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Estrogen Pretreatment of Organotypic Hippocampal Slices Protects Neurons against Oxygen-Glucose Deprivation with Akt Activation

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In several experimental models, estrogens protect neurons against ischemic insults. However, the recent clinical studies of hormone replacement showed negative results to prevent stroke. Therefore, optimal models to study estrogen replacement for neuroprotection are needed before its clinical application. Organotypic hippocampal slice under oxygen-glucose deprivation (OGD) has been established as a model of cerebral ischemia and has advantages to study drug effects. We investigated whether estrogen protected CA1 neurons and affected activation of Akt (pAkt) in CA1 region under OGD. Thus, rat hippocampal slices on day 7 of culture were treated with 17-β estradiol (E, 1 nM) for 7 days before 30 min OGD, and cell death of CA1 neurons was quantified by propidium iodide (PI) staining and expression of pAkt was studied by Western blot and immunofluorescence. PI intensity in slices treated with E was significantly reduced 72 hour after OGD compared to that of non-treated slices ($p < 0.05$). E pretreatment also increased the expression of pAkt 72 hour after OGD compared to that of no treatment ($p < 0.01$). These data suggest that estrogen pretreatment may rescue neurons from ischemic insults through the activation of Akt and also indicate that our model would be a useful alternative method to study the mechanisms and effects of estrogen replacement treatment for neuroprotection.

Key Words: Estrogen, Pretreatment, Akt activation, OGD, Organotypic hippocampal slice, Neuroprotection

INTRODUCTION

Epidemiologic studies show that overall stroke incidence in women is lower than men (Prencipe et al, 1997; Sudlow & Warlow, 1997) and women in reproductive years rarely experience stroke (Carolei et al, 1993). This gender difference, however, disappears in aged subjects (Manolio et al, 1996). Therefore, resistance to stroke in women during reproductive period can be attributable to endogenous estrogens (Hurn & Macrae, 2000). In accordance with epidemiologic results, experimental studies support that estrogens protect neurons in *in vitro* (Behl et al, 1995; Weaver et al, 1997; Vedder et al, 1999; Harms et al, 2001; Sribnick et al, 2004) and *in vivo* models (McCullough & Hurn, 2003) from cerebral ischemia. Under ischemic insults by middle cerebral artery occlusion in rodents, for instance, intact females have smaller infarct volumes than those in age-matched males, and the effect disappears in ovariectomized females (Alkayed et al, 1998; Park et al, 2006). Furthermore, estrogen replacement in ovariectomized females or estrogen pretreatment of males reduces brain damages (Toung et al, 1998; Rusa et al, 1999). The results of most *in vivo* studies indicate estrogens act as a potent neuroprotectant showing more than 50% reduction of infarct volume.

Cellular and molecular mechanisms by which estrogens protect neurons from ischemic insults are not well understood. The suggested mechanisms include anti-inflammation, vasodilation through eNOS activation, anti-oxidative effects, regulation of transcription and interaction with signaling pathways such as adenylate cyclase, protein kinase C, mitogen-activated protein kinase (MAPK) and phosphoinositol-3-kinase (PI-3K) (Singer et al, 1999; Toran-Allerand et al, 1999; Honda et al, 2001; Zhang et al, 2001). Generally, pharmacologic concentration of estrogens induces vasodilation and antioxidative effects (Behl et al, 1997; Pelligrino & Galea, 2001). On the other hand, physiologic level of estrogens protects neurons through estrogen receptor (ER)-mediated mechanisms, either genomic or non genomic, including regulation of transcription and interaction with other signal transduction pathways (Toran-Allerand et al, 1999; Green & Simpkins, 2000).

Organotypic hippocampal slice cultures have been established for an *in vitro* model of cerebral ischemia (Newell et al, 1995; Laake et al, 1999; Bonde et al, 2002; Jung et al, 2004). Oxygen-glucose deprivation (OGD), an ischemic like condition *in vitro*, for 30 to 45 min induces significant CA1 neuronal death at 24 or 72 h after OGD which is a

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ABBREVIATIONS: DG, dentate gyrus; E, 17-β estradiol; ER, estrogen receptor; MAPK, mitogen-activated protein kinase; OGD, oxygen-glucose deprivation; pAkt, phosphorylated Akt; PI, propidium iodide; PI-3K, phosphoinositol-3-kinase.

