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Acute estradiol protects CA1 neurons from ischemia-induced apoptotic cell death via the PI3K/Akt pathway

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Abstract

Global ischemia arising during cardiac arrest or cardiac surgery causes highly selective, delayed death of hippocampal CA1 neurons. Exogenous estradiol ameliorates global ischemia-induced neuronal death and cognitive impairment in male and female rodents. However, the molecular mechanisms by which a single acute injection of estradiol administered after the ischemic event intervenes in global ischemia-induced apoptotic cell death are unclear. Here we show that acute estradiol acts via the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling cascade to protect CA1 neurons in ovariectomized female rats. We demonstrate that global ischemia promotes early activation of glycogen synthase kinase-3 β (GSK3 β) and forkhead transcription factor of the O class (FOXO)3A, known Akt targets that are related to cell survival, and activation of caspase-3. Estradiol prevents ischemia-induced dephosphorylation and activation of GSK3 β and FOXO3A, and the caspase death cascade. These findings support a model whereby estradiol acts by activation of PI3K/Akt signaling to promote neuronal survival in the face of global ischemia.

Keywords

Estradiol; PI3K/Akt/signaling; Neuronal death; Apoptosis; Global ischemia

1. Introduction

Transient global brain ischemia arising during cardiac arrest, cardiac surgery or induced experimentally in animals via bilateral carotid artery occlusion, causes highly selective, delayed neuronal death and delayed neurological deficits (Graham and Chen, 2001; Liou et al., 2003; Lo et al., 2003; Zukin et al., 2004). Pyramidal neurons in the hippocampal CA1 are

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Section: 1. Cellular and Molecular Biology of Nervous Systems

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particularly vulnerable, whereas interneurons in this cell layer and pyramidal neurons in other hippocampal subfields survive. Histological evidence of CA1 pyramidal neuron degeneration is not observed until 2-3 days after global ischemia in rats (Graham and Chen, 2001; Liou et al., 2003; Lo et al., 2003; Zukin et al., 2004). Although the mechanisms underlying ischemia-induced death are as yet unclear, the substantial delay between insult and onset of death provides the opportunity to examine molecular events that destine these neurons to die.

Estradiol-17 β , the primary estrogen secreted by the ovaries, acts on neurons to increase spine density and synapse number (Gould et al., 1990; Hao et al., 2003; Pozzo-Miller et al., 1999; Prange-Kiel and Rune, 2006; Sato et al., 2007; Woolley and McEwen, 1992; Woolley and McEwen, 1994), synaptic connectivity sustained by N-methyl-D-aspartate (NMDA) receptor activation (Srivastava et al., 2008), NMDA receptor NR1 subunit expression (Gazzaley et al., 1996), NR2B subunit mRNA and the number of NR2B binding sites (Cyr et al., 2001), the magnitude of long-term potentiation (LTP) (Cordoba Montoya and Carrer, 1997; Gupta et al., 2001; Smith and McMahon, 2005; Smith and McMahon, 2006) and potentiates kainate-elicited currents in CA1 pyramidal neurons (Gu and Moss, 1998; Moss and Gu, 1999). Furthermore, estrogens afford neuroprotection of cortical neurons in primary culture against chemically-induced neuronal death (Harms et al., 2001; Honda et al., 2000), in rat hippocampal organotypic cultures (Cimarosti et al., 2005b; Cimarosti et al., 2005a) and in experimental models of global and focal ischemia (Amantea et al., 2005; Behl 2002; Garcia-Segura et al., 2001; Wise, 2003) and ameliorate the cognitive deficits associated with ischemic cell death (Gulinello et al., 2006; Plamondon et al., 2006; Sandstrom and Rowan, 2007; Soderstrom et al., 2009). Estrogen receptor (ER)- α and ER- β are expressed in the hippocampus where they could subserve the neuroprotective actions of estradiol (McEwen, 2002; Miller et al., 2005). Moreover, neuroprotection by estradiol may involve interactions with membrane-associated estrogen receptors, together with intracellular ERs, to activate cell signaling pathways that promote neuronal survival (Lebesgue et al., 2009).

Crosstalk between estradiol and growth factor signaling pathways is implicated in the cellular actions of estradiol. In the brain, estradiol activates extra-cellular-regulated kinases (ERK)/mitogen-activated protein kinase (MAPK) (Cardona-Gomez et al., 2002a) and phosphoinositide 3-kinase (PI3K) (Segars and Driggers, 2002), well-characterized intracellular signaling cascades implicated in neuronal plasticity and survival (Alessi et al., 1996; Behl, 2002; Bryant et al., 2006; Datta et al., 1999; Sweatt, 2004; Thomas and Huganir, 2004). Chronic estradiol at a physiological dose acts *via* classical ER- α and ER- β , insulin-like growth factor-1 receptors, ERK/MAPK and cAMP response element binding protein (CREB) signaling to promote neuronal survival after transient global ischemia (Jover-Mengual et al., 2007; Miller et al., 2005). A single injection of 17 β -estradiol administered to ovariectomized rats 2-4 days before ischemia also protects hippocampal neurons against ischemic damage via activation of CREB (Raval et al., 2009). Moreover, a single dose of estradiol administered immediately after reperfusion (acute estradiol) ameliorates global ischemia-induced neuronal death and cognitive deficits (Gulinello et al., 2006), but the mechanism of this protection has not been explored.

