Estradiol Differentially Regulates c-Fos after Focal Cerebral Ischemia

Shane W. Rau, Dena B. Dubal, Martina Böttner, and Phyllis M. Wise

Estrogen replacement therapy enhances mood, delays cognitive decline, and reduces the risk of neurodegeneration. Our laboratory has shown previously that pretreatment with low physiological levels of estradiol protects against middle cerebral artery occlusion (MCAO)-induced brain injury during late phases of neuronal cell death. Immediate early genes (IEGs) are induced by various forms of brain injury, and their induction is known to be a critical step in programmed cell death. The current study tested the hypothesis that the ability of estradiol to reduce MCAO-induced cell death involves attenuation of expression of one or more IEGs. We examined the effects of estradiol on the temporospatial pattern of IEG expression and the modulation of this pattern by estradiol replacement. Rats were ovariectomized and treated with either vehicle or low physiological concentrations of estradiol. One week later, rats underwent MCAO and brains were collected 1, 4, 8, 16, and 24 hr later. We assessed IEG mRNAs in discrete regions of brain by RT-PCR at 24 hr. We examined expression of c-Fos mRNA and protein in greater detail using in situ hybridization and immunohistochemistry to delineate the time course and specific regions of cortex in which estradiol influenced its expression. Our results reveal that c-fos, fosB, c-jun, and junB levels were upregulated at 24 hr. Furthermore, estradiol selectively affected the expression of c-Fos mRNA and protein by attenuating the injury-induced increase in a time- and region-specific manner. Our findings strongly suggest that the ability of estradiol to protect against MCAO-induced cell death involves attenuation of c-Fos induction.

Key words: estradiol; immediate early gene; middle cerebral artery occlusion; stroke; apoptosis; cell death

Introduction

Stroke is the third leading cause of death in the United States (American Heart Association, 2001). The age-related increase in stroke risk is compounded in women. In the 45–54 year age group, stroke prevalence in women is half that in men; however, in the 65–74 year group, women and men exhibit almost equal stroke prevalence (American Heart Association, 2001). In the last century, the average life expectancy has increased from 47 to 77 years (Futterman and Lemberg, 2000), but the average age of women at the menopause has remained fixed at 51 years (Brambilla and McKinlay, 1989; Luoto et al., 1994; Bromberger et al., 1997). Therefore, a larger proportion of women are living an increasing number of years in a postmenopausal, hypoestrogenic state.

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). 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); 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). 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, various 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; Rusu et al., 1999; Sawada and Shimohama, 2000; Dubal and Wise, 2001; Kim et al., 2001; Mendelovitsch et al., 2001; Jover et al., 2002).

Our laboratory has shown that pretreatment with low physiological levels of estradiol protects against permanent middle cerebral artery occlusion (MCAO) (Dubal et al., 1998). Protection is specific to the late phase of injury and to the cortex, which is known to exhibit programmed cell death (PCD) (Chopp and Li, 1996; Li et al., 1998; Namura et al., 1998; Guegan and Sola, 2000; Sharp et al., 2000). The protective effects of estradiol have been associated with alterations in the expression of multiple genes (Dubal et al., 1999). Immediate early genes (IEGs) are elevated in ischemic injury (Kinouchi et al., 1994a) and have been...
shown to be involved in PCD cascades (Estus et al., 1994; Ferrer et al., 2000; Sharp et al., 2000).

The purpose of this study was to test the hypothesis that estradiol may attenuate ischemic injury, in part, by blocking increases in IEG expression in ischemic penumbra. We examined the effects of estradiol pretreatment and MCAO on IEG expression in the cerebral cortex of ovariectomized female rats that were treated with estradiol or vehicle. Our results show that estradiol selectively attenuates the injury-mediated increase in c-Fos expression.

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. Rats were bilaterally ovariectomized under methoxyflurane anesthesia to eliminate endogenous estradiol production and then implanted with a SILASTIC capsule 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 in the estrous cycle of rats (Smith et al., 1975). After 7 d, rats underwent MCAO or sham surgery. Rats were anesthetized with ketamine/acepromazine (80.0/0.52 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 previously described methods (Dubal et al., 1999). Briefly, a 4/0 monofilament suture was inserted through the internal carotid artery to the base of the middle cerebral artery. Sham surgical animals underwent the entire procedure except that the artery was not occluded.

