Published in final edited form as:

Free Radic Biol Med. 2012 June 1; 52(0): 2151–2160. doi:10.1016/j.freeradbiomed.2012.03.005.

17 β -Estradiol prevents cell death and mitochondrial dysfunction by estrogen receptor-dependent mechanism in astrocytes following oxygen-glucose deprivation/reperfusion

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Abstract

17 β -estradiol (E2) has been shown to protect against ischemic brain injury, yet its targets and the mechanisms are unclear. E2 may exert multiple regulatory actions on astrocytes that may greatly contribute to its ability to protect the brain. Mitochondria are recognized to play central roles in the development of injury during ischemia. Increasing evidence indicates that mitochondrial mechanisms are critically involved in E2-mediated protection. In this study, the effect of E2 and the role of mitochondria were evaluated in primary cultures of astrocytes subjected to an ischemia-like condition of oxygen-glucose deprivation (OGD)/reperfusion. We showed that E2 treatment significantly protects against OGD/reperfusion-induced cell death as determined by cell viability, apoptosis and lactate dehydrogenase leakage. The protective effects of E2 on astrocytic survival were blocked by an estrogen receptor (ER) antagonist (ICI 182,780), and were mimicked by an estrogen receptor (ER) agonist selective for ER α (PPT), but not by an ER agonist selective for ER β (DPN). OGD/reperfusion provoked mitochondria dysfunction as manifested by an increase of cellular reactive oxygen species production, loss of mitochondrial membrane potential and depletion of ATP. E2 pretreatment significantly inhibited OGD/reperfusion-induced mitochondrial dysfunction, and this effect was also blocked by ICI 182,780. Therefore, we concluded that E2 provides direct protection to astrocytes from ischemic injury by an ER-dependent mechanism, highlighting an important role for ER α . Estrogen protects against mitochondria dysfunction at the early phase of ischemic injury. However, overall implications for protection against brain ischemia and its complex sequelae await further exploration.

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Disclosure/Conflict of interest

The authors state no duality of interest.

Keywords

estrogen; protection; astrocyte; ischemic injury; mitochondria

Introduction

The female sex hormone estrogen exerts profound neuroprotective effects against ischemic brain injury, but the targets and underlying mechanisms of estrogen-mediated protection remain obscure [1, 2]. In the past few decades, most efforts have been devoted to understand how estrogen affects neurons in both *in vitro* and *in vivo* models, with less attention paid to astrocytes, the most abundant cell type in the brain [3]. Astrocytes function as the principal housekeeping cells of the central nervous system. They are dynamically involved in many important activities in brain, such as synaptic transmission, metabolic and ionic homeostasis, inflammatory response, antioxidant defense, structural and nutritive support of neurons, and formation and maintenance of the blood–brain barrier [4]. Because their end feet surround capillaries, astrocytes are the first cells to suffer ischemic insult among all types of brain cells [5, 6]. Astrocytes interact with neurons by cross-talk, both physiologically and pathologically [4, 7]. Proper astrocyte function is particularly important for neuronal survival under ischemic conditions. Dysfunction of astrocytes resulting from ischemic insult significantly influences the responses of other brain cells to injury [7, 8]. It is believed that astrocytes are critical determinants in stroke pathophysiology [9]. Thus, it is of great importance and significance to understand the role of astrocytes in estrogen-mediated protection against ischemic injury.

Astrocytes have been shown to be the targets of estrogen and are postulated to play a key role in estrogen-mediated protection of the brain [10, 11]. 17 β -estradiol (E2), the predominant form of estrogen, exerts multiple regulatory actions on astrocytes including, but not limited to, regulating astrocytic morphology and function, modulating the release of neurotrophic factors and inflammatory molecules [12]. Moreover, astrocytes have the ability to synthesize estrogen. It has been shown that expression of the E2 synthetic enzyme, aromatase and 17 β -estradiol production are both increased in astrocytes under pathological conditions [13]. E2 also has been found to regulate the synthesis of other steroids in astrocytes, such as progesterone [14, 15]. Many studies indicate that it is astrocytes that mediate the protective actions of E2 by releasing tumor growth factor β -1 [16, 17]. E2 has been shown to regulate the expression of aquaporin-4 expression in parenchymal reactive astrocytes and perivascular glial processes, and this may specifically relate to regulation of brain injury from ischemic stroke [18]. The protective effects of E2 against ischemic damage induced by middle cerebral artery occlusion are most prominent in the cortex, implicating astrocytes as a major targets for E2 protection against brain ischemic injury [19, 20]. Protective effects of E2 against ischemic insult *in vitro* also have been shown in astrocytes and cortical explant culture [21, 22]. However, the effects of E2 on astrocytes during ischemia are not completely understood.