Treatment of rat hippocampal organotypic cultures with estradiol induce the phosphorylation of the serine-threonine protein kinase B (Akt), an effector immediately downstream of PI3K (Cimarosti et al., 2005b) and a key player in the apoptotic neuronal death machinery after focal (Noshita et al., 2001; Noshita et al., 2003) and global (Endo et al., 2006; Miyawaki et al., 2009) cerebral ischemia. Several targets of Akt are involved in its ability to foster cell survival. Akt promotes cell survival, at least in part, by phosphorylation and inactivation of proapoptotic downstream targets such as glycogen synthase kinase-3 β (GSK3 β) (Cross et al., 1995; Endo et al., 2006), the pro-apoptotic forkhead transcription factor family member, forkhead transcription factor of the O class (FOXO)3A (Brunet et al., 2001; Kawano et al., 2002) and Bad (Datta et al., 1997; Miyawaki et al., 2008; Saito et al., 2003). Akt also controls a critical

prosurvival protein, β -catenin, by modulating the activity of GSK3 β . GSK3 β can promote cell injury (Crowder and Freeman, 2000) and increase caspase-3 activity (Koh et al., 2003), and these actions are reduced when Akt phosphorylates and inactivates GSK3 β (Nishimoto et al., 2008). There is evidence that estradiol acts via Akt to maintain FOXO3A phosphorylation and activation in the face of focal ischemia (Won et al., 2006).

The present study was undertaken to identify intracellular signaling cascades that mediate acute estradiol neuroprotection in global ischemia. We show that estradiol acts via PI3K/Akt signaling to promote survival of hippocampal CA1 pyramidal neurons after transient global ischemia. Global ischemia promotes a transient increase of Akt phosphorylation and decrease in the phosphorylation of Akt targets GSK3 β and FOXO3A in the hippocampal CA1 in the first few hours after ischemia. Estradiol prevents ischemia-induced dephosphorylation and activation of GSK3 β and FOXO3A and caspase-3 activation. Thus, estradiol administered acutely after ischemia maintains PI3K/Akt signaling, thereby promoting neuronal survival in the face of global ischemia.

2. Results

2.1. PI3K/Akt signaling is critical to estradiol protection of CA1 neurons

Estradiol acts via PI3K to afford protection of cortical neurons in primary culture (Harms et al., 2001; Honda et al., 2000; Honda et al., 2001) and in rat organotypically cultured hippocampal slices against chemically-induced neuronal death (Cimarosti et al., 2005b). We first examined a possible role for PI3K/Akt signaling in estradiol protection. Ovariectomized female rats were subjected to global ischemia or sham operation and immediately infused icv with estradiol (50 μ g) in vehicle or vehicle alone. Animals also received icv infusion of the PI3K inhibitor LY294002 (30 μ g) or vehicle into the lateral ventricle at 0 and 12 h after surgery. Global ischemia induced extensive death of pyramidal cells in the hippocampal CA1 at 7 days post-ischemia ($P < 0.001$ vs. sham; compare Fig. 1i,m,q with Fig. 1a,e,q). Estradiol did not detectably alter the appearance or number of CA1 neurons in sham-operated rats (Fig. 1c,g,q), but greatly reduced the ischemia-induced neuronal loss ($P < 0.01$ vs. ischemia, Fig. 1k,o,q). Plasma estradiol levels at 1 h after estradiol injection were 26.9 ± 3.0 pg/ml in the placebo group and 7895 ± 552 pg/ml in the estradiol group. The PI3K inhibitor LY294002 did not detectably alter the number or appearance of surviving neurons in sham-operated rats (Fig. 1b,f,d,h,q) or vehicle-treated animals subjected to ischemia (Fig. 1j,n,q), but abrogated the neuroprotective action of estradiol in the hippocampal CA1 ($P < 0.001$ vs. estradiol alone, Fig. 1l,p,q). These findings indicate that whereas LY294002 reverses the estradiol neuroprotection, it is itself neither toxic nor protective in the global ischemia model. Together, these findings indicate that PI3K/Akt signaling is critical to estradiol protection of hippocampal neurons in a rat model of global ischemia.

2.2. The PI3K inhibitor LY294002 attenuates global ischemia- induced increase in Akt phosphorylation in CA1

To examine the effects of the PI3K inhibitor LY294002 on the abundance and phosphorylation status of Akt, ovariectomized rats were subjected to global ischemia or sham operation, treated with the PI3K inhibitor LY294002 (30 μ g) or vehicle and examined for Akt and p-Akt abundance in CA1 after reperfusion. Global ischemia markedly increased phosphorylation of Akt at Ser473 in the CA1 pyramidal cell layer (increase to 354% of sham-operated, vehicle-infused control; $P < 0.01$; Fig. 2a,b). LY294002 did not affect Akt phosphorylation in sham-operated animals but significantly reversed the effects of ischemia on p-Akt in CA1 (Fig. 2a,b). These findings indicate that the dose of LY294002 used effectively decreased Akt signaling in the hippocampus after ischemia.

