for H&E and TUNEL studies ($n = 10$–14 per group), and the second group was used for the Western blotting studies ($n = 5$ per group). For all animals, after 7 d, rats underwent MCAO surgery. Rats were anesthetized with ketamine–acepromazine (80 − 0.52 mg/kg, i.p.). Body temperature was monitored with a rectal probe and maintained within 1°C of normothermia (36–38°C). The right middle cerebral artery was permanently occluded using methods described previously (Dubal et al., 1998). Briefly, a 4/0 monofilament suture was inserted through the internal carotid artery to the base of the middle cerebral artery. This occlusion leads to dramatically reduced blood flow in the striatum and overlying cortex.

A small number of animals did not survive the MCAO procedure or did not survive for the entire postsurgical period. These animals were excluded from additional study. This minor loss of animals is reflected in the lower reported numbers per experimental group for subsequent analyses. There was no significant difference in survival rates among the experimental groups. Animals were also excluded from final analyses if infarct size was ±2 SDs away from the group mean.

Tissue staining

Preparation. Brains were collected at 1, 4, 8, 16, and 24 hr after the onset of MCAO ($n = 6$–10 per experimental group). Brains were frozen on dry ice and stored at −80°C until 18 μm coronal sections from approximately bregma +2.2 through bregma −2.8 (Paxinos and Watson, 1997) were cut on a cryostat. This region contains the area that undergoes injury. Brain sections were mounted on slides and stored at −80°C until they were processed for H&E or TUNEL staining. Sections within a range from bregma +1.2 to bregma −0.8, where the infarct size was the largest, were used for these studies; this area will be referred to as the "test zone."

H&E staining. One slide of sections (two sections per slide) was taken from within the test zone of each animal. The sections were fixed with paraformaldehyde and stained with H&E to delineate the extent of ischemic injury (Bederson et al., 1986). The extent of the infarction was quantified with a computer-assisted imaging system (NIH Image, version 6.1). Previous studies using 2-mm-thick sections stained by 2% triphenyl–tetrazolium chloride (TTC) for quantification of infarct volume showed that the general size and shape of the infarction is relatively constant throughout the test zone (our unpublished observations). Therefore, infarct area from a single slide is representative of infarct throughout the test zone. In the current study, the area of infarct from a single slide multiplied by test zone thickness (2 mm) yields infarction volume.

TUNEL staining. One slide of sections (a slide adjacent to that taken for H&E staining, two sections per slide) was taken from within the test zone of each animal. TUNEL was performed on these sections according to the instructions of the manufacturer (*in situ* cell death detection kit, TMR red; catalog number 2 156 792; Roche Products, Hertforshire, UK). Briefly, the sections were fixed for 5 min in 4% paraformaldehyde. Sections underwent two washes in PBS and were incubated in permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate) for 30 min at 70°C. Sections were then washed twice in PBS followed by incubation for 60 min at 37°C in "TUNEL reaction mix" from the Roche Products kit. Sections underwent two more washes in PBS and were then immersed in Hoechst 33258 (Molecular Probes, Eugene, OR) solution (1:1000 dilution) for 10 min. Sections were finally rinsed in distilled water and coverslipped with anti-fade mounting medium. A positive control was performed by incubating slides for 60 min at 37°C in RQ–DNase solution (2 μl of RQ1 RNase-free DNase; catalog number M6101; Promega Madison, WI) before incubation in TUNEL reaction mix. A negative control was performed by incubating sections in reaction mix that contained only label solution and no TUNEL enzyme components. All areas of the brain exhibited TUNEL staining in the positive control slide. No cellular staining was observed in the negative control slide.

TUNEL-positive cells were counted in 20× microscope fields within frontal cortices (three fields per section) and parietal cortices (four fields per section) from a single section for each animal using an object counts array via BioQuant (Oxford, UK) software. Counts for all seven microscope fields were summed to yield a single cell count for each animal. The frontal and parietal cortical areas of tissue were chosen for cell counts because they represent peri-infarct cortex after MCAO in our paradigm. In the frontal cortex, two fields were counted that bordered on the central sulcus. One of these fields also encompassed the outer rim of cortical tissue, and the other included the outer rim of the corpus callosum. A third field encompassing the central layers of cortex was counted one 20× field lateral to the first two fields. In the parietal cortex, a field encompassing central layers of cortex lateral to the third frontal cortex field was counted. Next, one 20× field lateral to this first parietal field, two fields were counted, one encompassing the outer rim of cortex and the other including the border of the corpus callosum. The fourth parietal field encompassed the central layers of cortex, one 20× field lateral to the second and third parietal fields. Using this pattern of movement from field to field in each section, equal sampling was ensured from animal to animal.