Two separate groups of animals were collected by the above methods. These groups and the experiments performed with them will be referred to as experiments 1 and 2. Animals from experiment 1 were used for initial RT-PCR studies as well as for the in situ hybridization and immunohistochemistry studies. For experiment 1, brains were collected at 1, 4, 8, 16, or 24 hr after the onset of ischemia (n = 6–10 per experimental group). Animals from experiment 2 were used for the final set of RT-PCR studies. Only 24 hr brains were collected for experiment 2 (n = 6 per experimental group), and no sham animals were collected.

Reverse transcriptase-PCR studies

Microdissection

In the case of experiment 1, brains were fresh frozen on dry ice and stored at −80°C until coronal sections of these brains could be cut on a cryostat. In animals from the 24 hr group, three 200 μm sections were taken from the “test zone” (bregma +1.2 to bregma −0.8) (Paxinos and Watson, 1997) and stored on slides at −80°C until microdissection could be performed. The same anatomic area (i2, c2; see Fig. 1) was microdissected from each of the 200 μm sections for a given brain and combined as a single sample (e.g., one i2 sample and one c2 sample for each animal). For experiment 2, alternating 1 mm fresh sections of brain were collected using a brain matrix (Activational Systems) and then stained in 2% toluidine blue to visualize injury or frozen on dry ice and stored at −80°C for inclusion in RT-PCR studies. The area of the cortex analyzed for gene expression was selected using the following criteria. We first examined tissue from a 1 mm TTC-stained coronal section corresponding to the test zone. The adjacent frozen 1 mm section was then microdissected for use in gene expression studies. For all samples, anatomically similar regions were dissected, with care being taken to avoid collecting infarcted tissue from any sample (see Fig. 1). For experiment 1, only areas i2 and c2 were used. For experiment 2, all areas (i1, i2, c1, and c2) were microdissected and used.

cDNA preparation

Total RNA was isolated from microdissected samples by the method of Chomczynski and Sacchi (1987). Total RNA (0.5 μg) from each sample was reverse transcribed to produce cDNA using a protocol described previously (Dubal et al., 1999). The same procedure was performed on samples using a reaction solution without reverse transcriptase (RT) to check for genomic contamination.

PCR amplification

We used RT-PCR methods to determine relative changes in gene expression at the mRNA level as described previously (Dubal et al., 1999). Briefly, for each gene examined, we generated standard curves of cycle number to determine the optimum cycle number within the linear range for PCR amplification. This was determined to be between 21 and 28 cycles for all genes examined.

**Experiment 1.** For each gene, stock solutions were prepared containing 1.5 μCi MgCl2, 1× reaction buffer, 10 μCi of [32P]dCTP (3000 Ci/mmol) (NEN, Boston, MA), 0.5 μs dNTP mix, 1 μM each primer, and 1.5 U of Tag polymerase (Invitrogen). The stock solution was aliquoted (49 μl per tube), and 1/50 of cDNA (from reverse transcription reaction) was added to each sample tube. Samples were then thermocycled for PCR amplification (Touchdown thermocycler; Hybaid, Middlesex, UK) according to reaction conditions optimized for each separate gene. PCR products were then resolved by PAGE. The gels were dried, and products were visualized using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Experiment 2.** The RT-PCR procedures were all identical to experiment 1 with the following exceptions. (1) No radioactive dCTP, (2) −0.1 μl dNTP mix, (3) stock solution was aliquoted as 14 μl per tube, and (4) after products were resolved by PAGE, gels were stained in a solution of SYBR gold nucleic acid gel stain (Molecular Probes, Eugene, OR), and products were visualized using a PhosphorImager.