characteristic feature of global ischemia in rats and gerbils (Kirino & Sano, 1984; Kirino et al, 1984; Dirnagl et al, 1999). Furthermore, actions of intrinsic brain cells such as glia and neurons after ischemic insult can be investigated because there is no participation of cells infiltrating from peripheral blood that are involved in secondary damage after ischemia (Dirnagl et al, 1999; Skaper, 2003). Also, effects of chronic drug treatment such as estrogens can be observed since slices survive for several weeks (Raineteau et al, 2004). Therefore, organotypic hippocampal slices exposed to OGD are valuable means to study the phenomenon of delayed neuronal cell death and prevention of cell death by drugs. However, only a few studies on the effects of estrogen against ischemic insult have been carried out using organotypic hippocampal slices (Cimarosti et al, 2005a; Cimarosti et al, 2005b).

It should be noted that recent clinical trials (Wassertheil-Smoller et al, 2003; Anderson et al, 2004) and other experimental paradigms show that estrogens failed to reduce neuronal death, and that estrogen treatment even exacerbated cell damages (Harukuni et al, 2001; Nunez & McCarthy, 2003), while there are many studies to indicate that estrogens play a role in neuronal survival. The discrepancy between clinical and experimental studies indicates that dose, timing and duration of estrogen treatment would be critical in models used for experiments.

In the present study, we investigated whether estrogen protects neurons from OGD and also whether activation of Akt is involved in the effects of estrogen using rat organotypic hippocampal slice.

METHODS

Organotypic hippocampal slice culture

Organotypic hippocampal slice cultures were prepared according to the technique of Gahwiler (1988) and Jung et al (2004). The brains from 10-day-old Sprague-Dawley rats were aseptically removed and immersed in an ice-cold dissecting medium (pH 7.15) containing: 50% MEM without bicarbonate, 50% calcium and magnesium-free Hanks balanced salt solution, 20 mM HEPES and 7.5 g/l D-glucose. The hippocampi were dissected transversely (300 μm thickness) using a McIlwain tissue chopper. Five slices were placed on membrane units (0.4 μm, Millicell-CM, Millipore, MA, USA) and carefully separated from each other. The membrane units were placed into six-well trays, each well containing 1.2 ml of the culture medium, which is composed of 50% MEM with Earl's salts, 2 mM L-glutamine, 25% Earl's balanced salt solution, 25% normal horse serum, 6.5 g/l D-glucose, and 20 mM HEPES. The cultures were kept in an incubator at 37°C in 5% CO_2 and the media were changed twice a week. For treatment groups, 17 β-estradiol (0.1 to 10 nM) was added to the culture medium on the day 7 or 10 of culture and maintained until ischemic insult was induced.

Oxygen-glucose deprivation

This system is used to generate an ischemic-like condition *in vitro*. Oxygen-glucose deprivation (OGD) was induced according to the protocols of Strasser and Fischer (1995a; 1995b) with slight modifications (Bonde et al, 2002; Jung et al, 2004). After a 14-day culture, the slices for OGD were washed with fresh media, replaced with a buffer pregassed with 95%N_2/5% CO_2 OGD, [pH 7.4, containing NaCl (124 mM), KCl (4 mM), $CaCl_2$ (2 mM), KH_2PO_4 (1.25 mM), $NaHCO_3$ (25 mM), and mannitol (10 mM)] and then incubated in a 95%N_2/5% CO_2 incubator for 30 min. For reperfusion, the OGD slices were transferred to normal fresh culture medium which was maintained for 72 h. The control slices were incubated with fresh normal media for 30 min in the same way as with the OGD condition.

Assessment of neuronal cell death

For quantification of neuronal cell death in CA1 region, propidium iodide (PI) which penetrates damaged cell membrane was used (Strasser & Fischer, 1995b; Laake et al, 1999; Brana et al, 2002). Before imaging, PI (5 μg/ml, Sigma, Missouri, USA) was added into the culture medium. After 20 min, PI fluorescence images in CA1 regions of the slices were captured using a digital camera with fluorescence microscopy (Axiovert 200, Germany) and analyzed using an imaging software package (Axiovision LE 4.1, Carl Zeiss, Germany). The sensitivity of the camera and intensity of the excitation light was standardized so as to be identical between different experimental sets. The PI intensity, to indicate cell death was expressed as a percentage of the final fluorescence (F_{fin}) which was considered to be the fluorescence of 100% cell death (Strasser & Fischer, 1995b; Jung et al, 2004). Cell death (%) = $(F_t - F_0)/(F_{fin} - F_0) \times 100$ where F_t is the PI fluorescence of hippocampal slices measured at indicated time points after OGD reperfusion, and F_0 is the background fluorescence of the slices prior to OGD. F_{fin} was determined by PI intensity of the slices which were killed by 24 h of incubation at 4°C in the presence of PI.