Western blotting

Microdissection. Brains were collected at 4, 8, and 16 hr ($n = 3$–5 per experimental group). Alternating 1 mm sections of brain were collected using a brain matrix (Activational Systems, Warren, MI) and then stained in 2% TTC to visualize injury or frozen at −80°C for inclusion in Western blotting studies. We first examined tissue from a 1 mm TTC-stained coronal section from the test zone. The adjacent fresh, frozen 1 mm section was then microdissected, and a specific area (see Fig. 4, area X) of ipsilateral cortical tissue was removed for subsequent protein extraction and use in Western blotting studies. Area X was chosen so that live tissue from an identical anatomical area of cortical tissue ipsilateral to infarct could be directly compared between experimental groups without the risk of analyzing damaged tissue in any given animal. Previous studies from our laboratory (Dubal et al., 1999) have demonstrated injury-mediated changes in these tissue areas. Area X is also included within the area studied in the TUNEL studies and represents the edge of the tissue that is consistently alive after 24 hr of MCAO. We felt that this area would be an excellent region to explore time-dependent changes after an ischemic insult in both oil- and estradiol-treated animals without attempting to compare anatomically disparate regions of tissue.

Western blot analysis. Relative concentrations of protein were quantified by Western blotting using standard procedures. Total cell lysates were obtained from microdissected cortical tissue. Tissue was homogenized in lysis buffer [1× PBS, 1% Triton X-100, 0.1% SDS, 50 mM sodium fluoride, and 0.5% deoxycholate with complete protease inhibitor mixture (Roche Products)], and cellular debris was separated by centrifugation at 14,000 × *g* for 30 min at 4°C. Total protein (8 μg) was sepa-

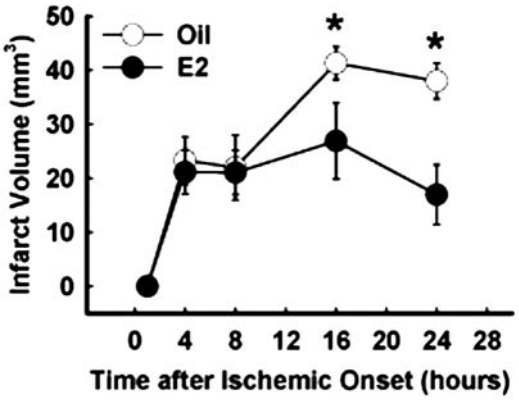

Figure 1. Estradiol (E2) protects the brain against ischemic injury. MCAO initiates an ischemic insult that develops into an extensive infarct over 24 hr. Data were analyzed using two-way ANOVA with dependent variables of time (1, 4, 8, 16, and 24 hr) and treatment (oil vs estradiol). There is an overall significant effect of time as well as an overall significant interaction between time and treatment. Using Newman–Keuls *post hoc* evaluation, there is a statistically significant difference between infarct volumes in oil- and estradiol-treated animals at 16 and 24 hr after MCAO (*$p < 0.05$; $n = 8$–10 per experimental group). Values represent mean ± SEM.

rated by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and incubated overnight at 4°C in 5% milk–Tris-buffered saline plus Tween 20 containing a mouse antispectrin antibody (1:5000, monoclonal antibody 1622; Chemicon, Temecula, CA). Blots were then washed three times for 10 min each and exposed to horseradish peroxidase-conjugated goat anti-mouse antibody (1:10,000; Jackson ImmunoResearch, West Grove, PA) for 1 hr. Blots were visualized using a Supersignal West Pico Chemiluminescent Substrate 34077 (Pierce, Rockford, IL) and a FujiFilm FLA-2000 imager (Fuji, Stamford, CT). The spectrin blots were then stripped and probed with rabbit anti-neurofilament 200 (N4142, 1:400; Sigma St. Louis, MO). Band intensity was detected and quantified by comparing relative signal intensity via NIH Image, version 6.1. All values for 120 kDa spectrin and 150 kDa spectrin fragments were normalized to full-length (240 kDa) spectrin and expressed as a ratio of fragment to full length. These ratios were then normalized to the control protein neurofilament 200 to account for any potential differences in lane loading.

