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Estrogen inhibits Fas-mediated apoptosis in experimental stroke

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Abstract

Estrogen is protective in experimental cerebral ischemia, yet the mechanism remains unclear. Fas-mediated apoptosis has been shown to be induced after cerebral ischemia and significantly contribute to ischemic brain damage. In this study, we tested if estrogen is protective against cerebral ischemia by suppressing Fas-mediated apoptosis. 17 β -estradiol-treated and untreated ovariectomized (OVX) female mice were subjected to 2 h middle cerebral artery occlusion (MCAO). Expression of Fas and Fas-associated death domain (FADD) were measured at 3, 6 and 12 h of reperfusion by RT-PCR and Western blot, respectively. Post-ischemic activities of caspase-8 and -3 activities, the two downstream effectors of Fas-induced apoptosis, were also assayed at same time points by ELISA. Finally, Fas antibody-induced cell death in primary cortical neurons was assayed by fluorescence activated cell sorter (FACS) in the presence and absence of estradiol. Our data showed that estradiol-treated OVX female mice sustained smaller infarct compared to untreated OVX mice. Ischemia upregulated Fas and FADD expression, and increased caspase-8 and -3 activities in OVX female mouse cortex, which were significantly attenuated by estradiol. Estradiol also significantly inhibited Fas antibody-induced neuronal cell apoptosis. Our data suggests that inhibition of ischemia-induced Fas-mediated apoptosis is an important mechanism of neuroprotection by estrogen in cerebral ischemia.

Keywords

Fas-mediated apoptosis; Estrogen; Neuroprotection; Ischemia

Introduction

The female sex hormone 17 β -estradiol has been shown to protect brain from ischemic damage, but the mechanisms of neuroprotection and signaling pathways used by estrogen are not completely understood (Hurn and Macrae, 2000). Recent clinical trials demonstrated that

hormone replacement therapy in postmenopausal women failed to reduce stroke risk and mortality, in contrast to early epidemiological studies and a wealth of experimental evidence (Hurn and Brass, 2003). The discrepancy between clinical and pre-clinical studies underscores the need to further study molecular targets of estrogen in brain and the molecular mechanisms of its neuroprotective action.

Mechanisms of neuronal cell death after cerebral ischemia have not been fully understood. Both necrotic and apoptotic cell death are present at the affected regions (Dirnagl et al., 1999). An increasing body of work has shown that Fas/FasL (Fas Ligand) play an important role in the pathology of ischemic stroke (Rosenbaum et al., 2000; Mehmet, 2001; Ruan et al., 2008; Liu et al., 2008). Fas (CD95/APO-1) belongs to the tumor necrosis factor (TNF) receptor superfamily. They both share a “death domain” and signal apoptosis through well established pathways. Upon activation, the cytoplasmic adaptor protein Fas-associated Death Domain (FADD) associates with the death domain of Fas, followed by recruitment and activation of caspase-8. These three molecules form the Death-Inducing Signalling Complex (DISC) that triggers apoptosis. Fas ligand (FasL) is also a critical component in death receptor-mediated apoptosis. Both Fas and FasL are upregulated by cerebral ischemia in brains of developing and adult mice (Felderhoff-Mueser et al., 2000, 2003; Matsuyama et al., 1995; Jin et al., 2001). Antibody-mediated neutralization of FasL reduces injury after stroke (Martin-Villalba et al., 2001). Moreover, mice with mutant Fas (lpr mice) sustained less ischemic damage compared to wild-type mice (Martin-Villalba et al., 1999; Rosenbaum et al., 2000). Although recent studies suggest that estrogen can reduce inflammation and Fas induction by LPS stimulation and hypoxia (Dimayuga et al., 2005; Gerstner et al., 2007), it is not clear whether estrogen also acts through the Fas/FADD pathway and protects brain from ischemia *in vivo*. Hence, in the current study, we will determine if estrogen protects brain from ischemic damage via inhibiting Fas/FADD-mediated apoptosis.

Materials and methods

Animals

Studies were approved by Animal Care and Use Committee at Nanjing University in Nanjing, China. Kunming female mice (25–30 g, n = 10/group) were purchased from a commercial source (Qinglongshan Animal Feeding Center, Nanjing, China).

