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Role of Signal Transducer and Activator of Transcription-3 in Estradiol-Mediated Neuroprotection

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Estradiol is protective in experimental cerebral ischemia, but the precise mechanisms remain unknown. Signal transducer and activator of transcription-3 (STAT3) is a transcription factor that is activated by estrogen, translocates to the nucleus, and induces the transcription of neuroprotective genes, such as *bcl-2*. We determined whether estradiol increases STAT3 activation in female rat brain after focal cerebral ischemia and whether STAT3 activation contributes to estradiol-mediated neuroprotection against ischemic brain injury. Ovariectomized (OVX) female rats with and without estradiol replacement were subjected to 2 h of middle cerebral artery occlusion (MCAO), and phosphorylated STAT3 (P-STAT3) and total STAT3 (T-STAT3) were quantified by Western blot analysis at 3 and 22 h of reperfusion. STAT3 activation was colocalized with neuronal and survival markers microtubule-associated protein 2 (MAP2) and Bcl-2 using immunohistochemistry. Infarct size was measured at 22 h after MCAO in estradiol-treated OVX animals in the presence and absence of STAT3 inhibitor cucurbitacin I (JSI-124) using 2,3,5-triphenyltetrazolium chloride staining. Estradiol increased P-STAT3 in the ischemic cortex cytosolic fraction at 3 h after MCAO without affecting T-STAT3. This was associated with increased P-STAT3 in the nuclear fraction, which remained elevated at 22 h after MCAO. The nuclear P-STAT3 colocalized with MAP2 and Bcl-2 within the peri-infarct zone. The P-STAT3 inhibitor JSI-124 abolished the protective effect of estradiol without affecting infarct size in untreated OVX rats. We conclude that estradiol increases STAT3 phosphorylation in neurons after MCAO and that STAT3 activation plays an important role in estradiol-mediated neuroprotection.

Key words: estrogen; STAT3; ischemia; neuroprotection; MCAO; JSI-124 (cucurbitacin I)

Introduction

We previously demonstrated that female rats sustain smaller infarcts after middle cerebral artery occlusion (MCAO) than male rats (Alkayed et al., 1998) and that chronic estrogen replacement that restores physiological levels of plasma estradiol is protective against ischemic brain injury in ovariectomized (OVX) (Rusa et al., 1999) and reproductively senescent (Alkayed et al., 2000) female rats. Estradiol exhibits acute (Bryant et al., 2005; Qiu et al., 2006) and chronic (O'Lone et al., 2004) effects. The chronic effects of estradiol are mediated via alterations in gene expression, the so-called "genomic" effects of estradiol (Xu et al., 2006). Acute, rapid effects of estradiol are believed to be mediated via so-called "nongenomic" mechanisms, such as changes in protein phosphorylation (Levin, 2002). However, the acute and chronic effects of estrogen are not mutually exclusive. Recent studies suggest that estrogen rapidly activates signal transduction pathways that ultimately lead to increased gene transcription (Bjornstrom

and Sjoberg, 2005). Specifically, in non-neural cell lines, estrogen induces the phosphorylation of signal transducer and activator of transcription-3 (STAT3) (Bjornstrom and Sjoberg, 2002). STAT proteins reside in the cytoplasm in an unphosphorylated dormant form. In response to a wide variety of external stimuli, including cytokines, hormones and growth factors, STATs are activated by tyrosine phosphorylation (Levy and Darnell, 2002). Phosphorylated STATs then dimerize and translocate to the nucleus where they bind specific DNA regions, referred to as STAT-inducible elements (SIEs), to induce gene transcription. There are six known STATs: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 (Levy and Darnell, 2002). Only STAT3 has been linked to estradiol's action, neuronal survival, and tissue protection from ischemia. Specifically, STAT3 phosphorylation is increased by estradiol in an aortic endothelial cell line (Bjornstrom and Sjoberg, 2002) and mediates survival effects of neurotrophins in motor (Dolcet et al., 2001) and sensory (Alonzi et al., 2001) neurons. In heart, STAT3 promotes tissue survival after myocardial infarction (Negoro et al., 2000) and plays an important role in tolerance to ischemia acquired by ischemic preconditioning (Hattori et al., 2001; Xuan et al., 2001). Finally, STAT3 induces the expression of neuroprotective genes, such as *bcl-2* (Linnik et al., 1995; Jia et al., 1996) and *bcl-x$_L$* (Parsadanian et al., 1998) (Grad et al., 2000; Stephanou et al., 2000). It is not known, however, whether estradiol enhances STAT3 phosphorylation in

brain regions affected by stroke or whether STAT3 activation contributes to estradiol's neuroprotective action in stroke.

Therefore, in the present study we tested the hypothesis that 17β-estradiol protects the brain against ischemic injury in part through STAT3 activation. We used Western blot with anti-STAT3 and anti-phospho-STAT3 antibodies to measure STAT3 expression and activation by estradiol in brain after MCAO. Furthermore, we used immunohistochemistry to determine the cell identity of phospho-STAT3-expressing cells. Finally, the STAT3 pharmacological inhibitor cucurbitacin I [JSI-124 (JSI)] (Blaskovich et al., 2003) was used to determine the role of STAT3 activation in infarct size reduction by estradiol. This is the first study to link the neuroprotective effect of estradiol against cerebral ischemia to STAT3.