One of the most important functions of astrocytes is energy support, largely depending on mitochondria. Mitochondria are unique organelles involved in energy production and cell life-death regulation. During mitochondrial energy production, an inevitable product, reactive oxygen species (ROS), is generated in the mitochondrial matrix [23]. It has been shown that mitochondrial energy production is decreased while ROS production is increased in brain cells under pathological conditions, which may account for the etiology and development of age-related diseases in central nervous system, including stroke [24]. Excessive ROS can affect mitochondrial enzymes, lipids, and DNA, causing deleterious effects leading to mitochondrial dysfunction. It has been suggested that the degree of

mitochondrial impairment in cerebral ischemia may be a critical determinant of the final extent of neuronal injury [25].

Increasing evidence suggests that E2 regulates mitochondrial function, which may play a central role in the protective action of E2 [26–28]. We previously found that E2 decreases mitochondrial oxidative stress in cerebral blood vessels as well as in brain tissue under physiological conditions [29–31]. More recently, we found E2 to protect mitochondrial function in cerebral endothelial cells following ischemic insult *in vitro* [32]. In astrocytes, recent studies have shown that E2 influences mitochondrial gene expression and respiratory chain activity, and regulates mitochondrial function [33, 34]. However, little is known about the effects of E2 on mitochondria in astrocytes during ischemia. To this end, the present study was designed to investigate the impact of physiological levels of E2 on cell viability and mitochondrial function under ischemic-like conditions of oxygen-glucose deprivation (OGD)/reperfusion in primary cultured astrocytes. In addition, by using specific estrogen receptor (ER) antagonist and agonists, the specific role of ER in E2-mediated effects was evaluated.

Materials and methods

Primary astrocyte cultures

Primary cultures of mouse cerebral cortical astrocytes were prepared as described previously with few modifications [35]. Briefly, meninges-free cortices were collected from 1 to 3 day old Swiss Webster mice. Cells were dispersed by mechanical and enzymatic dissociation using a solution containing 0.05% trypsin (Invitrogen, Carlsbad, CA). Cells were then suspended in plating medium consisting of minimal essential medium (MEM) (Gibco, Grand Island, NY) containing 10% fetal bovine serum (Hyclone, Logan, UT), 10% equine serum (Hyclone), 2 mM glutamine, and 0.5% penicillin/50 U streptomycin. Single-cell suspension was plated at a density of one hemisphere per 10 ml plating medium. Cultures were incubated at 37°C in a 95/5% mixture of atmospheric air and CO₂. After 3 days, the medium was changed to astrocyte growth medium (MEM supplemented with 10% equine serum and 2 mM glutamine), and this was repeated twice weekly thereafter. All animal experimental procedures were carried out in accordance with a protocol approved by the Institutional Animal Care and Use Committee. Cells were seeded in 24-well plates for cell death measurement; in 12-well plates for ATP assay; and in glass-bottom culture dishes (MatTek, Ashland, MA) coated with 0.5 mg/ml poly-L-lysine (Sigma-Aldrich, St Louis, MO) and 20 µg/ml laminin (Sigma-Aldrich) for cellular ROS and mitochondrial membrane potential assay. Cells were used for experiments after 3 weeks.

To identify astrocytes, the presence in cultured cells of glial fibrillary acidic protein (GFAP), a specific marker for astrocytes, was determined using an antibody to GFAP (Sigma-Aldrich) and Hoechst 33342 (Invitrogen). Analysis showed that at least 95% of cells in the culture were GFAP-positive.

Drug treatment

Prior to experiments, culture medium was replaced with phenol red-free MEM (Invitrogen) supplemented with 1% charcoal-stripped fetal bovine serum (Invitrogen), and cells were incubated overnight. The next day cells were treated with either E2 (Sigma-Aldrich) at physiological concentration (10 nM), 10 nM PPT (4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol; Tocris Cookson, Ellisville, MI), or 10 nM DPN (2,3-bis(4-hydroxyphenyl)-propionitrile; Tocris) for various times as indicated before OGD/reperfusion exposure. Ethanol alone was used as vehicle control. In some experiments, cells were treated with 100 nM ICI-182,780 (Tocris) for 0.5 h, and exposure to this ER antagonist was continued during

subsequent E2 or vehicle treatment. All drugs were dissolved in ethanol as stock solution at 10 mM. Further dilution was made using culture medium. The final concentration of ethanol in culture medium never exceeded 0.02%, this had no effect by itself. The concentrations of these drugs were maintained during OGD and reperfusion.

OGD/Reperfusion

OGD/reperfusion was performed as reported previously [32]. Briefly, shortly before OGD exposure, cells were washed three times with deoxygenated, glucose-free, balanced salt solution (BSS 0.0) containing the following (in mM): NaCl, 116; CaCl₂, 1.8; MgSO₄, 0.8; KCl, 5.4; NaH₂PO₄, 1; NaHCO₃, 14.7, and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 10; pH, 7.4. Cultures were then incubated with BSS 0.0 and immediately transferred to a humidified anaerobic chamber (Reming Bioinstrument, Redfield, NY) perfused with 95% N₂/5% CO₂ at 37°C. The oxygen level inside the anaerobic chamber was monitored with an oxygen sensor and was kept lower than 0.5% (0.3–0.5%) throughout the experiment. Control cells were placed in a normoxic incubator, and their medium was changed to BSS 5.5, which was identical to BSS 0.0 but supplemented with 5.5 mM glucose. After OGD, the medium was quickly replaced with glucose-containing, phenol red-free MEM or BSS 5.5 (live imaging experiments only), and cells were returned to normoxia. The length of the OGD and periods varied depending on the parameter measured.