Ovariectomy (OVX) and estrogen replacement

Ovariectomy in mice was performed 1 week before MCAO, and estradiol was replaced by implanting a silastic capsule (0.06200ID/0.12500OD) filled with either 0.035 ml of sesame oil or 17 β -estradiol (180 μ g/ml; Sigma, St. Louis, MO, USA) (McCullough et al., 2003). Serum estradiol level was measured by radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA, USA). OVX+estradiol, 58.1 $\pm$ 7.2 pg/ml vs OVX, 14.3 $\pm$ 0.2 pg/ml).

Middle cerebral artery occlusion (MCAO) in mice

Transient focal cerebral ischemia was induced in spontaneously breathing mice. The animals were anesthetized with an intraperitoneal injection of Sodium Pentobarbital (1%) at a dose of 45 mg/kg. Body temperature was maintained at 37 $\pm$ 0.5 $^{\circ}$ C by a heating lamp and heating pad. Middle cerebral artery occlusion (MCAO) was achieved by the intraluminal filament methods as previously described (Alkayed et al., 2001). Briefly, a midline neck incision was made under a dissecting microscope; the right common carotid artery and external carotid artery (ECA) were isolated. The ECA was ligated with 6–0 silk suture distal from carotid bifurcation and ECA branch was then cut distal to ligation point. Another 6–0 silk suture was tied loosely around ECA close to the common carotid artery. A 6–0 monofilament nylon suture with heat-rounded tip was introduced into a small incision on ECA and advanced to the origin of the

middle cerebral artery (6 mm from internal carotid/pterygopalatine artery). The silk suture around the ECA stump was tied tightly to prevent bleeding and secure the nylon suture. Mice were subjected to 2 h of occlusion and cerebral infarction was determined at 24 h reperfusion by 2, 3, 5-triphenyltetrazolium chloride (TTC) histology. Slices were photographed, and images were analyzed with image-analysis software (OSIRIS 4.19, Switzerland). Infarct volume in all slices was expressed as a percentage of the contralateral hemisphere after correcting for edema as previously described (Zhang et al., 2007).

Primary cortical neuronal culture

Primary cortical cultured neurons were prepared as previously described (Xu et al., 2003). Cells were prepared from fetal brain tissue on gestational day 16–17. The cortices of the fetus were carefully dissected with care to avoid including the hippocampus and striatum. Separate cortices were treated with trypsin for 4 min at 37 °C, triturated with a pipette and then with a flamed glass pipette. After dissociation, the cells were counted using trypan blue exclusion to determine the number of live cells. Cells were plated onto 24-well poly-D-lysine coated plastic plates at 1×10^6 cells per well. The growth medium was composed of serum-free, phenol red-free and estrogen-free Neurobasal medium supplemented with B27 (2.0 ml to 100 ml Neurobasal. Invitrogen, Carlsbad, CA), with no antibiotics.

Fluorescence Activated Cell Sorter (FACS) analysis of Fas induced apoptosis

Neurons at 10 day in vitro were preincubated with 10 nM 17 β -estradiol (Sigma, St. Louis, MO) for 15 min and then exposed to 2 μ g/ml anti-Fas monoclonal antibody (BD Biosciences Pharmingen, U.S.A) for 24 h. Neurons were harvested, pre-washed with staining buffer and resuspended in staining buffer containing 7-amino-actinomycin D (7-AAD) (20 μ g/ml) at a concentration of 1×10^6 cells/ml. The cell suspension was placed at 4 °C in the dark for 30 min. The cells were rewashed with staining buffer before they were added to the flow cytometer (Becton Dickinson, San Jose, CA).

Western blotting for Fas and FADD protein

Cortical tissue was dissected from ipsilateral and contralateral hemispheres of estrogen-treated or non-treated OVX Kunming mice subjected to 2 h MCAO followed by 3, 6 and 12 h reperfusion. Protein was extracted by homogenizing brain tissue in 50 mM Tris-HCl (pH 7.4) buffer containing 4 mM EDTA, 25 mM KCl, 1 mM PMSF, 1 μ g/ml leupeptin and pepstatin, 1 mM sodium orthovanadate, and 1% Triton X-100. The homogenate was centrifuged at 15,000 g for 5 min. Supernatant was collected and protein concentration in the supernatant was measured using the Bradford method. Extracted protein was separated by SDS-PAGE and blotted onto Immobilon membranes. Membranes were probed with primary antibodies against Fas (Pharmingen, 1:1000) or FADD (raised in our Lab, 1:500), and finally detected with enhanced chemiluminescence as previously described (Xu et al., 2003). α -Tubulin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as loading controls. The developed films were scanned and quantified by a densitometer (Bio-Rad, Hercules, CA).