All of the oligonucleotide sequence pairs used for gene amplification generated PCR products of expected sizes. Primers used for both experiments are as follows: L-27A sense primer, 5′-ATCGTAAAGCCGGCAAGC-3′, and antisense primer, 5′-GGAGCACTCTTCCATTGTT-3′ (214 bp) (Hoshimaru et al., 1996); c-fos sense primer, 5′-ATCTAACGGAGAGAAGC-3′, and antisense primer, 5′-CTGGAATTCCGACCAATTGTT-3′ (161 bp) (Estus et al., 1997); fosB sense primer, 5′-GAGATCCGGCGAGCTGCTACAAA-3′, and antisense primer 5′-TGGGCAATTCCGTCGTCGCA-3′ (58 bp) (Estus et al., 1997); junB sense primer, 5′-GGGAATTCACCCCAACTTGCTCAA-3′, and antisense primer 5′-GGAATTCACCACTTGACCCCG-3′ (69 bp) (Estus et al., 1997); c-jun sense primer, 5′-ACTCAAATTTCTTGTGCCACCCAAA-3′, and antisense primer 5′-GAGGAAATCCAGCAACTTGCCGAAGA-3′ (64 bp) (Estus et al., 1997); junD sense primer, 5′-GGGAATTCACCCCAACTTGCTCAA-3′, and antisense primer 5′-GGAATTCACCCCAACTTGACCCCG-3′ (128 bp) (Estus et al., 1997).

**In situ hybridization**

**Tissue preparation**

In addition to the 200 μm sections taken from the test zone in experiment 1 animals, 18 μm coronal sections were also taken for use in both the in situ hybridization and immunohistochemistry studies that follow. The sections were mounted on slides and stored at −80°C until they were processed for in situ hybridization or immunohistochemistry.

**Probe preparation**

The c-fos probe was generated using a template plasmid containing a 1352 bp EcoRI–XhoI fragment described previously (Curran et al., 1987) (generously provided by Dr. Robert Steiner, University of Washington School of Medicine, Seattle, WA). A riboprobe was transcribed in the presence of 13.3 μM α-thio[35S]labeled UTP.

**Hybridization**

Coronal sections (18 μm) from each animal (two sections per slide, one slide per animal) were processed simultaneously in a single-label in situ hybridization assay according to the method of Wise et al. (1992), with modifications. Briefly, sections were fixed in phosphate-buffered 4% paraformaldehyde, treated with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, and dehydrated. Hybridization buffer (50 μl) containing 600 ng/ml labeled c-fos cRNA was applied to each slide. Slides were coverslipped and incubated in humid chambers at 45°C for 18 hr. Sections were treated with RNase A (25 μg/ml), washed under conditions of increasing stringency, including a 1 hr wash at 60°C in 0.2× SSC, dehy-
drated in ethanol containing 300 mM ammonium acetate, and air dried. Slides were then apposed to film for 29 hr.

Slides from the test zone were used in this experiment. Three spatially juxtaposed circular areas of frontal and parietal cortices from one section per animal were analyzed on the films using pixel count and area arrays via BIOQUANT software (see Fig. 5). Densitometric values from the three regions were combined to yield a single value per animal. The three regions extended from the central sulcus laterally to the region of a typical infract border from a 24 hr oil-treated animal. This allowed for identical areas of analysis in oil- and estradiol-treated rats across time points without the risk of analyzing dead, infarcted tissue.

**Immunocytochemistry**

The entire immunohistochemical procedure was performed on slide-mounted sections, with one slide (two sections per slide) being used per animal. Sections were fixed in phosphate-buffered 4% paraformaldehyde. On day 1, sections were washed with 0.05 M Tris, pH 7.4. This was followed by a 1 hr blocking step at room temperature (Tris, 1% Triton X-100, 10% heat inactivated horse serum) and then incubation in blocking buffer containing a polyclonal antibody directed against a peptide mapping to the amino terminus of c-Fos p62 of human origin (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA; c-Fos(4), sc-52) overnight at 4°C. Sections were washed again in Tris and then incubated for 1 hr in blocking buffer containing biotin-conjugated secondary anti-rabbit anti-IgG (1:1000; Jackson ImmunoResearch, West Grove, PA). After another wash step the sections were incubated for 1 hr in avidin–biotin complex (ABC) solution for 1 hr (Vectastain Kit, Vector Laboratories, Burlingame, CA). Antibody complexes were visualized with nickel-enhanced DAB (3,3’-diaminobenzidine). In control experiments, immunohistochemical reactions lacking either c-Fos primary antibody or the secondary antibody were performed and showed no immunoreactive cells. Additionally, immunohistochemical reactions using c-Fos primary antibody preabsorbed with 10-fold excess c-Fos blocking peptide (Santa Cruz Biotechnology; sc-52P) showed no immunoreactivity demonstrating the specificity of the antibody. c-Fos immunoreactive cells were counted within ipsilateral and contralateral frontalparietal cortex from one section per animal using an object counts array via BIOQUANT software in seven separate 20× scope fields. 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 in 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. In 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, in one 20× field lateral to the previous two fields. Using this pattern of movement from field to field in each section, equal sampling was ensured from animal to animal. The counts from all seven fields were combined to obtain a single cell number for each coronal section.