Determination of cell death

Cell death was evaluated by determination of cell viability, apoptosis and lactate dehydrogenase (LDH) leakage. Briefly, cell viability was assessed by the MTT assay. MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide] (Invitrogen) was added into cell cultures at the final concentration of 5 mg/ml after completion of reperfusion. Cultures were then incubated at 37°C for 4 h. Subsequently, cells were lysed overnight in dimethyl sulfoxide (DMSO), enabling release of a blue formazan product which was then evaluated in a plate reader (absorbance, 570 nm). Data were normalized to the values from control cultures without OGD which were considered as 100% survival.

Apoptotic cells were determined by Hoechst 33258 staining as previously reported [36]. Following exposure to OGD/reperfusion, cells were washed twice in PBS and fixed in 4% formaldehyde at 4 °C overnight. The fixed cells were then washed and labeled with Hoechst 33258 (5 µg/ml, Invitrogen) at room temperature in the dark for 10 minutes. Cell nuclei were observed and imaged by an inverted fluorescent microscope. The number of apoptotic nuclei was determined on at least six randomly selected areas from three coverslips of every experimental group. Data were expressed as a percentage of apoptotic cells relative to the total number of cells.

LDH leakage was detected using a commercial kit from Roche Applied Science (Indianapolis, IN). Immediately after OGD or OGD/reperfusion, supernatants of culture media were collected, and LDH activity was measured according to the manufacturer's instructions. Data were normalized against the total LDH release from full kill (FK) control cultures that were stimulated for 10 min with 0.25% Triton X-100 (Sigma-Aldrich), a reagent that damages the plasma membrane resulting in total release of LDH.

Expression of ER α and ER β in primary cultured mouse astrocytes

The expression of ER α and ER β in primary cultured mouse astrocytes was detected by western blotting. In brief, soluble lysates of astrocytes were mixed with sample buffer and NuPAGE Reducing Agent (Invitrogen). Extracted Proteins were separated in 12% SDS-PAGE gel, and were then electrically transferred to polyvinylidene difluoride (PVDF)

membranes. Afterward, the membranes were blocked in 5% nonfat dry milk diluted in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature. Western blots were probed with a rabbit anti-ER α antibodies (1: 5000; Santa Cruz Biotechnology Inc., Santa Cruz, CA), or a rabbit anti-ER β antibodies (1: 5000; Santa Cruz) overnight at 4°C. The membranes were then incubated with an IRDye secondary anti-rabbit antibody (Thermo Scientific, Rockford, IL) for 1 h. Protein bands were visualized using LI-COR Odyssey System (LI-COR Biotechnology, Lincoln, NE).

Cellular ROS production

After 60 min OGD, immediately before initiation of the reperfusion period, cells were loaded in the dark with 2 μ M hydroethidine (HET, Sigma-Aldrich) for 30 min at 37°C, and the concentration of HET was maintained in the medium throughout each experiment. According to preliminary experiments, it took about 30 min for this dye to sequester into the cell. Cells were excited at 485 nm, and fluorescence emission monitored at 560 nm. HET was visualized using a live imaging workstation with an inverted microscope (TE-200; Nikon, Tokyo, Japan) fitted with a xenon lamp, filter wheel, and a 40 $\times$ epifluorescence oil immersion objective. Images were acquired with a 12-bit digital CCD camera (Photometrics, Tucson, AZ). To minimize photo bleaching of the fluorescent dye, fluorescence intensity was attenuated with neutral density filters (Omega optical, Battleboro, VT), and the exposure time never exceeded 50 ms. Data were analyzed (after background subtraction from a cell-free region) with Metafluor 7.0 software (Universal Imaging, West Chester, PA). Measurements of fluorescence intensity were started 35 min after OGD, following dye loading in reperfusion medium. At that time no significant differences in fluorescence (F₀) were observed among the groups. Fluorescence intensity of each cell in a visual field was then recorded for the next 30 min of reperfusion. The value for each cell (F_t) was normalized to the fluorescence intensity at 35 min reperfusion for that cell (F₀).

Mitochondrial Membrane Potential

TMRM is a cell-permeant, cationic fluorescent dye that is readily sequestered by active mitochondria and has been recognized as one of the best dyes for detection of mitochondrial membrane potential [37]. Following 90 min OGD, cells were reintroduced to oxygen and glucose and loaded in the dark with 100 nM TMRM at 37°C for 25 min. Unsequestered dye was then thoroughly washed out. Following 30 min reperfusion, TMRM fluorescence (Ex/Em: 540/590 nm) in mitochondria was then measured in the presence of oxygen and glucose (reperfusion), 30 min after the end of OGD. For each cell, TMRM fluorescence intensity was quantified in the mitochondria-rich perinuclear region, both before and after exposure to the protonophoric uncoupler, FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; Sigma-Aldrich; 5 mM). FCCP was used to dissipate the membrane potential and define the baseline for analysis of mitochondrial potential by TMRM. Data for each cell were normalized to the fluorescence intensity in the same area after exposure to FCCP.