2.3. Estradiol increases Akt phosphorylation in CA1 neurons

To examine the effects of ischemia and estradiol on the abundance and phosphorylation status of Akt, ovariectomized rats were subjected to global ischemia or sham operation, treated with estradiol or vehicle and examined for Akt and p-Akt abundance in CA1 at 1, 3 and 24 h after reperfusion. Global ischemia significantly increased phosphorylation of Akt at Ser473 in the CA1 pyramidal cell layer, evident at 1 h after ischemia (increase to 193% of control (sham-operated, vehicle-infused); $P < 0.001$; Fig. 3a,b); at 3 and 24 h, p-Akt levels were not significantly different from controls (Fig. 3a,b). Estradiol significantly increased Akt phosphorylation in sham-operated animals at 1 h (increase to 139% of control, $P < 0.05$, Fig. 3a,b) but did not significantly alter Akt phosphorylation at times after global ischemia (Fig. 3a,b).

2.4. Estradiol prevents dephosphorylation and inactivation of ERK2 in postischemic CA1

Estradiol is an upstream regulator of ERK/MAPK signaling in hippocampal neurons (Bi et al., 2000; Cardona-Gomez et al., 2002b), and ERK/MAPK is critical to the ability of long-term estradiol pretreatment to protect hippocampal neurons after global ischemia (Jover-Mengual et al., 2007). To compare the effects of post-ischemic administration of estradiol with our previous work implicating this signaling pathway in estradiol's neuroprotective actions when hormone is provided chronically at low levels, we examined the status of ERK1/2 phosphorylation after acute estradiol administration. Ovariectomized rats were subjected to global ischemia or sham operation, treated with estradiol or vehicle, and protein samples from the CA1 were subjected to Western blot analysis and examined for ERK1/2 abundance and phosphorylation at 1 and 3 h after reperfusion. Global ischemia significantly reduced phosphorylation of ERK1 and ERK2 in CA1, evident at 1 h after ischemia (decrease by ~36% for p-ERK1, $P < 0.05$ vs. sham-operated animals; decrease by ~51% for p-ERK2, $P < 0.001$ vs. sham-operated animals, Fig. 4a,b,c); at 3 h, p-ERK1/2 levels were not significantly different from controls (Fig. 4a,b,c). Estradiol did not significantly alter ERK1 and ERK2 phosphorylation in sham-operated animals but prevented the early ischemia-induced dephosphorylation of ERK2 ($P < 0.01$ vs. ischemia at 1 h, Fig. 4a,c). In estradiol-treated animals, ischemia did not reduce phosphorylation of ERK1 at 1 h after reperfusion (Fig. 4a,b).

2.5. Estradiol increases GSK-3 β phosphorylation 3 h after ischemia in CA1 neurons

GSK-3 β is a non-receptor serine/threonine kinase and a downstream target of Akt implicated in estradiol neuroprotection (Mendez and Garcia-Segura, 2006). Akt phosphorylates GSK-3 β on serine 9 to render it inactive, thereby activating glycogen synthesis and preventing apoptosis. To examine the effects of estradiol treatment and ischemia on GSK-3 β abundance and phosphorylation status, rats were subjected to global ischemia or sham operation, administered a single, acute injection of estradiol or vehicle, and protein samples from the CA1 were subjected to Western blot analysis at 1 and 3 h after reperfusion. Global ischemia did not significantly change the levels of p-GSK3 β at any times examined (Fig. 5a,b). Estradiol significantly increased GSK-3 β phosphorylation at 3 h after ischemia ($P < 0.05$ vs. ischemia at 3 h, vehicle-treated, Fig. 5a,b).

2.6. Estradiol prevents ischemia-induced dephosphorylation and activation of FOXO3A in CA

A well-characterized downstream target of PI3K/Akt signaling is the transcription factor FOXO3A, which promotes transcription of genes implicated in death pathways (Brunet et al., 2001; Kawano et al., 2002; Miyawaki et al., 2008; Miyawaki et al., 2009). Akt directly phosphorylates FOXO family members and inhibits their ability to induce expression of death genes. Akt-induced phosphorylation of FOXO3A retains the molecule in the cytoplasm, away

from target genes in the nucleus. To examine whether estradiol regulates phosphorylation and inactivation of FOXO, ovariectomized rats were subjected to global ischemia or sham operation, treated with estradiol or vehicle and examined for FOXO3A and p-FOXO3A abundance in CA1 at 3 h after reperfusion. Global ischemia induced a significant decrease in p-FOXO3A (by ~37%, $P < 0.05$ vs. sham-operated animals, Fig. 6a,b), with no significant change in total FOXO3A abundance in the cytosolic fraction of CA1. Estradiol significantly increased FOXO3A phosphorylation in sham-operated animals (increase to 142% of control, $P < 0.05$, Fig. 6a,b) and prevented the ischemia-induced dephosphorylation and activation of FOXO3A at 3 h after ischemia in the vulnerable CA1 ($P < 0.01$ vs. vehicle-treated, Fig. 6a,b).