Materials and Methods

This study was conducted in accordance with the National Institutes of Health guidelines for care and use of animals in research, and the protocols were approved by the Animal Care and Use Committee of Oregon Health & Science University.

Ovariectomy and estrogen replacement. Ovariectomy was performed in rats 1–2 weeks before MCAO, and estrogen was replaced via 21-d-release pellets containing 25 μg of 17β-estradiol (Innovative Research of America, Toledo, OH) implanted subcutaneously on the day of ovariectomy. Plasma 17β-estradiol was measured by radioimmunoassay when the rats were killed, as described previously (Rusa et al., 1999; Alkayed et al., 2000).

Experimental stroke in rat. Focal cerebral ischemia was induced in rats using the intraluminal MCAO model, as described previously (Alkayed et al., 1998; Rusa et al., 1999). Briefly, ovariectomized female rats (10–12 weeks of age, 200–250 g body weight) with and without estradiol replacement were anesthetized with halothane (1% in O₂-enriched air via face mask) and instrumented with a femoral catheter for monitoring arterial blood pressure and gases. Brain tissue perfusion was monitored over the ipsilateral parietal cortex using a laser-Doppler probe (Moor Instruments, Oxford, UK) placed 6 mm lateral and 2 mm posterior to bregma to assess vascular occlusion and reperfusion. Head and body temperatures were controlled and monitored through a temporalis muscle and rectal thermoprobes. Vascular occlusion was achieved by advancing a 4–0 nylon filament with heat-rounded tip into the internal carotid artery until a drop was observed in laser-Doppler perfusion (LDP). LDP was continuously monitored during the occlusion period and over 30 min after reperfusion. Only animals with a >50% drop in LDP relative to preocclusion baseline were used in this study. Vascular occlusion was maintained for 2 h, and then the occluding filament was withdrawn to permit reperfusion, and the rat was recovered for either 3 or 22 h. Each animal was deeply anesthetized at the appropriate time point with halothane, blood samples were obtained by cardiac puncture for measurement of plasma 17β-estradiol, and the brains were collected and processed for subcellular fractionation, immunohistochemistry, or determination of infarct volume. To quantify infarct, the brain was harvested at 22 h of reperfusion, 2-mm-thick coronal brain sections (seven sections per brain) were stained with 2,3,5-triphenyltetrazolium chloride and fixed in 10% formalin, and infarcted areas were measured in digital images of brain slices using MCID image analysis software (InterFocus Imaging, Linton, UK). Infarct volume was calculated by integrating infarcted areas across the rostral–caudal axis and expressed as a percentage of the contralateral hemisphere to account for edema.

The STAT3 inhibitor JSI-124 was initially given intraperitoneally at a dose of 1 mg/kg at 15 min before MCAO. However, we observed that JSI induces slight fluctuations in arterial blood pressure during MCAO. To rule out an effect of these fluctuations on ischemic severity and infarct size, we also administered JSI at 15 min after MCAO.

Subcellular fractionation. Cortical tissue from the area shaded in Figure 3 (peri-ischemia; between +2 and −3 mm from bregma) was dissected at 3 and 22 h of reperfusion and flash frozen in 2-methyl butane over dry ice. Tissue was stored at −80°C until use. Nuclear and cytosolic fractions

were prepared as described previously (Sonnenberg et al., 1989; Takeuchi et al., 1993) with the following modifications. Cortical tissue (80–100 mg) was homogenized at low speed in 400–500 μl of ice-cold hypotonic lysis buffer containing Roche (Mannheim, Germany) complete Mini EDTA-free protease inhibitor, phosphatase inhibitor cocktails I and 2 (Sigma, St. Louis, MO), 1 mM PMSF, and 1 mM DTT. Nuclear fractions were dialyzed using Slide-A-Lyzer Mini dialysis units (Pierce, Rockford, IL). Protein concentrations were measured using the Pierce BCA kit. The purity of each fraction was evaluated by probing cytosolic and nuclear fractions with antibodies against the cytosolic protein AKT and the nuclear protein Histone-3. Both antibodies were obtained from Cell Signaling Technology (Danvers, MA).

Western blot. Phosphorylated (Tyr 705; s.c.-7993-R; P-STAT3) and total (s.c. H-190; T-STAT3) STAT3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). As a positive control for STAT3 activation, we used interferon-α-treated HeLa cell extracts (Cell Signaling Technology). Protein samples (50 μg) from nuclear or cytosolic fractions were boiled for 5 min in 1× sample buffer (Invitrogen, Carlsbad, CA) before loading onto a Criterion 4–15% Tris-HCl gel (Bio-Rad, Hercules, CA). Gels were transferred using the Criterion transfer system at 100 V for 45 min onto nitrocellulose membranes (Bio-Rad). Membranes were blocked in PBST (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4, and 0.1% Tween 20) with either 5% bovine serum albumin (BSA; P-STAT3) or 5% milk (T-STAT3) for 1 h. Membranes were incubated with the appropriate blocking solution containing the antibody at 1:1000 dilution overnight at 4°C. After washing, membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) diluted 1:5000 in corresponding blocking buffer. P-STAT3 blots were stripped for 1 h at room temperature in 62.5 mM Tris, pH 6.8, 2% SDS, and 0.7% (v/v) β-mercaptoethanol, followed by extensive washing in PBST before blocking and incubation with anti-STAT3 antibody. Immunoreactive bands were visualized using Super Signal Dura (Pierce) chemiluminescence. Band intensity was recorded and analyzed using the Kodak (Rochester, NY) Image Station and software (2000R). Gels were stained with Coomassie blue, photographed with a CoolSNAPcf digital camera (Roper Scientific, Tucson, AZ), and analyzed with MCID image analysis software (InterFocus Imaging). The P-STAT3 and T-STAT3 band densities were normalized to total protein load obtained from the Coomassie-stained gel.