Data analysis. Data for H&E and Western blotting experiments were analyzed by two-way ANOVA. For the TUNEL experiment, data were analyzed separately as an early phase (1–8 hr) and a late phase (16–24 hr) of injury. Significant effects or interactions were probed using Newman–Keuls *post hoc* comparisons. Differences were considered statistically significant when $p < 0.05$. All data are expressed as mean ± SEM.

Results

Estradiol pretreatment reduces infarction volume during the late phase of injury

We analyzed total infarction volume at 1, 4, 8, 16, and 24 hr to assess the temporal pattern of cell death (Fig. 1). In both oil- and estradiol-treated animals, infarction is not detectable by H&E staining at 1 hr after MCAO, but we detected a dramatic rise in infarct volume from 1 to 4 hr that was maintained through the 8 hr time point. During the late phase of ischemic injury, estradiol dramatically reduces infarction volume at 16 and 24 hr after MCAO when compared with oil-treated animals (Fig. 1) (*$p < 0.05$).

Estradiol pretreatment delays and attenuates DNA fragmentation during early and late phases of injury

We analyzed the temporal pattern of DNA fragmentation after MCAO by counting numbers of TUNEL-positive cells in coronal sections. TUNEL was specific for cells with fragmented DNA as illustrated by a differential staining by TUNEL versus the general

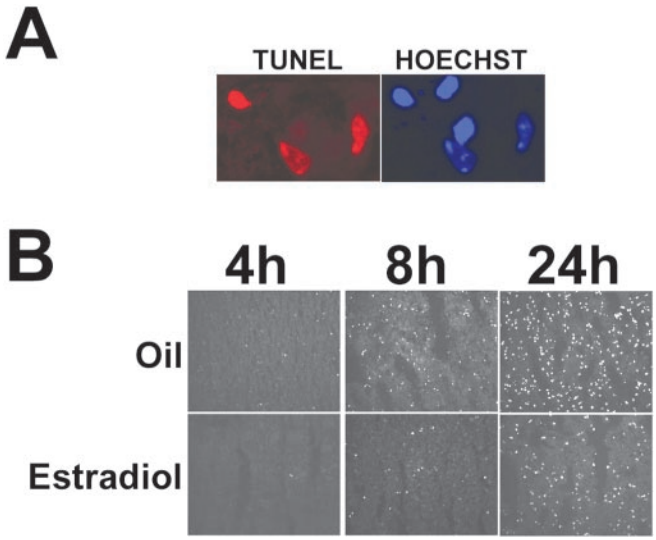

Figure 2. Composite of representative pictures from the ischemic cortex stained by the TUNEL technique and counterstained with Hoechst 33258. *A*, Microscope fields at 40× magnification showing cells that stained positive for TUNEL and the same field showing Hoechst counterstain. *B*, Representative 20× fields taken from within the parietal cortex of coronal brain sections stained by the TUNEL technique. These fields demonstrate TUNEL-positive cells from oil- and estradiol-treated animals at 4, 8, and 24 hr after the initiation of MCAO.