2.7. Estradiol blocks ischemia-induced activation of caspase-3 activity in CA1 neurons

Injurious stimuli such as global ischemia disrupt the integrity of the mitochondrial membrane, leading to the release of cytochrome c and activation of caspase-3, a “terminator” caspase implicated in the execution step of apoptosis (for review, see (Bredesen, 2008; Zúkin et al., 2004)). Global ischemia promotes cleavage of the biologically inactive precursor procaspase-3 to generate activated caspase-3 (Jover et al., 2002); ischemia-induced caspase-3 activity is maximal at 24 h after insult (Tanaka et al., 2004). To directly measure caspase-3-like functional activity after ischemia, we labeled brain sections with FAM-DEVD-FMK, a fluorescein-tagged analogue of the caspase inhibitor zDEVD-FMK, at 24 h. FAM-DEVD-FMK enters cells and binds irreversibly to catalytically active caspase-3, and thus provides a fluorescent indicator of the abundance of active caspase-3. In brain sections from control (sham-operated, vehicle-infused) animals, caspase activity was low (Fig. 7a,b, g). Global ischemia induced a 16-fold increase in caspase activity in the hippocampal CA1, evident at 24 h ($P < 0.05$ vs. control; Fig. 7c,d,g). The increase in caspase activity was subfield-specific in that it was not observed in the resistant CA3 or dentate gyrus. Acute estradiol treatment blocked the ischemia-induced elevation of caspase-3 activity in CA1 ($P < 0.05$ vs. vehicle-treated, Fig. 7e,f,g).

3. Discussion

These findings provide clear evidence implicating the Akt pathway as a critical cellular mediator of the neuroprotection afforded by a supraphysiological dose of estradiol administered at the onset of reperfusion in a clinically relevant model of global ischemia. We now have evidence that icv administration of a much lower dose (2 μ g) is just as effective as the high dose (unpublished observations) and that LY 294002 also blocks protection by the low dose. These results are in agreement with findings of others that Akt is critical to cell survival after cerebral ischemia and indicate that hormone administration after an ischemic event can maintain Akt signaling. Activation of Akt and suppression of GSK3 β mediates neuroprotection of vulnerable hippocampal CA1 neurons after transient global ischemia by overexpression of copper/zinc-superoxide dismutase (Endo et al., 2006) or by ischemic preconditioning (Miyawaki et al., 2008; Yano et al., 2001). Estradiol acts via PI3K to afford protection of cultured cortical neurons subjected to chemically-induced death (Harms et al., 2001; Honda et al., 2000) and of neurons in organotypically cultured hippocampal slices subjected to oxygen-glucose deprivation (Cimarosti et al., 2005b). PI3K/Akt signaling participates in the neuroprotective actions of estradiol pretreatment in gerbils subjected to focal ischemia (Koh et al., 2006). We now document the involvement of Akt in the neuroprotection afforded by a single, acute injection of estradiol delivered at the time of reperfusion in a clinically relevant model of global ischemia in rats.

PI3K/Akt and its downstream targets in acute estradiol neuroprotection

Our findings are consistent with the hypothesis that a high dose of estradiol administered immediately after induction of global ischemia acts via PI3K/Akt signaling to promote survival

of post-ischemic neurons. Administration of the PI3K inhibitor LY294002 blocks the ability of estradiol to promote survival of CA1 pyramidal neurons in the post-ischemic hippocampus. The finding that LY294002 inhibits Akt phosphorylation in CA1 after global ischemia and blocks estradiol protection documents a role for PI3K signaling in preservation of ischemic hippocampal neurons and is consistent with studies in organotypic cultures of rat hippocampus subjected to oxygen and glucose deprivation (Cimarosti et al., 2005b). Ischemia promotes a transient increase of Akt phosphorylation in the hippocampal CA1, while phosphorylation of GSK3 β and FOXO3A (known Akt targets) decrease in the first few hours after ischemia, in confirmation of others (Endo et al., 2006; Namura et al., 2000; Yano et al., 2001). At later times, activation of caspase-3 is also evident.

It is notable that Akt phosphorylation is markedly enhanced, but p-Akt is not catalytically active, in post-ischemic hippocampal neurons. Global ischemia triggers hyperphosphorylation and activation of Akt, which in turn promotes induction of the endogenous inhibitor of Akt, carboxyl-terminal modulator protein (CTMP); upon induction, CTMP rapidly binds Akt and extinguishes Akt activity (Miyawaki et al., 2009). A possible scenario is that estradiol suppresses expression of CTMP (or other negative regulators of Akt), enabling p-Akt to be activated in postischemic CA1 and promote phosphorylation and inactivation of downstream targets of Akt implicated in the apoptotic cell death, such as GSK-3 β and FOXO3A.

Estradiol administered icv immediately after reperfusion prevents ischemia-induced dephosphorylation and activation of GSK3 β and FOXO3A as well as caspase-3 activation. These findings are consistent with the evidence that binding of estradiol to ER- α leads to formation of a macromolecular signaling complex that recruits downstream signaling molecules such as the regulatory subunit of PI3K (Kahlert et al., 2000; Mendez et al., 2003). However, this study did not identify the cellular mediator of estradiol action when given acutely. Estradiol can activate both ER- α and ER- β as they have similar (nM) affinity for estradiol. Moreover, we have implicated both receptors in the neuroprotective actions of estradiol when administered systemically for 2 weeks prior to global ischemia (see Miller et al., 2005).

Neuroprotective pretreatments such as estradiol and ischemic preconditioning can reduce global ischemia-induced cell death by activation of Akt and subsequent inactivation of its downstream target, the proapoptotic protein Bad (Koh et al., 2006; Miyawaki et al., 2008). Our results extend these findings by demonstrating that acute estradiol also regulates two other downstream targets of Akt implicated in the apoptotic cell death, GSK-3 β and FOXO3A. These molecules as well as another Akt target, mTOR, have been implicated in estradiol protection in a focal ischemia model (Won et al., 2006; Koh et al., 2006; Koh et al., 2008). Taken together, these observations support a model whereby estradiol administered acutely after insult acts via PI3K/Akt and downstream signaling molecules to promote neuronal survival in the face of ischemic insults.

