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PRETREATMENT WITH A SINGLE ESTRADIOL-17 β BOLUS ACTIVATES CYCLIC-AMP RESPONSE ELEMENT BINDING PROTEIN AND PROTECTS CA1 NEURONS AGAINST GLOBAL CEREBRAL ISCHEMIA

A. P. Raval^{a,*}, I. Saul^a, K. R. Dave^a, R. A. Defazio^a, M. A. Perez-Pinzon^{a,b}, and H. Bramlett^{b,c}

^aCerebral Vascular Disease Research Center, Department of Neurology (D4-5), P.O. Box 016960, University of Miami School of Medicine, Miami, FL 33101, USA

^bNeuroscience Program, Leonard M. Miller School of Medicine, University of Miami, Miami, FL 33101, USA

^cDepartment of Neurological Surgery, Leonard M. Miller School of Medicine, University of Miami, Miami, FL 33101, USA

Abstract

Estradiol-17 β is released from the ovaries in a cyclic manner during the normal estrous cycle in rats. During the transition from the diestrous to proestrous stage, the 17 β -estradiol increases in blood circulation. We hypothesized that a higher serum level of endogenous 17 β -estradiol would protect hippocampal pyramidal neurons against global cerebral ischemia via activation of the cyclic-AMP response element binding protein (CREB)-mediated signaling cascade. Furthermore, we asked if a single 17 β -estradiol bolus provides protection against ischemia in the absence of endogenous estradiol. To test these hypotheses, rats were subjected to global cerebral ischemia at different stages of the estrous cycle. Ischemia was produced by bilateral carotid occlusion and systemic hypotension. Brains were examined for histopathology at 7 days of reperfusion. Higher serum levels of 17 β -estradiol (at proestrus and estrus stages) correlated with increased immunoreactivity of pCREB in hippocampus and ischemic tolerance. At diestrus, when circulating gonadal hormone concentrations were lowest, the pCREB protein content of hippocampus was reduced and showed the least number of normal neurons after ischemia compared to other stages of the estrous cycle. A similar phosphorylation pattern was also observed for mitogen-activated protein kinase (MAPK) and calcium-calmodulin-dependent protein kinase (CaMKII) in hippocampus. The cyclic variation in ovarian hormones did not reflect phosphorylation of protein kinase B (Akt). To test the efficacy of a single bolus of 17 β -estradiol before ischemia, ovariectomized rats were treated with 17 β -estradiol (5/10/50 μ g/kg) or vehicle (oil) and 48/72/96 h later rats were exposed to cerebral ischemia. A single 17 β -estradiol bolus treatment in ovariectomized rats significantly increased CREB mRNA activation and protected CA1 pyramidal neurons against ischemia. These results suggest that an exogenous bolus of 17 β -estradiol to ovariectomized rats protects hippocampus against ischemia via activation of the CREB pathway in a manner similar to the endogenous estrous cycle.

Keywords

in vivo; signal transduction; cerebral blood flow; neuroprotection; cyclic-AMP response element binding protein; hormone replacement therapy

The decline in circulating estrogen in post-menopausal women dramatically increases the risk of stroke (Sacco et al., 1997; Hurn and Macrae, 2000; Bushnell et al., 2008). There is strong evidence that chronic 17 β -estradiol treatment has both potent and long-lasting effects on improved pathophysiological outcome after brain ischemia in experimental animal models (Alkayed et al., 2000; Jover et al., 2002; McCullough and Hurn, 2003).

The Women's Estrogen for Stroke Trial (WEST) and Women's Health Initiative (WHI) were initiated based on promising preclinical data regarding neuroprotection. The outcomes from this trial were, however, unsuccessful and questions emerged about the safety of chronic estrogen treatment and the timing of initiation of estrogen therapy in women. A recent experimental study in ovariectomized (OvX) rats focused on defining the appropriate time to initiate the estrogen therapy and demonstrated that estrogen replacement after an extended period of hypoestrogenicity (10 weeks after ovariectomy) prevented estrogen from protecting the brain against ischemic injury. This study helped explain one aspect of WHI results that reported no beneficial effect of estrogen therapy against ischemic stroke: the majority of the subjects received estrogen therapy after an extended period of hypoestrogenicity (years after onset of menopause) (Suzuki et al., 2007). In this context, due to concerns regarding the safety of chronic estrogen treatment, we have focused on the identification of alternative estrogen treatment strategies for post-menopausal women.