Cellular ATP measurement

Immediately at the end of 6 h OGD exposure, cells were washed with phosphate-buffered saline and lysed with ATP-releasing buffer containing 100 mM potassium phosphate at pH 7.8, 2 mM EDTA, 1 mM dithiothreitol (DTT) and 1% Triton X-100. Cellular ATP levels were measured using a commercial ATP assay kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. A Novostar cell-based microplate reader (BMG LABTECH GmbH, Durham, NC) was used to measure the luminescence signal of the samples in Nunc 96-well plates. ATP standards were used each time to generate a calibration curve. ATP concentration in the astrocyte sample was normalized to the protein

concentration as determined by the bicinchoninic acid protein assay using a MicroBCA kit (Thermo Scientific).

Statistical Analysis

All data were expressed as mean $\pm$ SEM. Statistical analysis was performed by one-way ANOVA followed by Newman-Keuls *post hoc* analysis using Origin 7.5 and GraphPad Prism 5.0. $P < 0.05$ was considered statistically significant.

Results

E2 attenuates OGD/reperfusion-induced cell death in primary cultures of astrocytes

Astrocyte cell death is one of the common ultimate consequences under ischemic conditions where energy depletion and metabolic disruption are severe [7]. In the present study, the model of OGD/reperfusion was applied in primary cultures of cortical astrocytes to simulate ischemic insult. 6 h OGD/24 h reperfusion treatment resulted in obvious cell death in astrocytes; cell viability of vehicle-treated cells exposed to OGD/reperfusion was decreased by 36.5% compared to normoxic control cells. The same OGD/reperfusion exposure caused similar decreases of 35.7% and 32.0% in cell viability of astrocytes pretreated with 10 nM E2 for 0.5 and 12 h, respectively. In contrast, pretreatment of cells with E2 for either 24 or 48 h effectively inhibited OGD/reperfusion-induced reduction of cell viability; astrocyte cell death in these two groups was 17.4% and 12.7%, respectively, significantly less than in vehicle-controlled cells (Fig. 1).

Many studies have shown that astrocytes undergo apoptosis by ischemic insult which may greatly contribute to the outcome of stroke [6, 38]. In the present study, apoptotic cells were determined by Hoechst 33258 staining, which allows determination and quantification of cells with fragmented and condensed chromatin. Fig. 2 shows that 6 h OGD plus 24 h reperfusion increased apoptosis in astrocytes (the percentages of apoptotic cells in culture with and without OGD/reperfusion were 33.0% and 0.7%, respectively). E2 treatment significantly attenuated the OGD/reperfusion-induced increase of apoptosis (apoptotic cell ratio, 12.8%).

The protective effect of E2 against OGD/reperfusion-induced cell death was further confirmed by the results from the LDH leakage assay. LDH leakage into the extracellular space indicates cell membrane damage, and LDH leakage has been widely used for detection of cell death. As shown in Fig. 3 OGD and subsequent reperfusion significantly increased LDH leakage. The LDH activities of media collected at 6 h OGD and 24 h reperfusion were increased 4.1- and 4.8-fold compared to the corresponding normoxic controls. LDH leakage from astrocytes pretreated with 10 nM E2 for 48 h was significantly reduced compared with those from vehicle-treated cells during both the 6 h period of OGD (40.2% reduction; Fig. 3A) and the 24 h reperfusion period (40.0% reduction; Fig. 3B).

Involvement of ER in E2-mediated protection

Effects of E2 could be mediated either by ER-dependent or -independent mechanisms, or both [39]. We showed that both classic isoforms of ER, ER α and ER β , are expressed in rodent cortical astrocytes (Fig. 4A). This finding is in agreement with others' reports [16, 40]. To test whether ERs were involved in E2-mediated astrocytic protection against ischemic injury, an ER antagonist, ICI 182,780, was used. As shown in Figs. 2 and 3 the application of ICI 182,780 completely blocked the protective effects of E2 on OGD/reperfusion-induced apoptosis and LDH leakage, while ICI-182,780 by itself had no effect. Following OGD/reperfusion, the apoptotic cell ratio in cultures treated with E2 plus ICI 182,780 was 31.8%, similar to values from vehicle-controlled cultures (Fig. 2). In the LDH

assay, ICI 182,780 was also found to abolish the protective effects of E2 against LDH leakage during 6 h OGD and during 24 h reperfusion (Fig. 3).

To specifically determine the roles of ER α and ER β , two selective agonists, PPT and DPN, were applied. As shown in Fig. 4B, only the ER α -selective agonist PPT was able to partially mimic the effect of E2 in decreasing OGD/reperfusion-induced LDH leakage, while the ER β selective agonist DPN had no significant effect. The effects of ER agonists were further confirmed by the cell viability assay, and similar findings were obtained. Only PPT, but not DPN, could simulate the effects of E2 to inhibit OGD/reperfusion-induced reduction of cell viability in astrocytes (Fig. 4C). The concentration used for both PPT and DPN was 10 nM, which has been shown to selectively activate the respective ERs [29, 32, 41]. Each agent at this concentration had no effect on cell death by itself in the absence of OGD (data not shown).