Estradiol/ERK/MAPK interactions in estradiol neuroprotection

In addition to acting through the PI3K/Akt pathway, estradiol is known to activate MAPK signaling in CA1 neurons. Long-term pretreatment with estradiol at physiological levels ameliorates global ischemia-induced CA1 neuronal death (Gulinello et al., 2006; Jover et al., 2002; Miller et al., 2005). ERK/MAPK signaling is critical to estradiol-induced phosphorylation and activation of CREB and protection of CA1 neurons in global ischemia (Jover-Mengual et al., 2007). Chronic estradiol increases basal phosphorylation of both ERK1 and ERK2 in hippocampal CA1 and prevents ischemia-induced dephosphorylation and inactivation of ERK1 and CREB, downregulation of Bcl-2 and activation of the caspase death cascade. In the present study, we examined the impact of a single, acute injection of estradiol given immediately after ischemia on ERK1/2 phosphorylation/activation. Acute estradiol

prevented ischemia-induced dephosphorylation of ERK2 in the early postischemic period. These findings suggest that estradiol can activate multiple signaling pathways, depending on the dose and mode of administration, which may converge on common downstream signaling molecules to promote survival of hippocampal neurons in response to transient global ischemia. Whether ERK/MAPK signaling interacts with the PI3/Akt pathway at some point or if they independently converge on a downstream target such as caspase is currently unknown.

In summary, our results indicate that the neuroprotective actions of estradiol administered at the onset of reperfusion in a clinically relevant model of transient global ischemia are mediated by PI3K/Akt signaling, which prevents ischemia-induced activation of GSK3 β and FOXO3A and the caspase death cascade. Thus, post-ischemia estrogen therapy may represent a viable strategy for rescue of neurons from global ischemia-induced cell death.

4. Experimental procedures

4.1. Animals

Age-matched female Sprague-Dawley rats weighing 100-150 g (Charles River, Wilmington, DE) at the time of ischemic insult were maintained in a temperature and light-controlled environment with a 14 h light/10 h dark cycle and were treated in accordance with the principles and procedures of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Protocols were approved by the Institutional Animal Care and Use Committee of the Albert Einstein College of Medicine. All female rats were ovariectomized under halothane anesthesia (4% for induction, followed by 1% for maintenance).

4.2. Global ischemia

Seven days after the ovariectomy, rats were subjected to global ischemia by four-vessel occlusion as described (Calderone et al., 2003). In brief, rats were fasted overnight and anesthetized with halothane (4% for induction, followed by 1% for maintenance). The vertebral arteries were subjected to electrocauterization, the common carotid arteries were exposed and isolated with a 3-0 silk thread, and the wound was sutured. Twenty-four hours later, the animals were anesthetized again, the wound was reopened and both carotid arteries were occluded for 10 min with nontraumatic aneurism clips, followed by reperfusion. Arteries were visually inspected to ensure adequate reflow. Sham-operated rats were subjected to the same anesthesia and surgical procedures as animals subjected to global ischemia (vertebral artery coagulation and carotid artery exposure), except that the carotid arteries were not occluded.

In all cases, anesthesia was discontinued immediately after initiation of carotid artery occlusion. The anesthesia was initiated again just after the non-traumatic aneurism clips were removed and maintained until the icv injections were complete (see below). In total, animals were under anesthesia 5 min before carotid artery occlusion and again for about 15 min beginning just after reperfusion to inject drugs. Body temperature was monitored and maintained at 37.5 ± 0.5 °C with a rectal thermistor and heat lamp until recovery from anesthesia. Animals that failed to show complete loss of the righting reflex and dilation of the pupils from 2 min after occlusion was initiated until the end of occlusion and the rare animals that exhibited obvious behavioral manifestations (abnormal vocalization when handled, generalized convulsions, hypoactivity) or loss of > 20% body weight by 3-7 days after ischemia were excluded from the study. Ninety three rats were subjected to global ischemia. There were 3 deaths due to respiratory arrest; 9 other rats were excluded from the study because they failed to show neurological signs of ischemia (no loss of consciousness or incomplete dilation of the pupils during occlusion).

4.3. Estradiol and LY294002 administration

Halothane-anesthetized animals were injected with 50 µg of estradiol (cyclodextrin-encapsulated 17β-estradiol, water soluble, Sigma; Saint Louis, MO) or vehicle (2-hydroxypropyl-β-cyclodextrin, 1.08 mg, Sigma) in 5 µl of saline by unilateral injection into the right lateral ventricle at a flow rate of 5 µl/min immediately after reperfusion. Some animals were injected with the PI3K inhibitor LY294002 (30 µg, Promega; Madison, WI) or vehicle (5 µl of 50% DMSO) immediately after estradiol or vehicle injection and again 12 h later. Intracerebroventricular (icv) injections of 75% DMSO have no obvious harmful effects (Blevins et al., 2002; Jover-Mengual et al., 2007). Animals were positioned in a Kopf small animal stereotaxic frame with the incisor bar lowered 3.3 ± 0.4 mm below horizontal zero. A stainless steel cannula (28 gauge) was lowered stereotaxically into the right lateral ventricle to a position defined by the following coordinates: 0.92 mm posterior to bregma, 1.2 mm lateral to bregma, 3.6 mm below the skull surface according to the atlas of Paxinos and Watson (Paxinos 1998).