Western blot hybridization

Each set of experiments contained control and experimental samples (5 slices each), and they were simultaneously subjected to Western blot hybridization. Slices were lysed in a sodium dodecyl sulfate (SDS)-buffer (62 mM Tris-HCl, 1 mM EDTA, 2% SDS, pH 6.8~7.0) containing one tablet of a protease inhibitor cocktail (Complete Mini, Boehringer Mannheim, Germany) in 10 ml of solubilizing buffer, and then incubated for 30 min on ice and centrifuged at 15,000 for 10 min at 4°C. Protein concentration in the supernatant was determined (Bio-Rad Laboratories, Hercules, CA, USA), and 25 μg of protein was loaded for SDS-PAGE. Proteins were transferred onto a polyvinylidine difluoride (PVDF, Amersham Phamarcia Biotech Inc., NJ, USA) membranes using an electroblotting apparatus. Membranes were blocked overnight in TBS containing 0.1% Tween-20 and 5% dry milk, incubated overnight with phosphorylated Akt (pAkt, 1 : 1,000; Santa Cruz biotechnology, CA, USA) antibody and washed three times (30 min each) with TBS containing 0.1% Tween-20. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h each and then washed three times (30 min each) with TBS containing 0.1% Tween-20. Protein bands were visualized with the ECL Plus Western blotting detection system (Amersham Pharmacia Biotech, Buckinghamshire, England). The membrane is reblotted using antibody stripping solution (Chemicon international, CA, USA) according to the manufacturer's instruction to visualize actin (1 : 1,000; Santa Cruz biotechnology). For

quantification, densities of pAkt were normalized by corresponding blots for actin.

Immunofluorescence

The inserts were rinsed twice in cold 0.1 M phosphate buffer saline (PBS), followed by fixation for 2 h with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at 4°C, and slices were gently detached from the membrane. The slices were then incubated for 30 min in permeabilization buffer (0.5% Triton X-100 in PBS), and were transferred in 0.1 M PBS containing 3% normal serums (Vector Laboratories, CA, USA) and 1% bovine serum albumin (BSA) for 1h. Subsequently, slices were incubated overnight with pAkt antibody (1 : 250) in 1% normal serum and 0.5% BSA. On the following day, the slices were applied with secondary antibody conjugated with Alexa fluor 555 goat anti-rabbit IgG (1 : 1,000 dilution; Molecular Probes, Oregon, USA). PBS (0.1 M) was used to wash slices between all steps. The slices were mounted with a mounting medium (Vectashield, Vector Laboratories), and the images were taken using fluorescence microscopy (Axiovert 200, Germany).

Statistics

The data are expressed as mean±S.E.M. Multiple comparisons were evaluated by the analysis of variance, followed by post hoc Fisher's PLSD tests using statview program (statview 5 version, SAS Institute, USA). Differences were considered significant at $p<0.05$.

RESULTS

Estrogen pretreatment protects neurons from OGD

To determine whether estrogen reduces neuronal death induced by oxygen-glucose deprivation (OGD) in organotypic hippocamal slices, we treated the slices with 17β-estradiol (from 0.1 to 10 nM) for 3 days or 7 days before OGD. The durations and concentrations of estrogen treatment were based on earlier other *in vitro* as well as our *in vivo* studies (Teter et al, 1999; Rune et al, 2002; Sato et al, 2002; Park et al, 2006). After 30 min under OGD, PI intensity of CA1 regions at 24 h was significantly increased and further increased at 72 h compared to those of control (5.8 fold and 6.5 fold, respectively, $p<0.01$, Fig. 1). OGD selectively induced CA1 neuronal death at 72 h. However, in slices with estrogen pretreatment (1 nM) for 7 days, the increase of PI intensity in CA1 regions was reduced at 24 h and significantly decreased at 72 h compared to OGD without pretreatment (0.5 fold, $p<0.05$, Fig. 1), and pretreatment of estrogen at any concentrations for 3 days did not reduce PI intensity (data not shown). These findings indicate that pretreatment with relatively low dose of estrogen reduces CA1 neuronal death of organotypic hippocampal slices against OGD. Based on these results,