E2 suppresses ROS generation induced by OGD/reperfusion

Many studies have shown that ROS generation is increased in astrocytes under ischemic conditions using various models including OGD/reperfusion [24, 25, 42]. The increased ROS generation brings detrimental effects that may play a critical role during the process of cell death [4]. Mitochondria are a major source of cellular ROS. We previously showed that E2 suppresses mitochondrial ROS in whole brain tissue and in cerebral blood vessels under physiological conditions [29, 30], and inhibits the increase of mitochondrial ROS following ischemic insult [32]. Thus, we hypothesized that E2 would suppress ROS generation in astrocytes under ischemic condition.

Intracellular ROS levels were evaluated during the reperfusion period using the ROS-sensitive fluorescent dye HET. We found HET staining was not significantly different between the different groups of astrocytes at the initial time point for measurement (35 min reperfusion), most likely because it takes time for the dye to enter into the cell. For cells in the normoxic control condition, HET fluorescence slowly increased as the dye incubation continued. In contrast, cells exposed to OGD/reperfusion showed much more rapid ROS generation, with HET fluorescence increasing by 2.9-fold over a 30 min period ending 65 min after OGD. This is significantly higher than the corresponding values (increasing by 1.3-fold compared to the initial fluorescence) of normoxic controls. In contrast, cells treated with E2 showed more moderate increases in ROS levels, with the HET fluorescence increasing approximately 1.5-fold after 65 min reperfusion. Furthermore, addition of ICI 182,780 significantly blocked the effects of E2, with HET signal increasing by about 2.4-fold after 65 min reperfusion (Fig. 5).

E2 inhibits OGD/reperfusion-induced mitochondrial membrane potential loss

Proper mitochondrial function, including ATP generation and protein import, are largely dependent on maintenance of the mitochondrial membrane potential [43], and loss of mitochondrial membrane potential has been shown to be an important early event of ischemic injury [42, 44]. As shown in Fig. 6 cells in normoxic control conditions showed a typical TMRM mitochondrial pattern of perinuclear fluorescence. In contrast, TMRM intensity in cells exposed to 90 min OGD/30 min reperfusion was significantly decreased by 76.5% compared to normoxic control cells, indicating that mitochondrial membrane potential was severely depolarized. E2 treatment significantly inhibited the OGD/reperfusion-induced reduction of TMRM fluorescence. After 30 min of reperfusion, TMRM signals in E2-treated cells were approximately 57.5% of that in cells under normoxic control. Addition of ICI 182,780 abolished the effect of E2 resulting in a decrease in the TMRM signal by 67.3% compared to normoxic control-cells.

Effects on ATP Levels

Deprivation of glucose and oxygen induces disruption of cellular energy metabolism, ultimately causing ATP depletion [43]. To investigate the effects of E2 on OGD-induced ATP depletion, ATP levels in astrocyte cultures were determined after 6 h OGD exposure. As shown in Fig. 7 similar levels of ATP were found in cells treated with vehicle, E2, or E2 plus ICI 182, 780 under normoxic conditions. OGD treatment significantly reduced ATP levels by 44.9% compared to normoxic-control cells. Treatment with E2 alone exerted significant protective effects against OGD-induced ATP depletion. Following 6 h OGD, ATP levels in cultures treated with E2 were higher than vehicle-controlled cultures by 46.4%, although still lower than normoxic controls. The application of ICI 182,780 blocked the protective effects of E2 as evidenced by the finding that ATP levels in this group were similar to values from hypoxic vehicle-controlled cultures.

Discussion

Estrogen has proven protective properties against ischemic injury in animal models of stroke. However, the targets and the mechanisms of estrogen-mediated protective action still remain poorly understood. In the present study, the effects of E2 on primary cultures of astrocytes following ischemic insult were examined in an ischemia like *in vitro* model of oxygen and glucose deprivation. It was essential to select the time points where the impact of OGD was clear, but not so great that any protective effects of estrogen might be overwhelmed or obscured. Therefore, based on a previously published study from our laboratory concerning the effect of estrogen on cerebral endothelial cell viability [32] and some preliminary experiments in astrocytes, various time points were selected for determination of different parameters where change in each of the output measures could be determined, but this change was not so severe that possibility of seeing protective effects of estrogen would be eliminated. Our results demonstrate that E2 prevents OGD/reperfusion-induced cell death and mitochondrial dysfunction including enhanced ROS generation, mitochondrial membrane potential depolarization, and ATP depletion. Furthermore, we showed that the protective effects provided by E2 against *in vitro* ischemic injury were blocked by an ER antagonist, ICI 182,780, indicating that these effects were mediated by ER.