4.4. Histological analysis

Neuronal cell loss was assessed by histological examination of toluidine blue-stained brain sections at the level of the dorsal hippocampus from vehicle- and estradiol-infused animals killed at 7 days after ischemia. Animals were deeply anesthetized with pentobarbital (50 mg/kg, ip), blood was collected by cardiac puncture for assay of plasma estradiol levels (see below) and perfused transcardially with ice cold 4% paraformaldehyde in PBS (0.1 M, pH 7.4). Brains were removed and immersed in fixative (4°C overnight). Coronal sections (15 µm) were cut at the level of the dorsal hippocampus (3.3 to 4.0 mm posterior from bregma) with an electronic cryostat (Thermo Electron Corporation, Pittsburgh, PA), and every fourth section was collected and stained with toluidine blue. The number of surviving pyramidal neurons per 250-µm length of the medial CA1 pyramidal cell layer was counted bilaterally in 4 sections per animal as described (Jover-Mengual et al., 2007; Miller et al., 2005) under a light microscope at 40× magnification. Cell counts from the right and left hippocampus on each of the four sections were averaged to provide a single value (number of neurons/250 µm length) for each animal.

4.5. Serum estradiol assay

Tubes containing whole blood were placed on ice (10 min) and centrifuged at $300 \times g$ for 5 min. Serum was collected and stored (-20°C) until analyzed. Serum hormone levels were measured by fluoroimmunoassay using the DELPHIA estradiol assay (Perkin Elmer Life Sciences; Turku, Finland). All assays were performed in duplicate, and the mean value reported. The sensitivity of detection is 13 pg/ml. The inter- and intra-assay coefficients of variance are 10.1% and 4.1%, respectively.

4.6. Western blot analysis

For quantification of protein abundance in the hippocampal CA1, Western blot analysis was performed as described (Jover-Mengual et al., 2007). In brief, experimental and sham animals were deeply anesthetized with pentobarbital (50 mg/kg, ip), blood was collected by cardiac puncture for assay of plasma estradiol levels and killed by decapitation at 1, 3 and 24 h after reperfusion. Hippocampi were rapidly dissected, and transverse slices of dorsal hippocampus (1 mm) were cut with a McIlwain tissue chopper. The CA1 was rapidly micro-dissected, placed in ice-cold saline supplemented with protease inhibitor cocktail (1%, Sigma) and phosphatase inhibitor cocktail 1 (1%, Sigma) and homogenized in lysis buffer containing HEPES (5 mM), MgCl_2 (1 mM), EGTA (2 mM), dithiothreitol (1 mM), sucrose (10%), protease inhibitor cocktail (1%) and phosphatase inhibitor cocktail 1 (1%). Part of the sample from each animal was used to isolate cytosolic fraction by differential centrifugation.

Proteins from whole-cell lysates (p-Akt, Akt, p-ERK1/2, ERK1/2) and cytosolic fractions (p-GSK-3 β , GSK-3 β , p-FOXO3A, FOXO3A) were separated by SDS-PAGE and subjected to Western blot analysis. Protein concentration was determined by BCA protein assay kit (Pierce, Rockford, IL). Aliquots of protein (40-70 μ g) were dissolved in Laemmli sample buffer (0.025 M Tris-HCl, 5% glycerol, 1% SDS, 0.5% PBS, 0.1 M dithiothreitol, 2.5 mM β -mercaptoethanol, 1 mM PMSF, 0.5 mM NaHNO₃, pH 6.8), loaded on 10% polyacrylamide gels, subjected to electrophoresis and transferred to nitrocellulose membranes for immunolabeling with antibodies to p-Akt, Akt, p-GSK-3 β , GSK-3 β , p-FOXO3A, FOXO3A, p-ERK1/2 and ERK1/2. After incubation with primary and appropriate secondary antibodies, membranes were treated with enhanced chemiluminescence reagents (ECL, Amersham Life Science) and apposed to XAR-5 X-ray film (Eastman Kodak Co., Rochester, NY). Membranes were reprobbed with anti- β -actin antibody as a loading control.

To quantitate protein abundance, bands on Western blots were analyzed with a Scan Jet 4-C computing densitometer using NIH IMAGE 1.61 software. Band densities for p-Akt, p-GSK-3 β , p-FOXO3A, p-ERK1 and p-ERK2 were corrected for variations in loading and normalized to the corresponding band densities for total Akt, GSK-3 β , FOXO3A or total ERK1 and ERK2, respectively; normalized means were expressed as a percentage of the corresponding value for control (sham-operated, vehicle-infused) animals. Because of the large number of treatment groups, which included two surgical conditions (sham vs. ischemia), two hormone treatments (estradiol vs. vehicle) and multiple time points after surgery, it was not always possible to run samples for all conditions on a single gel. Therefore, to enable comparisons from experiment to experiment, band densities for all samples on a given gel were normalized to the band density for a sample from an animal treated with vehicle, subjected to sham operation and killed 1 h after surgery ("control"). Each gel included at least one sample from such a control animal to enable comparisons of data across different experiments, and a different control animal was prepared for each experiment.