RT-PCR analysis for FADD mRNA

Tissue sampling was the same as described above for Western blot. Total RNA was extracted using TRIzol (Invitrogen). cDNA was reverse transcribed from total RNA using oligo-dT primer and amplified with gene-specific primers. FADD mRNA levels were normalized to GAPDH and expressed as the ratios of FADD/GADPH. Primer sequences used in this study were: 5'-TGGCCTGGACCTGTTTCACG-3' (FADD forward primer), 5'-GACTTCGGGGGTACTTCTCCTCA-3' (FADD reverse primer); 5'-AACGACCCCTTCATTGAC-3' (GAPDH forward primer), and 5'-TCCACGACATACTCAGCAC-3' (GAPDH reverse primer). The thermal parameters were:

94 °C for 5 min, followed by 30 cycles of 94 °C 40 s, 58 °C 40 s, 72 °C 40 s and 72 °C 10 min. The PCR products were analyzed on 1.5% agarose gels and visualized by ethidium bromide. The size of the products was 287 bp for FADD and 191 bp for GAPDH. The gel was visualized with UV-transilluminator and photographed. Quantification of digital images of DNA bands was performed with spot densitometry.

Caspase-8 and -3 activity assay

Tissue sampling was the same as described above for Western blot. Activities of caspase-8 and -3 were measured using commercial kits (R&D Systems, Minneapolis, MN). Briefly, after digestion with 1 × trypsin, the homogenate of cortical tissue was transferred into a cell strainer. Single cells were collected by centrifugation at 250 g for 10 min and incubated with cell lysis buffer on ice for 10 min. Protein concentration was determined using the Bradford measurement. The assay is based on detection of cleavage of specific substrates IETD-AFC (AFC: 7-amino-4-trifluoromethyl coumarin) for caspase-8 and DEVD-AFC for caspase-3. The cleavage of the peptide by the caspase releases the fluorochrome that, when excited by light at 400 nm wavelength, emits fluorescence at 505 nm, which is quantified using fluorescence detector.

Statistical analysis

Data were expressed as the mean±SEM and analyzed with a t-test for two groups and one-way analysis of variance (ANOVA) with post hoc Newman–Keuls multiple range test for multiple groups. SigmaStat 11.5 (SPSS) was used for analysis. $p<0.05$ was considered statistically significant.

Results

Estrogen reduced infarct size after MCAO in mice

To confirm that estrogen has a neuroprotective effect *in vivo*, we examined the ability of estrogen to reduce infarct size after MCAO in mice. As expected, estrogen replacement (E2) reduced mice infarct size after MCAO (Fig. 1). (n = 10 per group, $p<0.05$).

Estrogen suppressed ischemia-induced Fas and FADD expression in mouse cortex

To investigate if Fas is involved in the pathological progress of stroke, Fas protein was measured by Western blot. Fas expressed at low levels in the cortex of OVX mice with or without estrogen treatment before MCAO (Fig. 2, sham). Protein levels were significantly induced by MCAO in the ipsilateral cortex of OVX mice at 3, 6 and 12 h reperfusion. The induction of Fas in OVX mice was significantly attenuated by estrogen replacement at all the time points examined (Fig. 2). Also, FADD mRNA levels were determined by RT-PCR (Fig. 3A). Cortical FADD mRNA was induced on the ischemic side in OVX mice at 3, 6 and 12 h after MCAO compared to both contralateral cortex and sham-operated animals. The peak induction of FADD mRNA occurred at 6 h after MCAO, which was significantly suppressed by Estrogen (Fig. 3B). In accordance with mRNA levels, FADD protein level also responded to estrogen (Fig. 3C). Low expression of FADD protein was detected in cortex of OVX mice before MCAO (data not shown). However, FADD protein level was significantly upregulated in cortex at 3, 6 and 12 h after MCAO, which was decreased by estrogen at 6 and 12 h of reperfusion. The experiments were performed at least in triplicate.