**Data analysis**

Data were analyzed by two- or three-way ANOVA, depending on the experiment. For time course experiments (in situ hybridization and immunohistochemistry), data were separated into an early-phase injury (1–8 hr) and a late-phase injury (16–24 hr). Significant interactions were probed using Newman–Keuls tests. All data are expressed as mean ± SEM. Differences were considered statistically significant when \( p < 0.05 \).

**Results**

**Estradiol specifically modulates injury-induced c-fos mRNA levels**

Figure 1 shows representative TTC-stained brain sections from an ovariecetomized oil- and estradiol-treated rat killed at 24 hr after MCAO. The outlined regions correspond to tissue that was microdissected from adjacent sections for RNA analysis by semi-quantitative RT-PCR. Tissue of animals from two separate experiments was used in RT-PCR studies. In experiment 1, mRNA levels within the i2 and c2 regions were quantified, whereas in experiment 2, mRNA levels were measured in i1, i2, c1, and c2 regions.

Baseline gene expression among the experimental groups was determined relative to the marker, ribosomal L-27A. L-27A gene expression did not change with injury or estradiol treatment in experiment 1 (Table 1) or experiment 2 (data not shown), indicating that the amount of live tissue was represented equally among treatment groups (ischemic and sham, oil and estradiol-treated animals). Values for the immediate early genes from experiment 1 (Table 1) show that estradiol or ischemia, or both, selectively alter gene expression after the induction of injury. None of the genes examined showed any estradiol-induced alterations in sham animals.

**Experiment 1**

Table 1 lists data for all IEGs examined in experiment 1. ANOVA analysis shows a significant effect of hemisphere for all IEGs \( (\ast p < 0.05) \) with the exception of junD. Levels of mRNA of c-fos, fosB, fosB, and c-jun increased significantly on the ipsilateral side compared with the contralateral side of injured animals. Estradiol significantly attenuated the injury-mediated increase in c-fos mRNA at 24 hr. ANOVA analysis of c-fos mRNA in injured animals showed a significant effect of hemisphere \( (\ast p < 0.05) \) and a significant effect of treatment \( (p < 0.05) \) with no interaction. Similar trends were observed in fosB and junB mRNA levels, but they did not reach statistical significance (Table 1). Because these trends were intriguing, we performed experiment 2, adding another microdissected area in each hemisphere (i1, c1).

**Experiment 2**

Figure 2 comprises a composite of PCR results illustrating representative expression of L-27A and IEGs from experiment 2. Figures 3 and 4 show c-fos, fosB, and junB levels on the ipsilateral and contralateral sides in oil- and estradiol-treated rats that have undergone MCAO. MCAO results in an upregulation of c-fos, fosB, and junB mRNA levels as illustrated by significant increases in ipsilateral compared with contralateral cortical regions \( (p < 0.05) \). Estradiol specifically attenuated c-fos mRNA expression in the i1 and c1 cortical regions at 24 hr after the onset of ischemia \( (\ast p < 0.05) \) (Fig. 3). In contrast, estradiol replacement did not affect injury-induced or baseline fosB or junB expression (Fig. 4).
Table 1. Experiment 1: gene expression of cellular marker L-27A and IEG family members