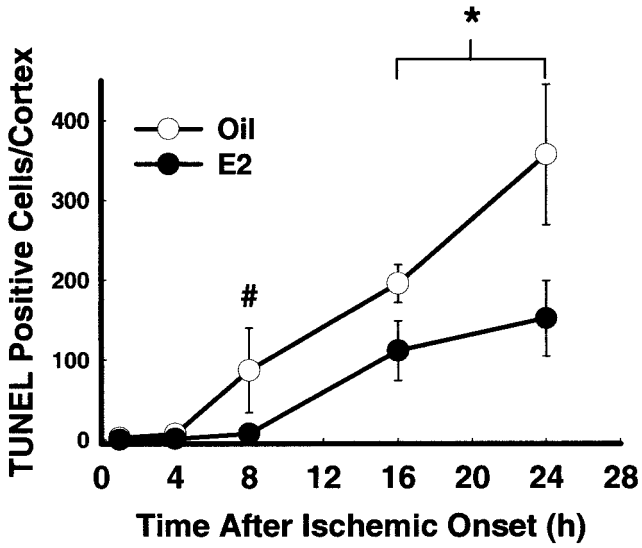

Figure 3. Estradiol (E2) delays and attenuates the number of TUNEL-positive cells in the ischemic cortex. The mean number of TUNEL-positive cells in the ischemic cortex rises dramatically after 4 hr in oil-treated animals and continues to rise through the remaining time points after ischemic injury. In estradiol-treated animals, the number of TUNEL-positive cells rises dramatically after 8 hr. ANOVAs were run for the early (1–8 hr; $n = 8$–10 per experimental group) and late (16–24 hr; $n = 8$–10 per experimental group) phases of injury. There is a main effect of treatment (oil vs estradiol) on the number of TUNEL-positive cells during early (#$p < 0.05$) and late (*$p < 0.05$) phases of ischemic injury. Values represent mean ± SEM.

nuclear dye Hoechst 33258 (Fig. 2*A*). In the ischemic cortex, very few TUNEL-positive cells were observed during the first 4 hr after ischemic onset (Figs. 2*B*, 3). In oil-treated animals, we observed a rapid increase in the number of TUNEL-positive cells per cortex between 4 and 8 hr, which continued throughout the remainder of the evolution of ischemic injury. In contrast, in estradiol-treated animals, this rise in TUNEL-positive cells was not observed until between the 8 and 16 hr time points after ischemic onset. Statistical analysis of TUNEL data was performed by sep-

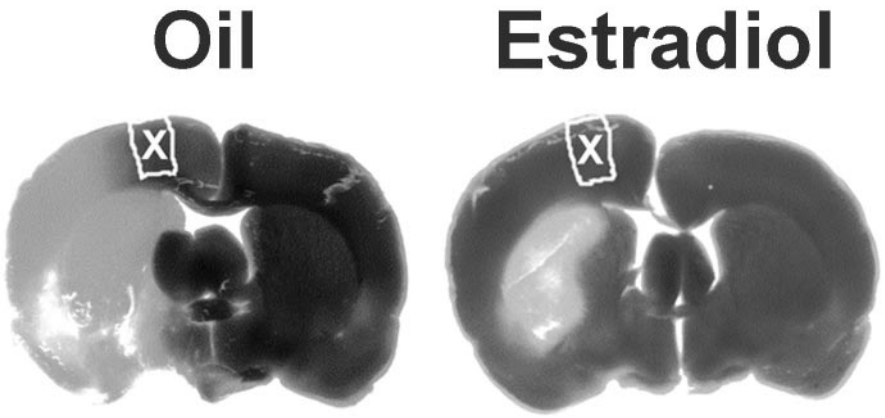

Figure 4. Representative images of sections from an oil- and estradiol-treated rat that underwent 24 hr of permanent middle cerebral artery occlusion. Infarcted tissue is light, whereas live tissue is dark. An adjacent 1 mm frozen coronal section was microdissected in anatomically equivalent areas (X) from oil- and estradiol-treated animals.

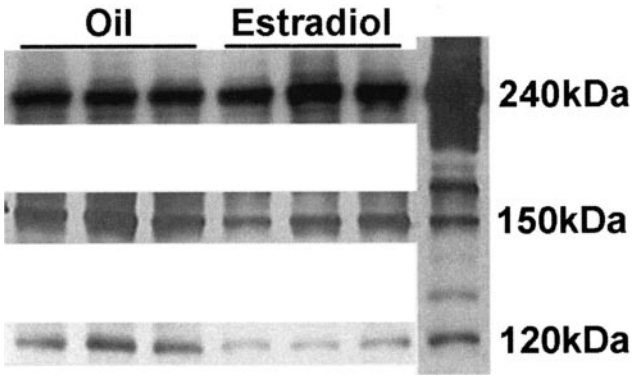

Figure 5. Composite of Western blot results showing representative lanes from oil- and estradiol-treated animals killed 4 hr after the initiation of MCAO. Proteins extracted from area X (Fig. 4) were analyzed by Western blotting to detect intact spectrin (240 kDa) and 120 kDa and 150 kDa spectrin breakdown products.