4.7. Antibodies

The following antibodies were used in this study: **1)** anti-phospho Akt rabbit polyclonal antibody, which recognizes Akt only when phosphorylated at Ser473 (1:1000; Cell Signaling Technology, Inc., Beverly, MA); **2)** anti-Akt rabbit polyclonal antibody, which recognizes total Akt (1:2000, Cell Signaling Technology); **3)** anti-phospho GSK-3 β rabbit polyclonal antibody, which recognizes GSK-3 β phosphorylated at Ser9 (1:1000; Cell Signaling Technology); **4)** anti-GSK-3 β mouse monoclonal antibody, which recognizes total GSK-3 β (1:1000, Biosource International, Inc., Camarillo, CA); **5)** anti-phospho FOXO3A (FKHRL1) rabbit polyclonal antibody, which recognizes FOXO3A phosphorylated at Ser253 (1:1000; Upstate Biotechnology, Inc.); **6)** anti-FOXO3A rabbit polyclonal antibody, which recognizes total FOXO3A (1:1000, Upstate Biotechnology, Inc.); **7)** anti-phospho MAPK (p-ERK1/2) mouse monoclonal antibody, which recognizes ERK1 and ERK2 phosphorylated on both at Thr202 and Tyr204 residues (clone 12D4, 1:5000, Upstate Biotechnology, Inc., Lake Placid, NY); **8)** anti-MAPK1/2 (ERK1/2) rabbit polyclonal antibody, which recognizes total ERK1/ERK2 (1:5000, Upstate Biotechnology, Inc.); **9)** anti- β -actin mouse monoclonal antibody, which recognizes an epitope located within the N-terminal domain of the β -isoform of actin (1:20000; Sigma, Saint Louis, MI). Secondary antibodies for Westerns were horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:5000, Amersham, Buckinghamshire, England, UK) for polyclonal antibodies, or sheep anti-mouse IgG (1:2500, Amersham) for monoclonal antibodies.

4.8. Caspase activity assay

Caspase activity assays were performed on fresh frozen brain sections using the APO LOGIX™ carboxy-fluorescein (FAM) caspase detection kit (Cell Technology, Minneapolis,

MN) according to manufacturer's instructions. FAM-DEVD-FMK is a carboxy-fluorescein-tagged analog of zDEVD-fluoromethyl ketone (FMK), a broad-spectrum cysteine protease inhibitor that enters cells and irreversibly binds activated caspases (Amstad et al., 2001; Bedner et al., 2000; Smolewski et al., 2001). FAM-DEVD-FMK exhibits higher affinity for caspase-3 than for caspase-8, caspase-7, caspase-10 or caspase-6 (Garcia-Calvo et al., 1998) and exhibits much lower affinity for the calpains than for caspases; thus, at 5 μ M FAM-DEVD-FMK is a relatively selective inhibitor of caspase-3. Moreover, FAM-DEVD-FMK labeling of CA1 neurons correlates well with caspase-3 activation, as assessed by Western blot analysis. In this study we therefore refer to FAM-DEVD-FMK labeling as indicative of caspase-3 activity. In brief, estradiol- and vehicle-injected animals were deeply anesthetized with pentobarbital (50 mg/kg, i.p.) and killed by decapitation at 24 h after ischemia or sham operation (control). Brains were removed, frozen and cut into sections (18 μ m) in the coronal plane of the dorsal hippocampus. Brain sections (3 per animal) were labeled with 5 μ M FAM-DEVD-FMK (1 h, 37°C), washed three times with 1 $\times$ Working Dilution Wash Buffer and viewed under a Nikon ECLIPSE TE-300 fluorescent microscope equipped with an image analysis system at an excitation wavelength of 488 nm and emission wavelength of 565 nm. Images were acquired with a SPOT RT CCD-cooled camera with Diagnostic Software version 3.0. For quantitation of caspase-3 activity, the fluorescence intensity within the entire hippocampal CA1 cell layer was analyzed using NIH Image 1.61. The mean fluorescence intensity of CA1 in the right and left hemisphere from each of the three sections was averaged to provide a single value for each animal.

4.9. Statistical analysis

The results were expressed as mean $\pm$ SEM. Data analysis was performed using GraphPad Prism 4.00. Statistical comparisons among groups were conducted using a two-way ANOVA with Bonferroni's multiple comparisons or t-test post hoc analysis (neuron counts, immunoblots and caspase-3 activity). T-test was used for the serum estradiol data. Differences were considered significant at $P < 0.05$.

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Abbreviations

Akt	protein kinase B
CREB	cAMP response element binding protein
ERK	extra-cellular-regulated kinases
FOXO	forkhead transcription factor of the O class
GSK3 β	glycogen synthase kinase-3 β
MAPK	mitogen-activated protein kinase
PI3K	phosphoinositide 3-kinase

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Fig. 1. The PI3K inhibitor LY294002 attenuates estradiol protection

Ovariectomized female rats were subjected to global ischemia (white and black bars) or sham operation (grey bars) and treated with estradiol or vehicle immediately upon reperfusion. Animals also received LY294002 or vehicle icv at 0 and 12 h after vehicle or estradiol injection ($n = 3-12$ animals per group). Global ischemia induced extensive death of pyramidal cells in the hippocampal CA1 at 7 days post-ischemia (**i, m, j, n, q**). LY294002 greatly reduced estradiol protection, assessed at 7 days after surgery (**l, p, q**). Scale bars: lower magnification, 400 μm; higher magnification, 40 μm. **so**, stratum oriens; **sp**, stratum pyramidale; **sr**, stratum radiatum. ***, $P < 0.001$ vs. all sham groups; ##, $P < 0.01$ ischemia+estradiol vs. ischemia and ###, $P < 0.001$ vs. ischemia+estradiol+LY294002).