Immunohistochemistry. Brains were harvested at 22 h, cut into 2 mm coronal sections, fixed with 4% paraformaldehyde for 4–5 h with gentle shaking at 4°C, and cryopreserved in 20% sucrose overnight at 4°C. Tissue blocks from ischemic (n = 4) or sham-operated (n = 2) animals were frozen in 2-methylbutane at −55°C, sectioned on a cryostat at 20 μm, and thaw mounted on Superfrost Plus glass slides (Fisher Scientific, Pittsburgh, PA). The sections were stored at −20°C until used. Sections were incubated at 4°C overnight with the following primary antisera: T-STAT3 (rabbit polyclonal s.c.-482; 1:2000; Santa Cruz Biotechnology), P-STAT3 (rabbit polyclonal 9131 Tyr 705; 1:1000–1:3000; Cell Signaling Technology), Bcl-2 (monoclonal M 0887; 1:100; Dako North America, Carpinteria, CA), microtubule-associated protein 2 (MAP2; chicken polyclonal CPCA-MAP2; 1:10,000; Encor Biotechnology, Alachua, FL) and Cy3-conjugated glial fibrillary acidic protein (GFAP) (monoclonal C 9205; 1:10,000–1:20,000; Sigma). Some sections were incubated with rabbit polyclonal antiserum to T-STAT3 or P-STAT3 that had been preadsorbed with the immunogen peptide [s.c.-482p (Santa Cruz Biotechnology) and 1195 (Cell Signaling Technology) for T-STAT3 and P-STAT3, respectively] at 10 μg/ml before application to the tissue section. The following day, sections were washed in PBS (0.1 M phosphate buffer, pH 7.4, and 0.15 M NaCl) for 30 min, pretreated with 5% normal goat serum for 1 h, and then incubated for 2 h at room temperature in biotinylated IgG [donkey anti-rabbit, 1:300; anti-mouse, 1:300 (Jackson ImmunoResearch); or anti-chicken (Vector Laboratories, Burlingame, CA), 5 μg/ml]. After rinsing in PBS, the sections were incubated for 1 h at room temperature in streptavidin-conjugated Cy3 (1:1000), rinsed for 3–6 h in PBS, and protected with a coverslip applied using a glycerol–glycine buffer (2:1; pH 8.6) containing 5% n-propyl gallate to reduce photobleaching (Giloh and Sedat, 1982). Both the primary and second-

Table 1. Physiological variables during middle cerebral artery occlusion

Group (n)	MAP[a] (mmHg)	Arterial blood pH	$PaCO_2$[b] (mmHg)	PaO_2[c] (mmHg)	Rectal Temp. (°C)	Head Temp. (°C)	Blood glucose (g/dl)	LDF[d]
OVX plus vehicle (11)	99 ± 3	7.43 ± 0.01	45 ± 1	132 ± 4	36.9 ± 0.1	37.1 ± 0.1	121 ± 6	37 ± 2
EST plus vehicle (8)	105 ± 3	7.47 ± 0.01	40 ± 2	120 ± 4	37.0 ± 0.1	36.8 ± 0.1	119 ± 7	40 ± 2
OVX plus JSI-124 (8)	93 ± 3	7.45 ± 0.01	45 ± 2	139 ± 3*	36.8 ± 0.1	36.9 ± 0.1	126 ± 8	34 ± 4
EST plus JSI-124 (11)	100 ± 3	7.43 ± 0.01	43 ± 2	129 ± 3	37.0 ± 0.1	37.0 ± 0.1	117 ± 6	30 ± 3

Values are mean ± SEM. EST, Estrogen-replaced; n, number of animals; Temp., temperature. *$p < 0.05$, ANOVA.

[a]Mean arterial blood pressure.

[b]Arterial CO_2 tension.

[c]Arterial O_2 tension.

[d]Laser-Doppler flowmetry.

ary antisera were diluted in Tris-(hydroxymethyl)aminomethane (0.5%; Sigma) in phosphate buffer containing 0.7% seaweed gelatin (Sigma), 0.4% Triton X-100 (Sigma), and 3% BSA (Sigma), adjusted to pH 7.6. For P-STAT3 staining, or colocalization of P-STAT3 with Bcl-2, MAP2, or GFAP, the procedure developed by Munzberg et al. (2003) was used with slight modification. The tissue was pretreated in 1% NaOH and 0.5–1% H_2O_2 in H_2O for 20 min, 0.3% glycine for 10 min, and 0.03% SDS for 10 min. Thereafter, the sections were blocked for 1 h with 5% normal goat serum in PBS/0.25% Triton X-100. The different antisera were diluted in the same blocking solution before being applied to the tissue sections overnight. Slides were analyzed and photographed using a Nikon (Tokyo, Japan) E800 microscope equipped with epifluorescence and a Nikon DS digital camera. Slides were also analyzed using confocal microscopy with a Leica (Bannockburn, IL) TCS SP confocal system using a 40× numerical aperture 1.25 PL APO objective. Individual sections, 0.488 μm apart, were imaged by sequential excitation with the 488 nm line of an Ar gas laser and the 561 nm line of a DPSS laser and projected into one plane.