arate two-way ANOVA for early phase data (1–8 hr) and late-phase data (16–24 hr). We hypothesized that the effect of estradiol on late-phase infarction volume may be accompanied by an early phase effect on the number of TUNEL-positive cells in ischemic cortex. Therefore, we believed it was important to explore the different phases of injury by separate analyses. Pretreatment with estradiol reduced the number of TUNEL-positive cells during both the early (1–8 hr) and late (16–24 hr) phases of injury (Figs. 2*B*, 3) ($^{\#}p < 0.05$; $^{*}p < 0.05$).

Estradiol pretreatment reduces injury-mediated SBDP120 but not SBDP150 spectrin fragments in peri-infarct cortex

We extracted proteins from the peri-infarct region of the ischemic cortex depicted in Figure 4 and determined relative amounts of spectrin and specific cleavage fragments (SBDP120 and SBDP150) of spectrin in oil- and estradiol-treated animals by Western blot analysis ($n = 3$ per experimental group). Estradiol pretreatment dramatically attenuates the level of SBDP120 at 4 hr after MCAO (Figs. 5, 6) but does not significantly alter the secondary rise in the level of SBDP120 observed at 16 hr (Fig. 6). Estradiol also did not affect the rise in SBDP150 observed at 16 hr (Fig. 7).

Discussion

The primary findings of this work are as follows: (1) estradiol delays and attenuates injury-mediated DNA fragmentation in ischemic cortex; (2) the effects of estradiol on DNA fragmentation are evident as early as 8 hr after MCAO and thus precede the observed reduction in cortical infarct volume at 16–24 hr; and (3) both attenuation of DNA fragmentation and reduced infarct volume are preceded by an estradiol-mediated reduction in the relative amount of the caspase-3-mediated spectrin breakdown product SBDP120 at 4 hr after MCAO. This temporal pattern of effects leads us to propose that estradiol modulates a cascade of actions. We suggest that the actions of estradiol involve an attenuation of caspase-3 activation that leads to a downstream reduction in programmed cell death and subsequently reduced infarct volume.

Our data both confirm a previously delineated cascade of events after focal cerebral ischemia and demonstrate a potential protective pathway used by estradiol. The time course of changes in PCD markers in these studies is consistent with observations from other models of brain injury. Guegan and Sola (2000) demonstrated an increase in cleavage fragments of poly(ADP)ribose polymerase, a substrate of caspases in PCD cascades, as early at 3 hr after the initiation of left middle cerebral artery coagulation. Nath et al. (2000) observed the SBDP120 as a marker of caspase activity after NMDA-induced cytotoxic injury in neonatal rats. These authors observed increased caspase activity at 6, 18, and 24 hr after NMDA injection and DNA laddering, characteristic of PCD, at 24 hr. Shibata et al. (2002) observed caspase-3 activations as early as 1 hr after MCAO in mice. To the best of our knowledge, the observation of an apparent biphasic alteration in caspase activity after MCAO is unique to our study. We speculate that the secondary rise in caspase activation may be related to a late rise in calpain activity, as suggested by the dramatic increase observed in the SBDP150 at 16 hr after MCAO initiation. Such an interaction has been described previously after UV-induced neuronal death, during which calpain activates the caspase-3 pathway (McCollum et al., 2002). Additionally, our findings are consistent with previous studies from our laboratory (Wilson et al., 2002) and others (Liu et al., 2001; Jover et al., 2002; Linford and Dorsa, 2002; Monroe et al., 2002) that established estradiol as an inhibitor of programmed cell death cascades.

Previous studies have shown that caspase-3 activation leads to DNA fragmentation and subsequently to expansion of infarct volume in MCAO models of ischemic injury (Namura et al., 1998; Guegan and Sola, 2000; Plesnila et al., 2001; Luo et al., 2002). Caspase-3 is known to be an executioner caspase and is the final common enzyme in a number of pathways of programmed cell death. Because of this unique position of caspase-3 in various PCD pathways, its activation opens up the possibility that multiple upstream effectors may be targets of estradiol-induced neuroprotection.