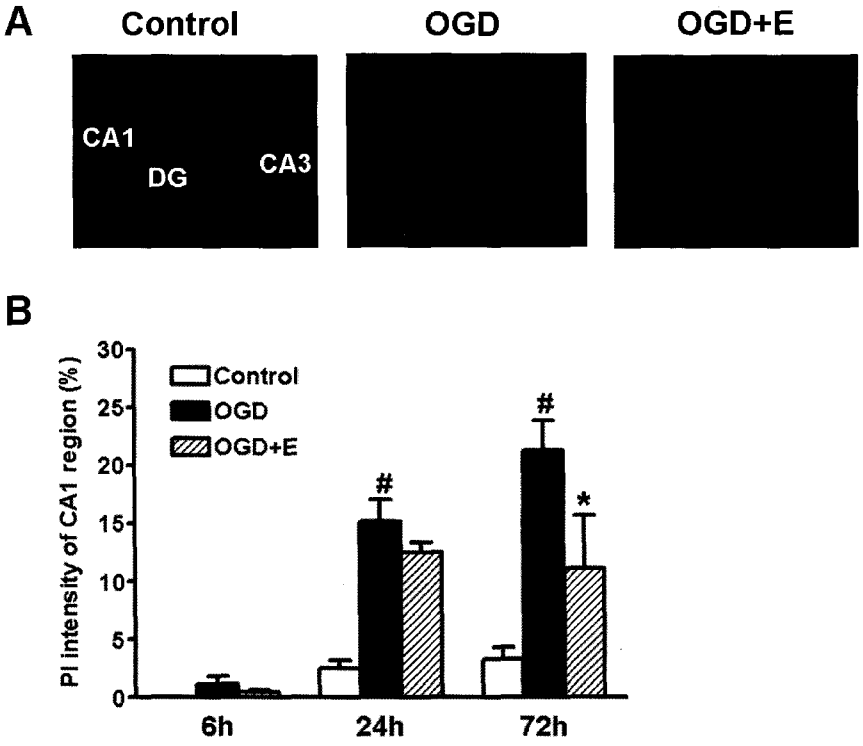

Fig. 1. Estrogen pretreatment reduced CA1 neuronal death induced by OGD. (A) PI intensity in CA1 region (CA1) at 72 h after 30 min of OGD. Slices were treated with 17β-estradiol (OGD+E, 1 nM) 7 days before OGD. CA3, CA3 region; DG, dentate gyrus. (B) Quantification of PI intensity at 6 h, 24 h and 72 h after 30 min OGD. Each value represents mean±S.E.M. ($n>5$, each group). $^{\#}p<0.01$ compared to control, $^{*}p<0.05$ compared to OGD (analysis of Variance and Fisher's PLSD test).

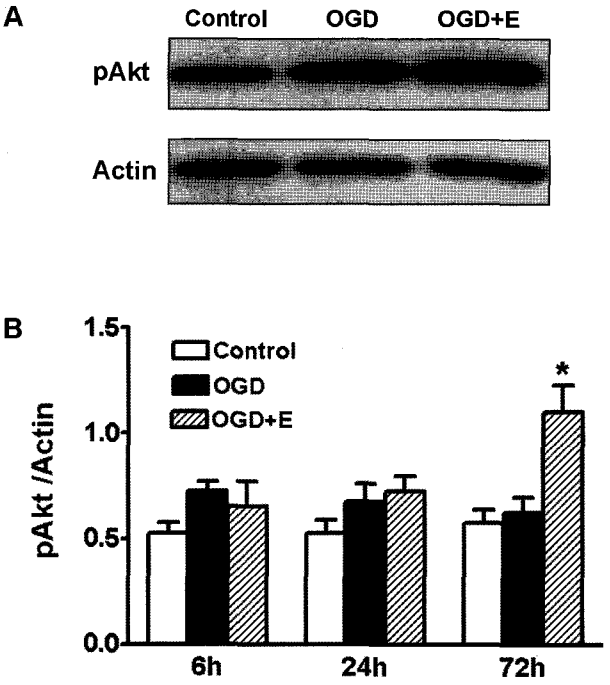

Fig. 2. Activation of Akt after OGD with estrogen pretreatment. (A) Expression of pAkt at 72 h after 30 min OGD by Western blot. Slices were treated with 17 β-estradiol (OGD + E, 1 nM) for 7 days before OGD. (B) Quantification of pAkt at 6 h, 24 h and 72 h (n=3, each group) after 30 min OGD. Actin was used as an internal control to normalize the densities of pAkt. Each value represents the mean ± S.E.M. *$p < 0.01$ compared to OGD (analysis of Variance and Fisher's PLSD test).