Statistical analysis. Physiological variables during MCAO and differences in infarct size were analyzed with one-way ANOVA and *post hoc* Newman–Keuls test. Densitometric measurements of P-STAT3 and T-STAT3 Western blots were evaluated separately with Student's *t* test, as were the differences in serum hormone levels. The criterion for statistical significance was $p < 0.05$. All values are reported as mean ± SEM.

Results

Physiological variables during MCAO were maintained within a physiological range (Table 1). At the time that the rats were killed, plasma 17β-estradiol was 70 ± 6 pg/ml in estradiol-replaced compared with 14 ± 1 pg/ml in untreated ovariectomized rats ($n = 18$ per group; $p < 0.001$). Mean laser-Doppler cortical perfusion during MCAO was similar among all experimental groups (Table 1).

Estradiol increases cytosolic STAT3 phosphorylation in cortex after MCAO

We first measured STAT3 phosphorylation in the cytosolic fraction prepared from the cerebral cortex at 3 h after MCAO. STAT3 phosphorylation was barely detectable in the cytosolic fractions from the contralateral side and sham-operated animals (data not shown). However, STAT3 phosphorylation was induced after MCAO in the cerebral cortex cytosolic fraction. Figure 1 also shows that estrogen replacement further enhanced STAT3 phosphorylation in postischemic cytosolic fractions compared with unreplaced OVX animals ($n = 5$ per group; $p < 0.05$). Estradiol did not change T-STAT3 protein at this early time point, suggesting that estradiol enhances the activation of STAT3 but does not alter the amount of STAT3 protein in the cerebral cortex after MCAO. Interestingly, STAT3 phosphorylation was not different between estradiol-treated and untreated groups at 22 h of reperfusion ($n = 5$ per group; data not shown), suggesting that STAT3 activation in the cytosol is transient.
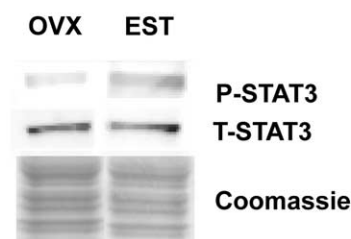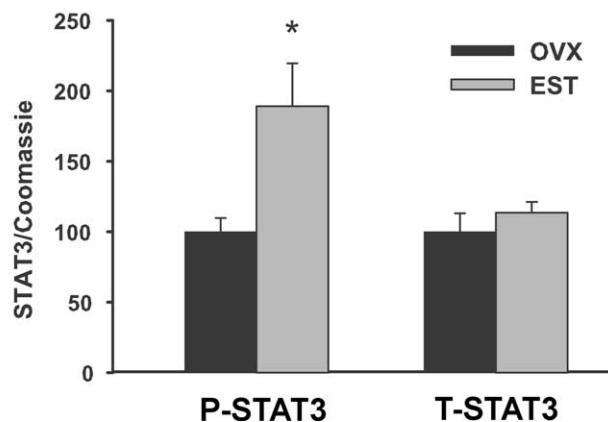
Figure 1. Estradiol replacement increases cytosolic STAT3 phosphorylation but not total STAT3 in the cerebral cortex after MCAO. Cytosolic extracts were prepared from OVX and estradiol-replaced (EST) female rat brains 3 h after MCAO, and Western blot was performed with an antibody against P-STAT3 or T-STAT3. **A**, Representative P-STAT3 and T-STAT3 blots and Coomassie-stained gel showing equal protein loading. **B**, Summary graph depicting the changes in cytosolic STAT3 relative to total protein load. All values were normalized to mean cytosolic P-STAT3 and T-STAT3 in ovariectomized animals, which were set at 100%. *$p < 0.05$ for the effect of EST on cytosolic P-STAT3 ($n = 5$ per group).