Our laboratory has explored various members of the Bcl-2 family of genes as potential targets for the actions of estradiol. The Bcl-2 family is composed of a number of intracellular proteins. These are central regulators of programmed cell death cascades. This family of proteins contains both pro-programmed and anti-programmed cell death factors. It is the balance of these factors at a given time that determines whether a cell will be moving toward life or death (for review, see Cory and Adams, 2002). In models of

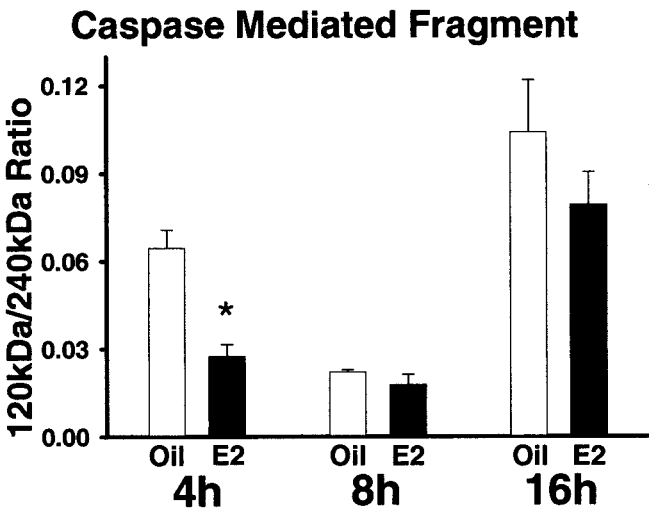

Figure 6. Estradiol (E2) reduces the caspase-mediated (120 kDa) spectrin breakdown product at 4 hr after MCAO onset in the peri-infarct ischemic cortex (*$p < 0.05$). Two-way ANOVA demonstrated an overall effect of treatment (oil vs estradiol). *Post hoc* Newman–Keuls reveals an effect of estradiol specific to the 4 hr time point (*$p < 0.05$; $n = 3$ per experimental group). Estradiol has no effect on levels of the 120 kDa spectrin breakdown product at 8 hr or on the secondary rise in this product at 16 hr after MCAO onset ($n = 3–4$ per experimental group). Relative levels for each sample are expressed as a ratio of 120 kDa fragment to full-length spectrin (240 kDa) normalized to neurofilament 200 from the same sample. Values represent mean ± SEM.

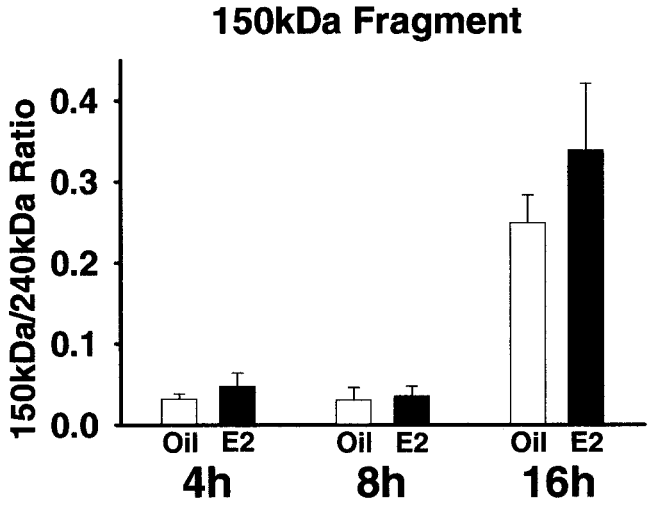

Figure 7. Estradiol (E2) does not affect the 150 kDa spectrin breakdown product in the peri-infarct ischemic cortex. The 150 kDa spectrin breakdown product shows a dramatic rise at 16 hr after MCAO onset. Two-way ANOVA demonstrates a significant overall effect of time but no effect of treatment and no interaction ($n = 3–4$ per experimental group). Relative levels for each sample are expressed as a ratio of 150 kDa fragment to full-length spectrin (240 kDa) normalized to neurofilament 200 from the same sample. Values represent mean ± SEM.