Estrogen suppresses Fas -induced apoptosis in neurons

To further determine if estrogen could inhibit Fas-induced apoptosis in neurons directly, various amounts of anti-Fas-antibody (0.5, 1, 2 and 4 µg/ml) were tested for their ability to induce apoptosis in cells at 0, 3, 6, 12, 24 and 48 h by FACS (data not shown). Peak induction

of neuronal apoptosis was detected in cell incubation with 2 µg/ml Fas-antibody at 24 h (Fig. 4). At this dose of Fas-antibody, estrogen (E2, 10 nM) decreased neuronal death by 50.0% ($p<0.05$). The experiments were performed at least in triplicate.

Estrogen suppressed caspases-8 and -3 activities induced by cerebral ischemia

Activities of two downstream effective caspases in Fas-mediated apoptosis, caspase-8 and -3 were assayed (Figs. 5A and B). Cortical caspase-8 and caspase-3 activities in ipsilateral side of OVX mice at 3, 6 and 12 h reperfusion after MCAO were significantly higher than those in contralateral sides ($p<0.05$), whereas estrogen-replaced mice exhibited lower cortical caspase-8 ($p<0.05$) and caspase-3 ($p<0.05$) activities in ipsilateral cortex compared to OVX mice without estrogen treatment. All assays were performed in at least three independent experiments.

Discussion

The major findings of our study are: 1) key components in Fas-mediated apoptosis (Fas, FADD and caspase-8) were induced in brain by cerebral ischemia, 2) post-ischemic induction of Fas and FADD was significantly reduced by estrogen, 3) neuronal apoptosis induced by Fas-antibody was inhibited by estrogen, and 4) the downstream effector of caspase-8, caspase-3, also was induced by cerebral ischemia and suppressed by Estrogen. These findings suggest that activation of Fas-mediated apoptosis could be a causative event leading to the ischemic brain damage, and that estrogen's neuroprotective properties might be in part mediated through its inhibitory effects on Fas/FADD/caspase-8-mediated apoptosis.

Experimental evidence suggests that the apoptotic pathway mediated by Fas contributes to cell death after stroke. Fas and FasL are upregulated by cerebral ischemia in brain of experimental mice (Felderhoff-Mueser et al., 2000, 2003; Matsuyama et al., 1995; Jin et al., 2001). Mice with mutant Fas, the so-called *lpr* mice, are protected against ischemic brain injury compared to wild-type controls (Martin-Villalba et al., 1999; Rosenbaum et al., 2000). The studies that investigate FADD expression pattern in brain after ischemia are still lacking. Here we report that FADD is expressed at low level in cerebral cortex under non-ischemic conditions and its expression was promptly induced by ischemia as early as 3 h reperfusion and lasted up to 12 h after MCAO in OVX mice. We also confirmed that experimental ischemia induced Fas expression, and found that FLIP expression remained unchanged in OVX mice after MCAO (data not shown). The fact that ischemia induced positive regulators of Fas-mediated apoptosis (Fas and FADD), but not the negative regulator FLIP, suggests that Fas/FADD-mediated apoptosis is an endogenous mechanism of brain damage after ischemia. Our data showed that estrogen not only reduced Fas induction but also inhibited FADD during ischemia, suggesting that estrogen protects brain through multiple targets on Fas-mediated apoptotic pathway.

Previous studies showed that Fas plays a critical role in the apoptosis process during T cell development (Bharhani et al., 2006; Saito et al., 2007). Monoclonal antibodies recognizing Fas such as Jo2 have cytolytic activity on cell expressing Fas. The cell death caused by anti-Fas antibodies is characteristic of apoptosis and suggests that the lethal effects are a result of interaction of antibody with a functional Fas antigen. We further confirmed our *in vivo* findings and showed that anti-Fas antibody was sufficient to induce cell death in primary cultured neurons and 17β-estradiol reduced its expression.