Estradiol increases nuclear STAT3 phosphorylation in cortex after MCAO

Because STAT3 is translocated to the nucleus after activation, we then determined whether estradiol increases nuclear STAT3 phosphorylation. As in the cytosolic fractions, very little P-STAT3 was observed in nuclear fractions from the contralateral side. However, in agreement with the effect of estradiol on the cytosolic fractions, estradiol increased ipsilateral nuclear P-STAT3 at 3 h after MCAO (data not shown). In contrast to the cytosolic fraction, nuclear P-STAT3 was also increased at 22 h after MCAO (Fig. 2) ($n = 5$; $p < 0.05$), suggesting that estradiol
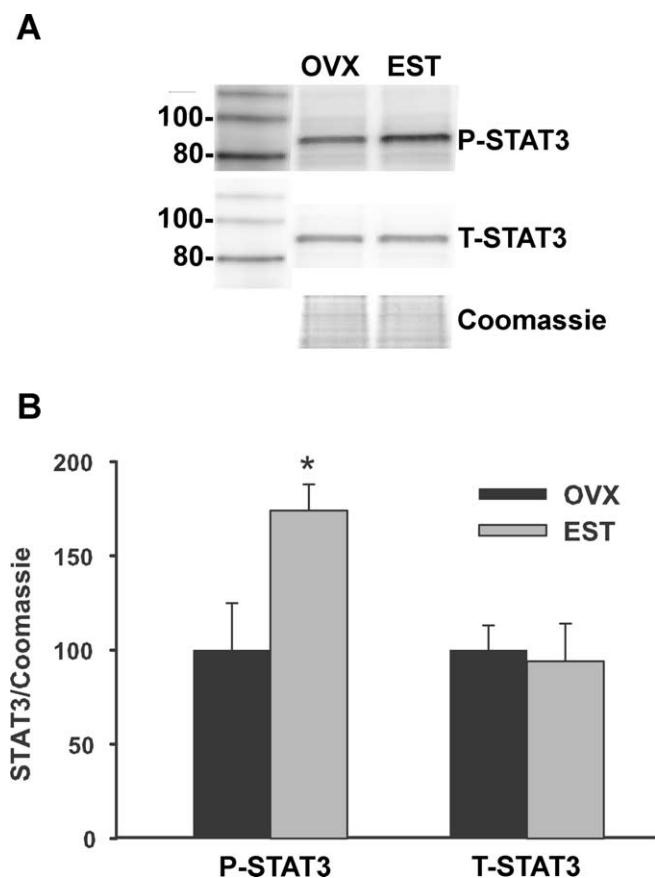
Figure 2. Estradiol replacement increases nuclear P-STAT3 but not T-STAT3 after MCAO. Nuclear extracts were prepared from the cerebral cortex of OVX and estradiol-replaced (EST) female rat brains at 22 h after MCAO, and Western blot was performed with an antibody against P-STAT3 or T-STAT3. **A**, Representative P-STAT3 and T-STAT3 blots and Coomassie-stained gel. **B**, Summary graph depicting the changes in nuclear STAT3 relative to total protein load. All values were normalized to mean nuclear P-STAT3 and T-STAT3 in ovariectomized animals, which were set at 100%. *$p < 0.05$ for the effect of EST on nuclear P-STAT3; $n = 5$ per group for P-STAT3; $n = 4$ for T-STAT3.

induces early translocation of STAT3 to the nucleus and that STAT3 remains activated in the nucleus for 24 h after MCAO. In accordance with increased protein translocation, T-STAT3 was also increased in the nuclear fraction at 3 h, although this difference was not statistically significant ($p = 0.09$; $n = 5$) and disappeared at 22 h after MCAO ($n = 4$).

STAT3 activation in ischemic brain colocalizes with neuronal and survival markers
To determine cell type and subcellular localization of P-STAT3, double immunofluorescence labeling was performed in estradiol-replaced animals with an anti-P-STAT3 antibody and cell type-specific antibodies MAP2 (neuronal marker) (Fig. 3B) and GFAP (astrocytic marker; data not shown). In agreement with Western blot analysis, P-STAT3 was detected at very low levels in the contralateral side (Fig. 3B) (representative of $n = 4$) and sham-operated animals ($n = 2$; data not shown). In the ischemic core, no MAP2 or P-STAT3 immunofluorescence was detected, indicating widespread cell death. In contrast, in the peri-ischemic region, strong P-STAT3 immunofluorescence was observed in numerous nuclei, which predominantly colocalized with MAP2 (Fig. 3B) (representative of $n = 4$), although a few P-STAT3-expressing cells were also GFAP positive (data not shown), suggesting that within 24 h after MCAO, STAT3 is selec-

tively activated in peri-infarct neurons. Sections incubated with rabbit polyclonal antiserum to P-STAT3 that had been preabsorbed with the immunogen showed no immunoreactivity, confirming the specificity of the anti-phospho-STAT3 antibody. We then examined whether cells expressing P-STAT3 also express Bcl-2 (Fig. 3C). In the ischemic core, no Bcl-2 or P-STAT3 immunofluorescence was detected. Neurons in the peri-ischemic region were immunoreactive for both P-STAT3 and Bcl-2 ($n = 4$ independent observations), suggesting that P-STAT3 and Bcl-2 expression colocalize in the same cells.

STAT3 inhibition abolishes protection by estradiol
To determine whether estradiol-induced STAT3 phosphorylation contributes to the neuroprotective effect of estradiol, we evaluated the effect of the P-STAT3 inhibitor JSI-124 on infarct size after MCAO in estradiol-treated and untreated OVX rats. STAT3 phosphorylation was significantly reduced by JSI-124 to $57 \pm 10.5\%$ ($n = 6$) after ischemia (Fig. 4) relative to vehicle ($100 \pm 15.2\%$; 40% DMSO in water; $n = 4$; $p < 0.05$), suggesting that the inhibitor crosses the blood–brain barrier and effectively inhibits STAT3 phosphorylation in brain after MCAO. More importantly, JSI significantly increased infarct size at 22 h after MCAO in estradiol-treated rats ($n = 8$ in vehicle and $n = 11$ JSI; $p < 0.05$). Interestingly, JSI-124 had no effect on infarct size in untreated ovariectomized rats ($n = 11$ vehicle; $n = 8$ JSI), consistent with the lower level of STAT3 phosphorylation in OVX rats. Because there were no differences in infarct size between the animals treated with JSI before ($n = 4$) or after ($n = 7$) MCAO, animals from the two groups were combined for this analysis (Fig. 5).