<table>
<thead>
<tr>
<th></th>
<th>Oil Ipsi</th>
<th>Estradiol Ipsi</th>
<th>Oil Contra</th>
<th>Estradiol Contra</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-27A</td>
<td>1.28 ± 0.13</td>
<td>1.31 ± 0.10</td>
<td>1.29 ± 0.03</td>
<td>1.36 ± 0.05</td>
</tr>
<tr>
<td>c-fos</td>
<td>2.46 ± 0.22*</td>
<td>1.85 ± 0.28**</td>
<td>1.16 ± 0.18</td>
<td>0.98 ± 0.14**</td>
</tr>
<tr>
<td>fosB</td>
<td>2.60 ± 0.25*</td>
<td>1.89 ± 0.33*</td>
<td>0.91 ± 0.11</td>
<td>0.70 ± 0.06</td>
</tr>
<tr>
<td>junB</td>
<td>1.50 ± 0.14*</td>
<td>1.19 ± 0.12*</td>
<td>0.96 ± 0.10</td>
<td>0.94 ± 0.09</td>
</tr>
<tr>
<td>c-jun</td>
<td>1.53 ± 0.20*</td>
<td>1.51 ± 0.17*</td>
<td>0.98 ± 0.06</td>
<td>1.04 ± 0.06</td>
</tr>
<tr>
<td>junD</td>
<td>1.19 ± 0.17</td>
<td>1.08 ± 0.05</td>
<td>1.23 ± 0.10</td>
<td>1.04 ± 0.08</td>
</tr>
</tbody>
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Tissue samples were taken from oil- and estradiol-treated MCAO and sham animals at 24 hr after surgery. Ipsi and Contra refer to areas i2 and c2, respectively (Fig. 1). Baseline levels of L-27A were not affected by ischemia or by estradiol treatment, indicating that the overall amount of live tissue is equally represented among experimental groups. Gene expression of IEG family members in sham and injured rat cortex were normalized to L-27A values. The expression of specific genes increased in ipsilateral ischemic cortex (*p < 0.05). Estradiol attenuated the injury-mediated increase in c-fos mRNA levels (**p < 0.05). ANOVA demonstrates effect of treatment and of hemisphere with no interaction. Estradiol did not alter expression of any genes in sham animals.

Estradiol attenuates injury-induced c-fos mRNA in cortex during the late phase of ischemic injury

To further explore the estradiol-induced modulation of the temporospatial pattern of c-fos gene expression after injury, in situ hybridization was performed on coronal sections of the same animals with a radiolabeled probe to c-fos. In all three areas (Fig. 5) of ischemic cortex that were examined in oil- and estradiol-treated animals, c-fos mRNA levels exhibited a dramatic rise between 1 and 4 hr after ischemic onset. It should be noted here that c-fos expression is absent in the striatum at all time points examined (data not shown). In estradiol-treated animals, the late phase (16–24 hr) injury-mediated increase in c-fos mRNA levels is attenuated in comparison their oil-treated counterparts (Fig. 6) (*p < 0.05).
MCAO results in an increase in all IEGs that we examined, except for one. Our studies demonstrate three important findings. First, by 24 hr, estradiol-treated animals show a peak expression of c-Fos immunoreactive brain sections. In oil- and estradiol-treated animals that underwent 24 hr of permanent MCAO hybridized with radioactive c-fos riboprobe. Areas 1, 2, and 3 demarcate regions from which densitometric measurements were taken using pixel count and area arrays via BIOQUANT software.

The effects of MCAO and estradiol on c-fos mRNA are translated into parallel changes in the c-Fos protein expression pattern

Figure 7 shows representative microscopic fields of c-Fos-immunoreactive brain sections. In oil- and estradiol-treated animals, few c-Fos-positive cells were detected at 1 hr after ischemic onset. In contrast, the number of c-Fos-immunoreactive cells rose dramatically during the early phase of injury in both oil- and estradiol-treated animals. This rise continued in oil-treated animals to an even higher level at 16 hr, where it reached a plateau. In estradiol-treated animals, the number of c-Fos-immunoreactive cells reached a plateau at 8 hr and was significantly lower than oil-treated animals during the late phase of injury (16 and 24 hr) (Fig. 8) (* p < 0.05). c-Fos expression by immunohistochemistry is absent from the ischemic striatum (data not shown).