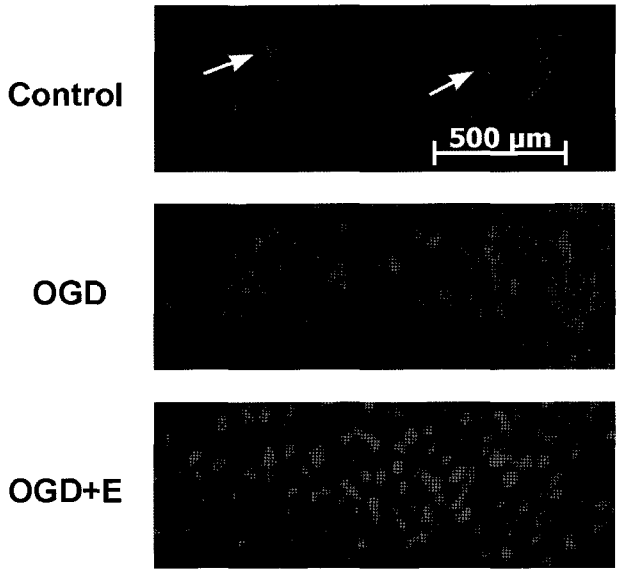

Fig. 3. Increased expression of pAkt in CA1 region by estrogen pretreatment. Immunoreactivity of pAkt was shown in CA1 region at 72 h after 30 min OGD. Arrows indicate pAkt positive staining cells. 17 β-estradiol (OGD + E, 1 nM) was applied for 7 days before OGD. Three experiments were done independently.

the regimen of 1 nM estrogen pretreatment for 7 days was used for further studies.

Activation of Akt by estrogen pretreatment

To determine whether the protection exerted by estrogen was related to activation of Akt, we examined the expression of phosphorylated Akt (pAkt), which is an activated form, in whole slices by Western blot. In control, the basal expression of pAkt was not changed during 72 h. Compared to the control, OGD slightly induced pAkt at 6 h and the expression of pAkt was gradually reduced during 24 h and 72 h, however, there was no statistical significance between the control and OGD (Fig. 2). Estrogen pretreatment also induced pAkt expression at 6 h compared to control, but not as much as that of OGD without pretreatment. However, activation of Akt by estrogen was further increased at 24 h, and the increase was much greater at 72 h than that of OGD without treatment (1.7 fold, $p < 0.01$, Fig. 2). The increase of Akt activation was inversely correlated with PI intensity in estrogen pretreated slices at 24 h and 72h after OGD.

Next, we examined the expression of pAkt in slices at 72 h by immunofluorescence in order to find out whether pAkt was expressed in CA1 regions where neuronal death was reduced by estrogen. In control, a few cells with neuronal shape showed pAkt expression mainly in cytoplasm (arrows, the first panel in Fig. 3), whereas the number of positive cells with pAkt in CA1 region of OGD slices was slightly increased. Morphologically, staining mainly in nuclei in CA1 region of OGD slices appeared to be more condensed than the control. Expression of pAkt in CA1 regions of estrogen pretreated slices was markedly increased compared to OGD or control (Fig. 3).

DISCUSSION

Current study was undertaken to investigate whether estrogen protects CA1 neurons of organotypic hippocampal slices against ischemic-like condition, OGD, and found that pretreatment of the slices with relatively low dose estrogen reduced delayed CA1 neuronal death, and the protective effects were accompanied with activation of Akt in CA1 region. Taken together, the results suggest that estrogen may protect neurons against ischemia in organotypic hippocampal slices by enhancing activation of Akt.

In the model used to study estrogen effects, doses, time schedule and duration of estrogen should be considered. There is abundant evidence in several studies that acute or chronic treatment with variable doses of estrogen effectively rescue neurons against ischemic insults. However, optimum doses and duration of estrogen pretreatment are not clear (McCullough & Hurn, 2003). For instance, chronic pretreatment with physiologic doses of estrogen reduced infarct volume in ovariectomized female rats, while large doses administered immediately before insult had no effect (Rusa et al, 1999). Furthermore, two different studies show that estrogen pretreatment with either physiologic or supraphysiologic concentrations even exacerbates neuronal damages (Harukuni et al, 2001; Bingham et al, 2005). These negative effects of estrogen suggest that basic experimental studies for the role of estrogen in ischemic insults should closely be investigated before estrogen is clinically applied for neuroprotection. Using organotypic hippocam-