E2 is a pleiotropic hormone in the central nervous system targeting multiple sites to exert its protective effects. Astrocytes have been shown to play a critical role in E2-mediated protection under several different experimental conditions of brain injury, including ischemia [10, 12, 45]. OGD/reperfusion has been widely used as an *in vitro* ischemic model to simulate *in vivo* ischemia where oxygen and glucose are in deficit [43]. It has been shown that OGD induces cell death in primary cultured astrocytes [21, 42]. The present study showed that E2 significantly prevented OGD-induced cell death requiring a pretreatment duration not less than 24 h. This is consistent with the findings that long term pretreatment of E2 is required to protect against injury-induced cell death in cultured cortical explants as well as *in vivo* ischemic damage induced by middle cerebral artery occlusion [22, 46]. Similarly, studies from our laboratory and others found that short term (less than 24 h) E2 exposure failed to protect against OGD or chemical-induced cell death in other brain cell types, such as neurons and cerebral endothelial cells [32]. In contrast, one study reported that only short-term (0.5 h) pretreatment with E2 effectively protects against OGD-induced cell death in astrocytes [21]. One possible explanation for these different effects of E2 concerns different cell culture systems. The primary cultures of astrocytes used in latter study were 10 to 14 days *in vitro*; these have been shown to be more resistant to OGD in our preliminary studies as well as in studies of others [6, 42]. Therefore, it is possible that E2 would be more effective in eliciting a protective effect with younger astrocyte cultures. An important caveat to our study is that it focuses on the impact of ischemia on astrocytes in

culture at the earliest points in the evolution of stroke. How the protective effect of estrogen on astrocytes that we describe may carry over into brain protection given the complex interactions among cell types requires further exploration. Furthermore, time-dependent changes in astrocyte function that occur after the initial ischemic insult further complicate our ability to predict how our results may translate into *in vivo* effects. Nevertheless, our results using cultured astrocytes may help to inform future investigations.

E2 may act through multiple and diverse mechanisms, genomic and nongenomic, receptor and non-receptor mediated to exert protective effects [1, 26, 39]. Astrocytes have been found to express ERs, including ER α and ER β . In the present study, we conclude that the protective effects of E2 in astrocytes were ER-mediated mainly based on the following three points: firstly, all E2 protective effects against OGD-induced cell death and mitochondrial dysfunction were blocked by the ER antagonist, ICI 182,780. Second, the E2 concentration we used is 10 nM, a concentration similar to circulating levels of E2 found in cycling mice and consistent with the known affinity of E2 for its receptors. E2 at this physiological concentration exerts effects mainly through ER-dependent mechanisms, while E2 at micromolar concentrations shows antioxidant properties that are independent of ERs [26, 27]. Third, the protective action of E2 on astrocyte viability during ischemia can be imitated by an ER agonist selective for ER α , PPT, further supporting the important role of ER in E2-mediated effects. However, a study of neuronal cell death in ischemia/reperfusion found that much higher estrogen concentrations can also protect, albeit through different mechanisms that may not depend on classic estrogen receptors [47].

ER α and ER β are similar in their structures and ability to bind estradiol, but they are different in their cellular distribution and may have distinct roles [39]. Although the specific roles of ER α and ER β are unclear, increasing evidence suggests that ER α may play a critical role in the protective effect of E2 against brain injury including ischemic stroke [48]. It has been shown that the expression of ER α is increased in the cortex of ovariectomized animals after ischemia, while the expression of ER β remains unchanged, suggesting that astrocytic ER α may have a more important influence on ischemic injury [49]. By using transgenic mice with targeted deletion of ER α or ER β , many studies have shown that the presence of ER α , but not ER β , is a prerequisite for E2 to exert protective action against ischemic injury [50]. An *in vitro* study reported that the expression of ER α is decreased by hypoxia and glucose deprivation while ER β remains unchanged in astrocytes, and indicated that ER α may limit estrogen-mediated signaling during ischemic conditions [51]. In the present study, the protective effect of E2 on OGD-induced cell death was mimicked by an ER agonist selective for ER α (PPT), but not an ER agonist selective for ER β (DPN). This finding is in agreement with what we found previously that PPT, but not DPN, mimics the effects of E2 in cerebral endothelial cells under normoxic and ischemic conditions [29, 32]. Interestingly, a recent study showed that 5 min E2 treatment increases the level of ER α , but 24–48 h E2 treatment reduces the expression of ER α in astrocytes [52]. The different response of ER α expression to E2 treatment with different treatment durations may partially account for why at least 24 h pretreatment of E2 is required for the protective action of E2 against ischemic injury. Taken together, our results as well as others' studies particularly highlight an important role for ER α in E2-mediated protection against ischemic injury in astrocytes.