ischemia, the loss of Bcl-2 has been associated with an exacerbation of injury (Krajewski et al., 1995; Sato et al., 1998), whereas overexpression of this factor can protect against a number of toxic stimuli (Martinou et al., 1994; Choi, 1996; Kitagawa et al., 1998b; Yang et al., 1998). Previous work in our laboratory has shown that estradiol can maintain levels of *bcl-2* mRNA in the ischemic cortex (Dubal et al., 1999). The effects of estradiol in our model are specific to *bcl-2*, because estradiol had no effect on the expression profiles of *bax*, *bcl-x_L*, *bcl-x_S*, *bad* (Dubal et al., 1999), or *bim* (Wise, 2003). The ability of estradiol to maintain the level of *bcl-2* mRNA in the ischemic cortex may explain the attenuation of caspase activity and overall reduction in PCD that we observed.

Another pathway to consider upstream of caspase-3 is a neuroprotective pathway involving Akt, a serine–threonine kinase. Activated Akt phosphorylates and inhibits the actions of several programmed cell death mediators, such as Bad and caspase-9, and thereby prevents programmed cell death in several cell types, including neurons (Crowder and Freeman, 1998). Estradiol has been shown to directly activate Akt in the cortex (Singh, 2001) and to enhance Akt activation in cortical explant cultures after ischemic injury (Wilson et al., 2002).

The results of the current study enhance our understanding of the mechanisms involved in estradiol-mediated neuroprotection. Estrogens have been established as potent neuroprotective and neurotrophic factors (Calakos and Scheller, 1994; Toran-Allerand et al., 1999; Green and Simpkins, 2000; Hurn and Macrae, 2000; Brinton, 2001; Garcia-Segura et al., 2001; Wise et al., 2001). Specifically, clinical studies have demonstrated that estrogens enhance mood and cognition and delay cognitive decline (Paganini-Hill et al., 1988; Kawas et al., 1997; Sherwin, 1999). Furthermore, many studies suggest that estrogens are able to protect against neurodegenerative diseases, such as Alzheimer's disease (Fillit, 1994; Paganini-Hill and Henderson, 1994, 1996; Henderson et al., 1996; Tang et al., 1996; Henderson and Paganini-Hill, 1997; Kawas et al., 1997; Asthana et al., 1999; Waring et al., 1999) and injury associated with stroke or stress (Paganini-Hill, 1995; Schmidt et al., 1996; Komesaroff et al., 1999). However, several recent studies have failed to demonstrate an amelioration of cognitive dysfunction in women already suffering from Alzheimer's disease (Marder and Sano, 2000; Roof and Hall, 2000; Wang et al., 2000). In addition, the Women's Health Initiative reported an increased risk for stroke, among other conditions (Writing Group for the Women's Health Initiative Investigators, 2002). Although the clinical literature gives conflicting results, a variety of *in vivo* and *in vitro* basic science studies provide striking evidence for cellular and molecular mechanisms underlying clear neuroprotective and neurotrophic actions of estradiol. Estrogens attenuate neuronal injury associated with cerebral ischemia and brain trauma in young and aging male and female rodents (Hall et al., 1991; Behl et al., 1997; Alkayed et al., 1998, 2000; Dubal et al., 1998; Miller et al., 1998; Toung et al., 1998; Rusa et al., 1999; Sawada and Shimohama, 2000; Dubal and Wise, 2001; Kim et al., 2001; Mendelowitsch et al., 2001; Jover et al., 2002).

A potential source of the clinical versus basic science discrepancies in the effects of estrogen on neuronal injury may be differences in the preparation and amount of estrogen being used in the various clinical trials and those used in basic science studies. For example, none of the clinical trials currently reported in the literature use a treatment paradigm that mimics our paradigm of estradiol pretreatment with basal concentrations of the hormone. The clinical literature has focused on estrogen–progesterone combination treatment or the use of estrogen-only treatments containing more than basal concentrations relative to estrogen concentrations across the reproductive cycle. A clinical trial using basal levels of 17β-estradiol in women in the early postmenopausal period may yield more favorable results.

The potential for attenuation of programmed cell death demonstrated by our current studies makes estradiol a very powerful potential therapeutic for a variety of CNS injuries and neurodegenerative diseases if given in the appropriate concentrations and at the appropriate time. An understanding of the differential modulation of programmed cell death in various tissues under

varying conditions will be essential to the development of the therapeutic potential of estradiol.

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