pal slice cultures, a few studies showed the effects of estrogen on noxious insults (Sato et al, 2002; Cimarosti et al, 2005b; Goodenough et al, 2005). For OGD, only one study has been reported. Cimarosti et al (2005b) showed that pretreatment of estrogen (10 nM) for 7 days significantly reduced CA1 damage at 24 h after 60 min of OGD. Compared to theirs, however, our results showed that lower concentration (1 nM) of estrogen still protected neurons effectively from delayed cell death induced OGD in organotypic hippocampal slices. Based on the results of estrogen-alone study in clinical trial (Anderson et al, 2004), the Food and Drug Administration in the USA requests that estrogens should be prescribed at the lowest effective dose and for the shortest duration to avoid harmful effects (Stefanick, 2005). Considering the findings in the current study, our model can be useful to study the neuroprotective mechanisms of estrogen in pre-menopausal females or in females treated with low-dose estrogen replacement.

The role of Akt activation in neuronal survival has been well demonstrated. Activated Akt phosphorylates, thus directly inhibiting BAD, caspase-9 and GSK-3β, mediators of apoptosis (Datta et al, 1997; del Peso et al, 1997; Pap & Cooper, 2002). Also, Akt activation induces survival genes such as Bcl-2 through regulation of cyclic AMP response element binding protein (CREB) (Pugazhenthi et al, 2000). Recent studies showed that estrogens induced activation of Akt and pharmacologic inhibitions of Akt reversed the positive effects of estrogen, suggesting involvement of Akt pathway in estrogen-mediated neuroprotection (Honda et al, 2000; Honda et al, 2001; Wilson et al, 2002; Yu et al, 2004; Cimarosti et al, 2005b; Dhandapani et al, 2005). Our results also support the beneficial effects of estrogen against ischemic insults with Akt activation. Some studies showed that activation of Akt was estrogen receptor (ER) dependent via non-genomic mechanism which was verified by using specific ER antagonists and showing rapid phosphorylation of Akt within 5 to 15 min by estrogen pretreatment, followed by the return of pAkt level to baseline within hours before insults (Honda et al, 2000; Honda et al, 2001; Dhandapani et al, 2005). In our study, pAkt expression by estrogen pretreatment was not increased at early time point but at later time point of 72 h after OGD compared to that of no treatment, suggesting that delayed activation of Akt by estrogen rather than early rapid induction is involved in neuroprotection. Consistent with our findings, Wilson et al (2002) showed that basal expression of pAkt was not changed by estrogen pretreatment, and that Akt activation was increased in estrogen treated cortical explants after insults. Therefore, it is possible that genomic pathways, ER dependent or not, are involved in Akt activation.

There are a few limitations in our study. First of all, we did not show direct relationship between Akt activation and neuroprotective effects of estrogen. Although pAkt expression was inversely correlated to PI intensity in our results, it is possible that other protective signaling pathways such as adenylate cyclase and/or MAPK or transcriptional regulation related to neuronal survival by classical genomic pathway can also be involved (Zhou et al, 1996; Dubal et al, 1999; Singer et al, 1999; Toran-Allerand et al, 1999; Rau et al, 2003). Further studies by using specific pharmacologic inhibitors of signaling pathways are needed to define the exact role of Akt activation in estrogen induced neuronal survival. In addition, our current study did not define which cell types expressed pAkt by estrogen. Akt activations by estrogen in several cell types of brain have been reported. For example, eNOS expression by estrogen has been suggested to be related with rapid activation of Akt in blood vessels (Hisamoto et al, 2001; Stirone et al, 2005), and cortical astrocytes showed the increase of pAkt expression by estrogen pretreatment (Dhandapani et al, 2005). Except experiments using neuronal cultures (Honda et al, 2000; Honda et al, 2001; Yu et al, 2004), pAkt positive cell types in cortical explants and organotypic hippocampal slices (Wilson et al, 2002; Cimarosti et al, 2005b) were not specified. In our results, the expression of pAkt in control CA1 regions showed neuronal shape, however, the pattern and shape of pAkt positive cells after OGD were different from those in control. Therefore, we could not rule out the possibility of positive staining in cells such as astrocytes or microglia.

In summary, pretreatment of organotypic hippocampal slices with low dose of 17β-estradiol protected neurons from delayed CA1 neuronal death and increased activation of Akt in CA1 region after OGD insults. Activation of Akt by estrogen may be in part an underlying mechanism of neuroprotection.

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