Fig. 2. The PI3K inhibitor LY294002 attenuates global ischemia- induced increase in Akt phosphorylation in CA1

Representative Western blots (a) and relative abundance of p-Akt (b) in CA1 whole-cell lysates from rats subjected to sham surgery or ischemia, infused icv with vehicle or LY294002 immediately upon reperfusion. Westerns were probed with antibodies to p-Ser473-Akt and Akt. Ischemia markedly increased phosphorylation of Akt (a,b). LY294002 significantly attenuated the ischemia-induced increase in p-Akt (a,b). Data are representative of 3-6 animals per group. There was no effect on total protein when expressed relative to β -actin in the same sample (data not shown). Values for ischemic rats were normalized to the corresponding values

for control (sham-operated, vehicle-infused) animals. ***, $P < 0.001$ vs. control and ##, $P < 0.01$ ischemia vs. ischemia+LY294002).

Fig. 3. Estradiol and global ischemia transiently increase Akt phosphorylation in CA1
Representative Western blots (**a**) and relative abundance of p-Akt (**b**) in CA1 whole-cell lysates from rats subjected to sham surgery or ischemia, infused icv with vehicle or estradiol and killed at 1, 3, or 24 h after surgery. Ischemia transiently increased p-Akt at 1 h (**a,b**). Estradiol significantly enhanced Akt (**b**) phosphorylation in sham-operated females but did not significantly alter Akt phosphorylation in ischemic rats (**b**). Neither ischemia nor estradiol treatment altered levels of p-Akt in CA1 at 3 or 24 h after ischemia. Data are representative of 3-11 animals per group. Values for ischemic rats were normalized to the corresponding values for control (sham-operated, vehicle-infused) animals. *, $P < 0.05$; ***, $P < 0.001$ vs. sham-operated, vehicle-infused rats.

Fig. 4. Estradiol prevents ischemia-induced dephosphorylation of ERK2 in CA1

Representative Western blots (**a**) and relative abundance of p-ERK1 (**b**) and p-ERK2 (**c**) in CA1 whole-cell lysates from rats subjected to sham operation or global ischemia and vehicle- and estradiol-treated at 1 and 3 h after surgery. Westerns were probed with antibodies to p-ERK1/2 and ERK1/2. Ischemia induced dephosphorylation of ERK1 (**a,b**), and ERK2 (**a,c**). Estradiol did not significantly change ERK1 (**b**) and ERK2 (**c**) phosphorylation in shams but maintained levels of p-ERK2 (**c**) in ischemic rats and prevented the effect of ischemia on p-ERK1 (**b**). Data are representative of 6-12 animals per group. Values for ischemic rats were normalized to the corresponding sham value. *, $P < 0.05$; ***, $P < 0.001$, vs. all sham groups; #, $P < 0.01$ ischemia+estradiol vs. ischemia+vehicle-infused rats.

Fig. 5. Estradiol prevents ischemia-induced inhibition of GSK-3β phosphorylation in hippocampal CA1

Representative Western blots (a) and relative abundance of p-GSK-3β (b) in the cytosolic fraction of CA1 from rats subjected to sham operation or global ischemia, infused icv with vehicle or estradiol and killed at 1 and 3 h after surgery. Westerns were probed with antibodies to p-Ser9-GSK-3β and GSK-3β. Global ischemia did not change the levels of p-GSK3β at any times examined (a,b). Estradiol significantly increased GSK-3β phosphorylation 3 h after global ischemia (a,b). Data are representative of 6-12 animals per group. Band densities for experimental animals were normalized to the corresponding sham value. *, $P < 0.05$.

Fig. 6. Estradiol prevents ischemia-induced FOXO3A dephosphorylation

Representative Western blots (a) and relative p-FOXO3A abundance (b) in the cytosolic fraction of CA1 of rats subjected to sham operation or ischemia, infused icv with vehicle or estradiol and killed at 3 h after surgery. Westerns were probed with antibodies to p-FOXO3A and FOXO3A. Ischemia promoted dephosphorylation of FOXO3A in the cytosolic fraction of CA1 neurons. Estradiol maintained the phosphorylated, inactivated state of FOXO3A in the CA1 of ischemic rats. Data are representative of 6-9 animals per group. Values for experimental animals were normalized to the corresponding sham value. *, $P < 0.05$ vs. sham-operated, vehicle-infused rats; ##, $P < 0.01$ ischemia+estradiol vs. ischemia+vehicle-infused rats.

Fig. 7. Estradiol blocks ischemia-induced caspase-3-like activity in CA1

Representative brain sections at the level of the dorsal hippocampus from control (sham-operated) (a,b) and experimental animals subjected to global ischemia (c-f) labeled with FAM-DEVD-FMK, a cell-permeant, irreversible inhibitor of caspases and calpains. In control brain caspase activity was undetectable (a,b,g). Global ischemia activated caspase in CA1 neurons, evident at 24 h (c,d,g). Estradiol prevented ischemia-induced caspase-3 activity (e,f,g). Data are representative of 3-5 animals per group. Abbreviations as in Fig. 1. Scale bars: lower magnification, 400 μ m; higher magnification, 50 μ m. *, $P < 0.05$ vs. sham and #, $P < 0.05$ vs. ischemia+estradiol.