Numerous mechanisms have been proposed and are under investigation in order to understand the neuroprotective properties of estrogen. We have previously shown that estrogen is neuroprotective against ischemic damage both *in vitro* and *in vivo* (Xu et al., 2006). Another study showed that estrogen can inhibit cell apoptosis through upregulation of post-ischemic bcl-2 (Alkayed et al., 2001). Other studies indicated that FasL has pathological function on

stroke and mutation of FasL protects brain from ischemic injury (Rosenbaum et al., 2000; Mehmet, 2001; Liu et al., 2008). This study provided additional evidence that estrogen can reduce cell death during ischemia through inhibiting Fas-mediated apoptotic pathway. Previous research has shown that estradiol is protective in experimental stroke at both physiological and pharmacological concentrations (Hoffman et al., 2006; Merchenthaler et al., 2003; Yang et al., 2000). However, the mechanisms underlying neuroprotection by physiological vs pharmacological doses of estradiol are likely different. When administered several days before cerebral ischemia, as in our study, physiological levels of estradiol likely attenuate brain injury by acting through the classical nuclear estrogen receptors to suppress neuronal apoptosis and other mechanisms via estradiol's genomic actions. At pharmacological doses, on the other hand, estradiol also displays acute neuroprotective effects even when administered 3 h after vascular occlusion in rodent stroke models, but the mechanisms of protection in this case are likely related to estradiol's rapid effects on membrane-associated receptors, ion channels and signal transduction pathways, culminating in such protective actions of estradiol as vasodilation, and anti-inflammatory and antioxidant actions. Thereby, estrogen is a potent pleiotropic hormone that exhibits an array of actions through multiple mechanisms.

In conclusion, our study suggests that estrogen inhibition of Fas-mediated apoptosis after cerebral ischemia is an important mechanism of neuroprotection, and that disruption of Fas-mediated signaling by pharmacological inhibition of key components in this pathway may represent a more targeted and mechanism-based therapeutic strategy against brain damage following stroke.

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Fig. 1. Estrogen reduced mice infarct size after MCAO. Female mice were ovariectomized and 17β -estradiol was implanted by a silastic capsule. Ischemia was induced by MCAO for 2 h. Panel A shows actual images, and bar graph (B) shows data summary. *versus OVX ($p < 0.05$).

Fig. 2.

Estrogen suppressed ischemia-induced upregulation of Fas protein in OVX mice. Upper panel is representative Western blot, and the lower panel is a summary graph of Fas protein levels relative to GAPDH. Significant induction of Fas expression was detected at 3, 6 and 12 h reperfusion after MCAO. Induction of Fas was attenuated by estrogen at all the time points.

*versus sham; **versus non-treated OVX mice ($p < 0.05$).

Fig. 3.

Estrogen inhibited ischemia-induced FADD expression in OVX mice. Levels of FADD mRNA and protein were measured with RT-PCR (A and B) or Western blot (C), respectively. Final results were normalized to GAPDH levels. (A) FADD mRNA levels were significantly higher in ischemic cortex of OVX mice at 3, 6 and 12 h reperfusion compared to contralateral cortex (control) as well as those in sham-treated mice without MCAO. (B) Estrogen replacement attenuates ischemia-upregulated FADD mRNA in ipsilateral cortex of OVX mice at 6 h reperfusion. (C) Estrogen-replaced mice exhibited significantly lower FADD protein levels in ipsilateral cortex compared to OVX mice at 6 and 12 h reperfusion. *versus control; **versus non-treated OVX mice ($p < 0.05$).

Fig. 4. Estrogen inhibited Fas antibody-induced apoptosis in primary cortical neuronal cultures. Cells incubated with 2 $\mu\text{g/ml}$ Fas antibody exhibited 19.23% increased apoptosis compared to control cells at 24 h after incubation, which was decreased by 50.0% in the presence of estrogen (E2, 10 nM). *versus control group; **versus Fas induced group without estrogen ($p < 0.05$).

Fig. 5.

Estrogen suppressed ischemia-induced caspase-8 and -3 activities. Panel A shows caspase-8 activities and panel B shows caspase-3 activities. Activities of both caspases were induced by ischemia at all the time points, which were significantly reduced by estrogen (E2). *versus control; **versus non-treated OVX mice ($p < 0.05$).