Discussion

Our studies demonstrate three important findings. First, by 24 hr, MCAO results in an increase in all IEGs that we examined, except for one. Second, c-Fos mRNA and protein increase within regions of the cerebral cortex in a time- and region-specific manner. Finally, estradiol selectively modulates the injury-induced pattern of c-Fos expression, whereas it shows no significant effect on the other IEGs.

IEG expression is thought to be a crucial part of the programmed cell death pathway since Estus and colleagues (1994) found that induction of cJun and the Fos family were necessary to induce apoptosis in a paradigm of NGF-withdrawal-induced delayed cell death. Administration of antibodies against these IEGs on cultured dorsal root ganglion cells prevented the observed cell death. In this model, increased JunB or JunD expression was not a functionally critical step in the induction of programmed cell death. The same group has also explored IEG involvement in programmed cell death in primary cortical neuronal cultures. Injury of these cultures via amyloid β (Aβ) treatment leads to a robust, time-dependent induction of IEGs. c-fos, specifically, was shown to be expressed in a subset of neurons that exhibited condensed chromatin, a hallmark of apoptotic or programmed cell death (Estus et al., 1997). On the basis of these findings, the goal of the present studies was to assess whether estradiol replacement therapy provides protection against MCAO-induced programmed cell death by attenuating the expression of one or more IEGs.

Our results confirm and extend the findings of previous investigators who studied gene expression after focal cerebral ischemic injury and its modulation by neuroprotective factors. Studies have demonstrated increased c-Fos gene expression after focal ischemia in intact male and female animals (Uemura et al., 1991; Kinouchi et al., 1994a; Dietrich et al., 2000). Kinouchi et al. (1994) reported that decreased glutamatergic toxicity, afforded by treatment with MK-801, significantly attenuated MCAO-induced c-fos and junB mRNA levels at 4 hr after onset of ischemia. This effect was most pronounced in the cingulate cortex (Kinouchi et al., 1994b). Uemura et al. (1991) examined an injury-mediated increase in c-Fos immunoreactivity after focal ischemia and demonstrated a similar attenuation by treatment with MK-801 (Dietrich et al., 2000). Both Uemura and Kinouchi and their colleagues (Uemura et al., 1991; Kinouchi et al., 1994a) showed a lack of c-Fos expression in the striatum after MCAO, a finding mirrored by the current studies.

The present study combines multiple methods to probe changes in mRNA as well as protein levels across a number of time points. The results clearly reveal that c-fos, fosB, c-jun, and junB are also upregulated in our model of permanent middle cerebral artery occlusion and that c-Fos expression is selectively attenuated by a different neuroprotective factor, estradiol, known to protect against programmed cell death (Jover et al., 2002; Linford and Dorsa, 2002; Monroe et al., 2002; Wilson et al., 2002; Rau et al., 2003). The predominance of the protective effects of estradiol in the cingulate cortex (areas i1, c1), as opposed to the areas of the injured brain that are closer to the infarct (areas i2, c2) (Fig. 3), is reminiscent of the spatial distribution of the protective effects of MK-801 observed by Kinouchi and colleagues (Kinouchi et al., 1994b).

The point on which our results differ from several previous studies is the temporal pattern of c-Fos induction in relation to ischemic injury. Two separate studies using photochemical methods to elicit ischemic injury explored c-fos mRNA expression patterns by in situ hybridization. These studies illustrate peaks in c-fos expression from 2 to 4 hr with a return to baseline expression by 24 hr after the initiation of ischemic injury (Dietrich et al., 2000; Johansson et al., 2000). Another study demon-
strates a peak in c-fos expression as early as 0.5 hr, with return toward baseline by 4 hr after initiation of reperfusion after 2 hr of ischemic injury (Kinoshita et al., 2001). After ischemia induced by a two-vessel occlusion technique, Uemura and colleagues (Uemura et al., 1991) also demonstrated peak c-fos expression at 2–4 hr after initiation of ischemia, with a reduction in expression by 8 hr and return to baseline by 2 d. Finally, the study most similar to the work described in this manuscript was published by Kinouchi and colleagues (Kinouchi et al., 1994a). These authors looked at c-fos expression after permanent MCAO via an intraluminal suture technique. The authors demonstrated a peak in c-fos expression at 4 hr after artery occlusion, with a return toward baseline expression at 24 hr after artery occlusion (Kinouchi et al., 1994a). These previous studies used varying techniques for induction of ischemia, and all demonstrate an earlier peak in c-fos gene expression than our results. Additionally, our results contrast with these previous studies by showing sustained expression of c-Fos mRNA and protein to the endpoint of 24 hr. The common difference is that the current study uses ovariectomized female rats, whereas each previous study used intact male rats. This may suggest that sex or gonadal status influences the timing and duration of IEG expression after injury.