Discussion
The results of this study show that (1) estradiol increases STAT3 phosphorylation in cytosolic and nuclear fractions after MCAO in ovariectomized female rats, (2) STAT3 activation is localized in neurons and cells expressing Bcl-2 within the peri-ischemic region, and (3) STAT3 inhibition abolishes the reduction in infarct size by estradiol after MCAO. We conclude that STAT3 is an important mechanism used by estradiol to protect neurons after cerebral ischemia.

Estradiol is protective against ischemic brain damage via multiple mechanisms of actions (Suzuki et al., 2006), which include rapid, "nongenomic" effects and chronic, "genomic" effects (Bryant et al., 2006; Suzuki et al., 2006). In the classical view of estrogen signaling, the hormone binds its receptor [Fig. 6, estrogen receptor (ER)], which acts as a transcription factor that binds to a known consensus sequence referred to as estrogen response element (ERE) to induce gene transcription. It is now recognized that estradiol exhibits other effects, the so called "rapid" or nongenomic effects that may eventually affect gene expression, particularly if the target of estradiol's acute effect is a transcription factor such as CREB (cAMP response element-binding protein) (Wade and Dorsa, 2003) or STAT3 (Bjornstrom and Sjoberg, 2002).

STAT proteins are transcription factors that reside in the cytoplasm in an unphosphorylated dormant form. When activated by phosphorylation, STATs dimerize and translocate to the nucleus, where they bind specific DNA regions to induce gene transcription (Levy and Darnell, 2002). Based on previous reports linking estrogen to STAT3 activation *in vitro* (Bjornstrom and Sjoberg, 2002) and the role of STAT3 in neuronal survival (Alonzi et al., 2001; Schweizer et al., 2002), we here tested the hypothesis that estradiol increases STAT3 phosphorylation after ischemia *in*
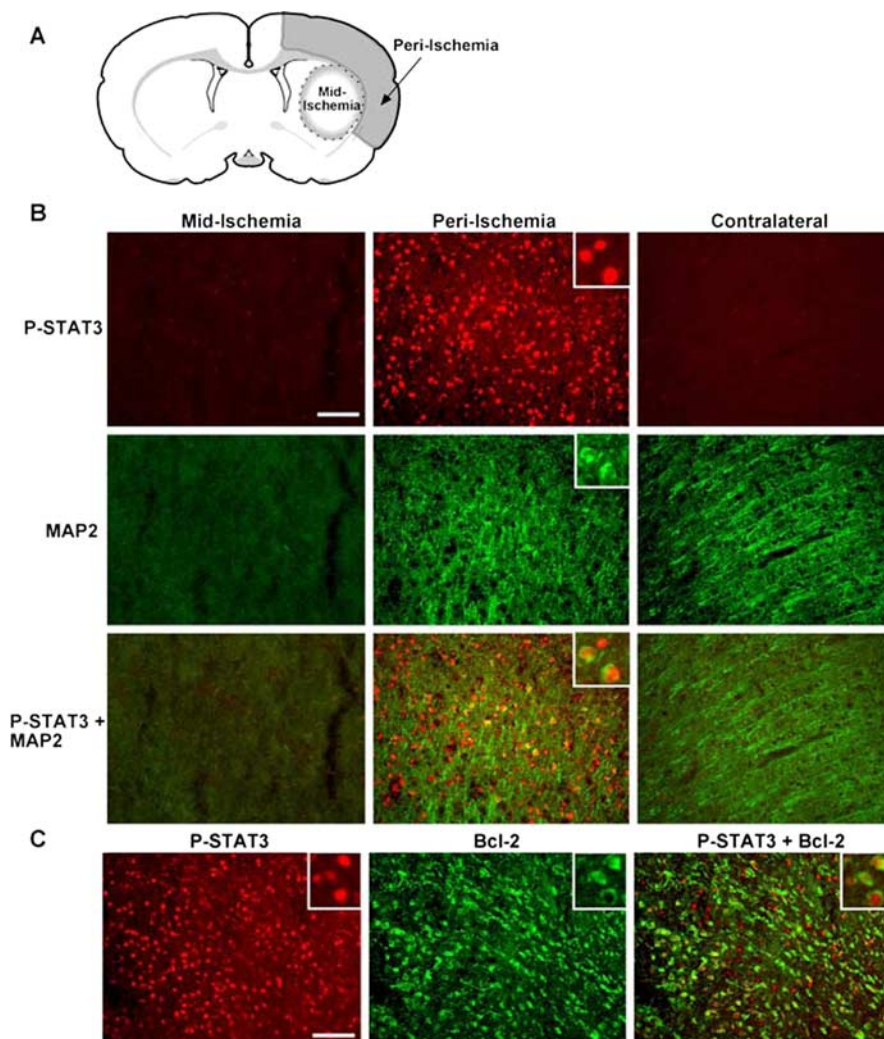
Figure 3. Colocalization of P-STAT3, MAP2, and Bcl-2 expression in the peri-ischemic cerebral cortex at 22 h after MCAO in estradiol-replaced OVX rats. *A*, Schematic drawing of a coronal rat brain slice illustrating the areas used for cortical dissections and immunohistochemical analysis (shaded area). The same designated areas were used for the contralateral side. *B*, Photomicrographs from midischemic (striatum) and peri-ischemic (cortex) regions on the ipsilateral and contralateral sides. The neuronal marker MAP2 (green) was highly expressed in most brain regions, including the peri-ischemic cortex and the contralateral cortex, but not in mid-ischemic tissue. P-STAT3 (red) was densely expressed in the peri-ischemic area only, which colocalized with MAP2. *C*, P-STAT3 (red) and Bcl-2 (green) exhibited a similar distribution pattern and colocalized in most peri-ischemic cortical cells. Insets illustrate higher-power examples of the immunoreactivity. Images are representative of four immunohistochemical runs from four different animals. Scale bars, 100 μm.
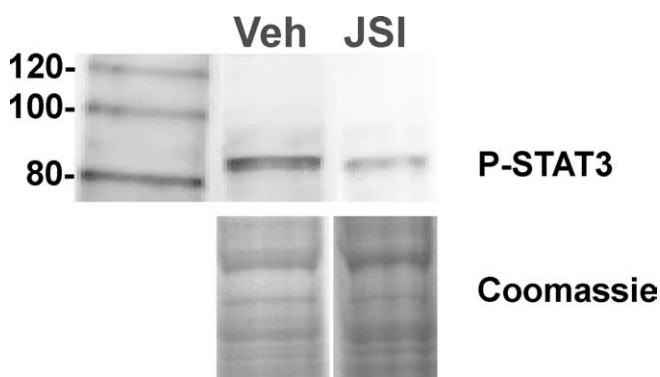
Figure 4. JSI-124 inhibits nuclear STAT3 phosphorylation in brain after systemic administration. Shown is Western blot analysis of P-STAT3 at 3 h after MCAO in estradiol-replaced OVX rats, which were injected with 1 mg/kg JSI-124 intraperitoneally 15 min before MCAO. Western blotting was performed on ipsilateral cortical tissue using an antibody against P-STAT3. Representative P-STAT3 blot with Coomassie-stained gel. Veh, Vehicle.