It is well known that mitochondria play a fundamental role in key cellular functions, including energy production, oxidative stress, and cell death. Mitochondria are particularly important for various types of brain cells due to the high metabolic demands of this organ. Estrogen may exert direct or indirect effects on mitochondrial function [12, 23]. A number of studies have suggested that mitochondria are critically involved in E2-mediated protection of neurons against insults induced by glutamate, glucose deprivation, beta-

amyloid peptide, hydrogen peroxide, and 3-nitropropionic acid [21, 26, 32, 39]. We previously showed that E2 increases levels of several mitochondrial respiratory chain proteins, including cytochrome c and complex IV subunits, in brain endothelial cells. Both *in vitro* and *in vivo* studies indicate that E2 reduces mitochondrial ROS production in neuronal cells and brain tissues [8, 29–31]. E2 also has been found to increase the expression or activity of the important mitochondrial-specific antioxidant enzyme, manganese superoxide dismutase, in cultured brain cells and brain tissues [29, 30]. These studies indicate that mitochondrial protection may be an important mechanism underlying the protective effects of E2. Thus, it could be expected that mitochondria also play a crucial role in protection by E2 of astrocytes.

Mitochondrial dysfunction is critically involved in the development of brain injury during cerebral ischemia. It has been implicated that mitochondria function as key modulators in the development of ischemic cell death in many brain cells including astrocytes [25]. Under ischemic conditions in which the delivery of oxygen and glucose are inadequate, mitochondrial oxidative phosphorylation is inhibited, resulting in more ROS generation [7, 25, 43]. Mitochondria are not only a primary source of ROS, but also a main target of ROS. Mitochondria undergo several prominent alterations in the early phase of ischemia that may contribute to eventual brain cell death, including changes in production of ROS, loss of mitochondrial membrane potential, and disruption of energy metabolism [43]. In the present study, our data in primary cultured astrocytes clearly showed that ROS were constantly increased after a relatively short-term OGD (up to 60 min) and reperfusion followed by a remarkable decrease of mitochondrial membrane potential and depletion of ATP. All of these effects were significantly inhibited by E2 in an ER-dependent manner. These findings are similar to those of our previous study in mouse brain endothelial cells and studies by others in human neuroblastoma cells [32, 44]. Taken together, these studies suggest that mitochondrial protection would be an important common mechanism for E2-mediated protection against ischemic brain injury. However, whether mitochondrial protection is a causative factor or a result of cellular protection is not yet known. Further studies will be necessary to investigate how E2 influences mitochondria under ischemia.

In conclusion, our results clearly demonstrate that estrogen protects cultured astrocytes from OGD/reperfusion-induced injury by ER-dependent mechanisms, and particularly highlight an important role of ER α in protecting astrocyte viability. Estrogen preserves mitochondrial function in the early phase of ischemic injury in astrocytes, indicating mitochondria as potential specific subcellular targets for estrogen-mediated action in the central nervous system during ischemia. Thus, strategies aimed to protect astrocytic mitochondria might be useful in developing therapies to attenuate ischemic brain injury.

Highlights

Primary cultured brain astrocytes were deprived of oxygen and glucose *in vitro*.
Estrogen protects from cell death via estrogen receptor alpha.
Estrogen inhibits reactive oxygen species production.
Estrogen preserves mitochondrial function and cell ATP.
The protective actions of estrogen are mediated by estrogen receptors.

Acknowledgments

This study was supported by the United States National Heart, Lung and Blood Institute (R01 HL-50775) and the National Natural Science Foundation of China (81102424, 81020108031, 31079630, and 30973558). Jiabin Guo is a recipient of Doctoral Scholarship from China Scholarship Council (20073020).

We thank Dr. Hongzheng. Yin and Allan Jay Acab for technical help in cell culture and live imaging experiments. We also thank Dr. Douglas Wallace and the U.C. Irvine Center for Molecular and Mitochondrial Medicine and Genetics for use of facilities.

Abbreviations

BSS	balanced salt solution
DPN	2,3-bis(4-hydroxyphenyl)-propionitrile
E2	17 β -estradiol
ER	estrogen receptor
FCCP	carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
FK	full kill
HET	hydroethidine
LDH	lactate dehydrogenase
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide
OGD	oxygen glucose deprivation
PPT	4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)-trisphenol
ROS	reactive oxygen species
TMRM	tetramethylrhodamine methyl ester

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Fig. 1.

E2 attenuates OGD/reperfusion-induced reduction of cell viability. Primary cultured cortical astrocytes were pretreated with either vehicle (0.02% ethanol) or E2 (10 nM) for various times (0.5–48 h) before exposure to 6 h OGD and 24 h reperfusion; all drug treatments were continuously maintained throughout OGD and reperfusion. Cell viability was determined by MTT conversion method. Data are the mean $\pm$ SEM from a representative experiment performed in quadruplicate. The experiment was repeated at least three times, and similar results were obtained. *, Significantly different from control without OGD/reperfusion exposure (Ctrl). #, Significantly different from vehicle-treated cells exposed to OGD/reperfusion ($P < 0.05$).

Fig. 2.