Despite the difference in c-fos expression patterns, estradiol is able to reduce ischemic infarct size in both ovariectomized female and intact male rats. After transient cerebral ischemia, females sustained >50% less infarction than gonadally intact males and ovariectomized females (Alkayed et al., 1998). Our previous studies have shown profound neuroprotective effects after permanent cerebral ischemia in ovariectomized female rats with estradiol pretreatment (Dubal et al., 1998). Intact and castrate males have also been shown to be protected from ischemic injury by estradiol. Toung and colleagues (Toung et al., 1998) demonstrated neuroprotection in intact and castrate male rats by chronic implantation with estradiol pellets in a model of cerebral ischemia with reperfusion injury. The effects of estradiol in this model were independent of relative changes in cortical perfusion as measured by laser Doppler flowmetry (Toung et al., 1998). A separate group, using a model of permanent MCAO by electrocautery, demonstrated neuroprotection by an intravenous bolus of estradiol. Infarct volume was significantly reduced by estradiol given 30 min before or at the time of MCAO. The estrogen receptor antagonist, ICI-182,780, abolished the neuroprotective actions of estradiol in these studies (Saleh et al., 2001).

Therefore, 17β-estradiol can protect both the male and female brain from damage by ischemic insult, yet there appears to be a different reaction to ischemic injury in the ovariectomized female and the intact male brain as evidenced by differing patterns of c-fos expression. Further study is required to fully explain these intriguing observations.

Cortical c-Fos expression after ischemic injury is known to be linked to an electrical phenomenon known as cortical spreading depression, or alternatively, cortical spreading depolarization (CSD). CSDs contribute to the early phase of expansion of ischemic infarction beyond the ischemic core, as shown by positive correlation between their number and infarct size and also by reductions in their number and amplitude by MK-801 (Gill et al., 1992; Iijima et al., 1992; Dietrich et al., 2000; for review, see Sharp et al., 2000). A significant body of literature has demonstrated a causal link between posts ischemic IEG expression and CSD (for review, see Kiessling and Gass, 1994). Given the effects of estradiol on the temporospatial pattern of c-Fos expression in conjunction with its previously observed regionally specific pattern of protection, we speculate that estradiol may be attenuating the occurrence of CSDs in ischemic cortex.

The effects of estradiol on injury-induced c-Fos shed light on the mechanism of estradiol-induced protection, the stage of injury that is protected by estradiol, and the role of IEGs. Specifically, several studies have shown that c-Fos is induced in the cingulate cortex after focal ischemia in intact animals (Herrera and Robertson, 1989; Uemura et al., 1991; Kinouchi et al., 1994a;
Dietrich et al., 2000). These studies typically show a peak in c-Fos mRNA and protein expression between 1 and 4 hr after the initiation of injury, with a subsequent diminution of expression by 24 hr. Our studies clearly show that in the absence of the gonads, c-Fos expression continues to increase over the 24 hr period after MCAO. Pretreatment with estradiol, the major biologically active ovarian steroid, at levels that mimic the intact state attenuates the further increase that continues during the later time points in ovariecotomized rats.

In summary, our results clearly show that the protective effects of estradiol correlate with attenuation of c-Fos gene and protein expression. These findings suggest that the neuroprotective mechanism of estradiol involves a crucial pathway in the early stages of the recruitment of ischemic penumbra into the final infarction. Finally, these studies constitute a significant expansion of our understanding of the protective mechanisms of estradiol in the brain and add to the growing literature exploring potential roles for this powerful hormone.

References