vivo and that STAT3 activation contributes to estrogen-mediated neuroprotection. Our data demonstrate that STAT3 is activated in the cytoplasm as early as 3 h after MCAO and that estradiol significantly increases the level of phospho-STAT3 in cytosolic fractions without affecting total STAT3. To determine whether activated STAT3 in the cytosol translocates to the nucleus, we then measured phosphorylated and total STAT3 in the nuclear fractions. We found that estradiol also increases the level of activated STAT3 in the nuclear fractions. The increase in cytosolic STAT3 phosphorylation by estradiol was transient, because there was no difference between estradiol-treated and untreated groups at 22 h of reperfusion. However, the increase in nuclear STAT3 phosphorylation by estradiol was sustained for up to 22 h. These observations suggest that STAT3 is rapidly activated by ischemic stress in the cytosol, followed shortly by translocation to the nucleus, where activated STAT3 remains for at least 24 h, presumably to sustain long-term gene expression. As illustrated in Figure 6, which summarizes our working model of how estradiol uses STAT3 as a mediator of neuroprotection after ischemia, STAT3 phosphorylation is increased in the cytosol in response to estradiol. STAT3 then translocates to the nucleus, where it binds to consensus DNA sequences in neuroprotective genes such as *bcl-2*.

To determine whether STAT3 is activated specifically in neurons and whether STAT3 activation is associated with neuroprotective gene expression, we determined whether P-STAT3 immunoreactivity colocalized with MAP2 and Bcl-2. P-STAT3 colocalized with MAP2 immunoreactivity, suggesting that STAT3 is activated after MCAO in neurons. Furthermore, P-STAT3 colocalized with Bcl-2 within the peri-infarct region, consistent with reports suggesting *bcl-2* regulation by STAT3. Because STAT3 is activated in brain under stress conditions, such as ischemia, and estradiol further increases levels of P-STAT3, estradiol may preferentially use STAT3 as an alternative pathway to stimulate gene transcription after MCAO.