Effect of E2 on OGD/reperfusion-induced apoptosis in astrocytes. Astrocytes were pretreated with either vehicle (0.02% ethanol) or E2 (10 nM) for 48 h. Some astrocytes were pretreated with the ER antagonist ICI-182,780 (ICI; 100 nM) for 0.5 h, and the concentration of ICI was maintained during E2 or vehicle treatment. Cultures were then exposed to 6 h OGD and 24 h reperfusion. All drugs were continuously maintained during OGD and reperfusion. Immediately after OGD/reperfusion, cells were fixed and apoptotic cells were detected by Hoechst 33342 staining. (A) Representative fluorescence images shown are nucleus staining with Hoechst 33342. Scale bar, 100 μ m. (B) Quantitative analysis of apoptotic nuclei in cultures. Data are shown as mean $\pm$ SEM. *, Significantly

different from control without OGD/reperfusion exposure (Ctrl). #, Significantly different from vehicle-treated cells exposed to OGD/reperfusion ($P < 0.05$).

Fig. 3.

An ER antagonist, ICI-182,780 (100 nM), abolishes the protective effect of E2 during OGD (A) and reperfusion (B). Astrocytes were pretreated with either vehicle (0.02% ethanol) or E2 (10 nM) for 48 h. In some cells, ICI-182,780 (100 nM) was first applied for 0.5 h; the concentration of this antagonist was then maintained during E2 or vehicle treatment. Cells were then exposed to 6 h OGD and 24 h reperfusion. All drugs were continuously maintained during OGD and reperfusion. Media were sampled for measurement of LDH activity (A) immediately after 6 h OGD and (B) after 24 h reperfusion. Values shown are the mean $\pm$ SEM ($n = 4$) from a representative experiment. The experiment was repeated for at least three times, and similar results were obtained. *, Significantly different from control

without OGD/reperfusion exposure (Ctrl); #, Significantly different from vehicle-treated cells exposed to OGD/reperfusion ($P < 0.05$).

Fig. 4.

Effects of ER-selective agonists on OGD/reperfusion-induced cell death. A. Western blots show presence of both ERα and ERβ in cultured astrocytes. B and C: Astrocytes were pretreated with E2 (10 nM), PPT (10 nM), DPN (10 nM), or vehicle (0.02% ethanol) for 24 h. Subsequently, cells were exposed to 6 h OGD and 24 h reperfusion. All drugs were continuously maintained during OGD and reperfusion. Cell death was evaluated by LDH leakage (B) and cell viability assay (C). Values shown are the mean ± SEM (n = 4) from a representative experiment. Similar results were obtained from three independent experiments. *, Significantly different from control without OGD/reperfusion exposure

(Ctrl). #, Significantly different from vehicle-treated cells exposed to OGD/reperfusion ($P < 0.05$).

Fig. 5.

E2 suppresses ROS generation induced by OGD/reperfusion in astrocytes. Cells were pretreated with either vehicle (0.02% ethanol) or E2 (10 nM) for 24 h. Some astrocytes were pretreated with the ER antagonist ICI-182,780 (ICI; 100 nM) for 0.5 h, and the concentration of ICI was maintained during E2 or vehicle treatment. Cells were then exposed to 60 min OGD followed by reperfusion (Rep). All drugs were continuously maintained during OGD and reperfusion. Cellular ROS production was monitored by hydroethidine (HET) staining. The traces represent the time course of ROS generation during 35 to 65 min reperfusion. For each cell, HET fluorescence intensity (Ft) was normalized to fluorescence at 35 min reperfusion as F0 for that cell. Data shown are mean $\pm$ SEM of 18 to 33 cells from an experiment representative of 6 to 16 experiments.

Fig. 6. E2 inhibits OGD/reperfusion-induced mitochondrial membrane potential loss in astrocytes. Cells were pretreated with either vehicle (ethanol 0.02%) or 10 nM E2 for 24 h. Some astrocytes were pretreated with the ER antagonist ICI-182,780 (ICI; 100 nM) for 0.5 h, and the concentration of ICI was maintained during E2 or vehicle treatment. Cells were then exposed to 90 min OGD followed by reperfusion (Rep). All drugs were continuously maintained during OGD and reperfusion. Mitochondrial membrane potential was measured by TMRM. (A) Examples of TMRM in cells under normoxic control or exposed to 90 min OGD and 30 min reperfusion. Scale bar, 50 μ m. (B) Mitochondrial membrane potential changes were recorded during 30 to 50 min reperfusion. TMRM fluorescence intensity (Ft)

was normalized to fluorescence intensity in the same area after exposure to FCCP (F_{FCCP}). Data are expressed as mean $\pm$ SEM of 31 to 39 cells from a representative experiment. Similar results were obtained from at least 9 independent experiments.

Fig. 7.

E2 partially preserves cellular ATP levels in astrocytes exposed to 6 h OGD. Primary cultured cortical astrocytes were pretreated with either vehicle (0.02% ethanol) or E2 (10 nM) for 24 h before exposure to 6 h OGD. Some astrocytes were pretreated with the ER antagonist ICI-182,780 (ICI; 100 nM) for 0.5 h, and the concentration of ICI was maintained during E2 or vehicle treatment. Cells were then exposed to 6 h OGD; the concentrations of all drugs were continuously maintained. Values shown are mean $\pm$ SEM ($n = 4$) from a representative experiment. Similar results were obtained from three independent experiments. *, Significantly different from control without OGD/reperfusion (Ctrl); #, Significantly different from vehicle-treated cells exposed to OGD ($P < 0.05$).