To determine the role of STAT3 activation in estradiol-mediated neuroprotection, we combined estradiol replacement with the STAT3 inhibitor JSI-124. We demonstrated that JSI-124 reduces STAT3 phosphorylation in brain after systemic administration, suggesting that JSI-124 crosses the blood–brain barrier at sufficient concentrations to effectively inhibit STAT3 activation in brain. Using JSI-124, we then demonstrated that STAT3 inhibition abolishes the ability of estradiol to protect against ischemic brain injury, suggesting that STAT3 is an important player in the mechanism of neuroprotection by estradiol. JSI-124 did not alter infarct size in untreated ovariectomized female rats, suggesting that the effect of JSI-124 observed in our study was specific to
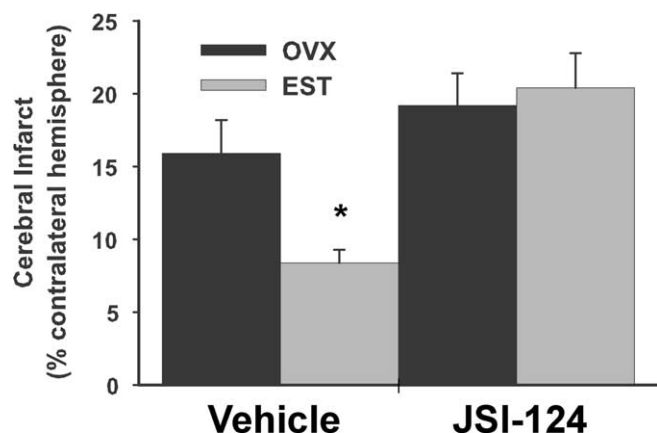
Figure 5. STAT3 inhibition abolishes the protective effect of estradiol. Infarct size was measured at 22 h after MCAO in estradiol-replaced (EST) and untreated OVX rats with and without the STAT3 inhibitor JSI-124 (1 mg/kg). The inhibitor was administered 15 min before or after MCAO. Because there were no differences, infarct size in the two groups was combined in this figure. Infarct size was significantly smaller in vehicle-treated EST than in the corresponding OVX group. However, the difference disappeared in the presence of JSI. $n = 11$ OVX, $n = 8$ EST, $n = 8$ OVX plus JSI-124, and $n = 11$ EST plus JSI-124 animals. *$p < 0.5$ compared with other groups.
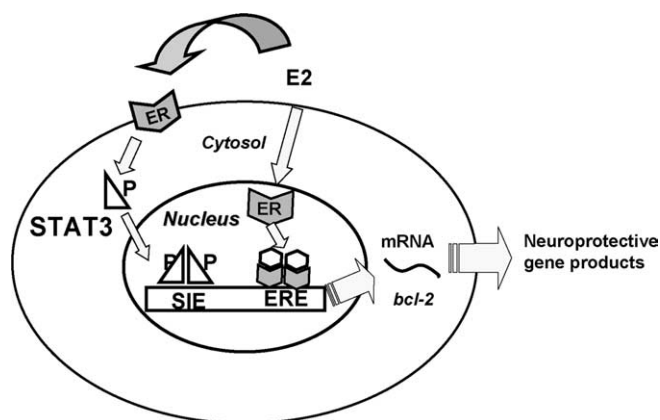
Figure 6. Working model for estradiol (E2)-mediated neuroprotection via STAT3. Estrogen induces STAT3 phosphorylation in the cytosol after cerebral ischemia. Activated STAT3 translocates to the nucleus and induces neuroprotective genes, such as *bcl-2*. Neuroprotection through estradiol-induced STAT3 phosphorylation may occur in parallel to or independent of the classical ER-mediated transcription via ERE. The SIE is the STAT3 consensus binding sequence.

estradiol-induced neuroprotection. JSI-124 has previously been demonstrated to selectively inhibit STAT3 phosphorylation and DNA binding without affecting other kinases involved in survival pathways such as AKT, ERK1/2 (extracellular signal-regulated protein kinase 1/2), JNK (c-Jun N-terminal protein kinase), and Src (Blaskovich et al., 2003). Furthermore, JSI-124 inhibits STAT3-dependent cancer cell proliferation both *in vitro* and *in vivo* without apparent toxicity (Blaskovich et al., 2003; Nefedova et al., 2005). In our study, JSI-124 caused fluctuations in arterial blood pressure when administered before MCAO. These fluctuations, however, disappeared when JSI-124 was administered after MCAO.

The significance of STAT3 to neuronal survival is highlighted by the observation that STAT3 gene deletion is lethal (Akira, 2000) and that neuronal survival is impaired after nerve lesion in neuron-specific STAT3-null mice (Schweizer et al., 2002). The role of STAT3 in neuronal survival is further supported by the observation that STAT3 is activated in brain after ischemic injury

(Justicia et al., 2000; Suzuki et al., 2001) and that multiple growth factors and cytokines use the STAT3 pathway to promote neuronal survival. For example, erythropoietin (EPO) protects the brain against ischemia-induced apoptosis (Ehrenreich et al., 2002; Solaroglu et al., 2003) and promotes regeneration of CNS neurons via STAT3 (Kretz et al., 2005). EPO also maintains Bcl-2, which correlates with cell survival after hypoxia/ischemia (Kumral et al., 2006). G-CSF (granulocyte colony stimulating factor) also protects the brain after an ischemic event (Solaroglu et al., 2006a). This protection correlates with increased phospho-STAT3 and increased Bcl-2 (Komine-Kobayashi et al., 2006; Solaroglu et al., 2006b) in surviving cells. Finally, blockade of IL-6 (interleukin-6) signaling reduces STAT3 phosphorylation in the peri-infarct area of the cortex, which is linked to increased apoptosis and infarct size (Yamashita et al., 2005). In contrast to our finding, a single report (Satriotomo et al., 2006) found that inhibiting STAT3 reduced brain damage after MCAO. In their study, total STAT3 expression was reduced by small interfering RNA, which reduces both P-STAT and T-STAT, compared with our study, in which STAT3 phosphorylation was reduced by JSI-124, but total protein was not changed. Furthermore, Satriotomo et al. (2006) showed that microglia/macrophages were the predominant cell type expressing P-STAT3 after ischemia, suggesting that STAT3 may play differential roles in different cell types.

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