In contrast to the well-documented experimental benefits of the chronic 17 β -estradiol treatment strategy, our recent study demonstrated that a single 17 β -estradiol bolus 48 h prior to ischemia induces neuroprotection in the hippocampal CA1 region in an *in vitro* model of global cerebral ischemia (Raval et al., 2006). We also showed that 17 β -estradiol pretreatment rescues the hippocampal CA1 region from subsequent ischemic damage via cyclic-AMP response element binding protein (CREB) activation (Raval et al., 2006). Further, phosphorylation of CREB required activation of mitogen-activated protein kinase (MAPK) and calcium-calmodulin-dependent protein kinase (CaMKII). Inhibition of either MAPK or CaMKII after 17 β -estradiol pretreatment reduced pCREB levels and abolished hippocampal protection against ischemia (Raval et al., 2006). A caveat of our study is that the organotypic slices are devoid of blood circulation and hippocampal cells are not exposed to the natural fluctuation of ovarian hormones in cultured conditions. Thus, in the current study employing a rat model of global cerebral ischemia, we aim to gain a better understanding of the cellular and molecular mechanisms by which endogenous estrogen fluctuations protect the brain in females.

In order to understand the mechanism(s) of neuroprotection induced by estrogen it is important to understand its pattern of release during the reproductive cycle. The estrous cycle in rat is 4–5 days (Hotchkiss and Knobil, 1994). The estrous cycle is divided into four main stages, namely: (1) proestrus, (2) estrus, (3) metestrus and (4) diestrus. During transition from the diestrus to the proestrous stage (follicular phase of the menstrual cycle in women) the 17 β -estradiol (most potent estrogen) level increases. The next transition from the proestrous to estrous stage is characterized by a pre-ovulatory surge of 17 β -estradiol and luteinizing hormone with the gradually increasing influence of progesterone corresponds to the ovulatory phase in humans. Subsequently, the transition from estrus to metestrus (luteal phase) is associated with higher titers of circulating progesterone. At diestrus, circulating gonadal hormone concentrations are lowest, which correlates to the late luteal phase and menstruation in the human female (Hotchkiss and Knobil, 1994).

Based on the fact that 17β -estradiol is released in cyclic fashion and that increasing titers of 17β -estradiol prevail during the transition from diestrus to proestrus, we hypothesize that a higher serum level of endogenous 17β -estradiol protects the hippocampus by activating the CREB-mediated signaling cascade in the CA1 region, thus protecting neurons against global cerebral ischemia. Furthermore, we asked if a single 17β -estradiol bolus provides protection against ischemia in the absence of endogenous estradiol. The knowledge gained from this study could be directed toward a new generation of single bolus 17β -estradiol therapeutic approaches.

EXPERIMENTAL PROCEDURES

All animal procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and were approved by the Animal Care and Use Committee of the University of Miami. According to these guidelines, efforts were made to minimize the number of animals and their discomfort.

Estrous cycle monitoring and ovariectomy

Female Sprague–Dawley rats 280 ± 20 g were used for the study. Daily vaginal smears of female rats were collected (between 9:00 and 10:00 AM) and identification of cell types was made microscopically (Marcondes et al., 2002). Only rats showing at least three consecutive normal period (4 days) estrous cycles were used for experiments. Ovariectomy was performed in the diestrus stage, using sterile procedures, by making a 1 cm incision on both sides of the back to expose the ovaries retroperitoneally. The ovaries were clamped and removed with the fallopian tubes being ligated, and the skin was then sutured. For sham-procedure, 1 cm incision on both sides of the back to expose the ovaries retroperitoneally were made and sutured without removal of ovaries. The plasma concentration of estradiol was measured by radioimmunoassay using a “Coat-a-Count” kit (Diagnostic Products Corporation) for all experimental groups (Fig. 1).

Production of cerebral ischemia

Rats were subjected to cerebral ischemia at different stages of the estrous cycle, at 7 days after ovariectomy and 48 h after 17β -estradiol (5, 10 or 50 $\mu\text{g/kg}$)\vehicle (peanut oil) treatment. Rats were anesthetized with 4% isoflurane and a 30:70 mixture of O_2 and nitrous oxide followed by endotracheal intubation. Isoflurane was subsequently lowered to 1.5%–2% for endovascular access. The femoral artery and vein were cannulated for blood pressure monitoring and blood gas analysis (PE-50 polyethylene catheter). Pancuronium (2 mg/kg) was injected (i.v.) followed by mechanical ventilation (60 breaths/min.) and lowering of isoflurane to 1.0%–0.5%. Physiological parameters including, PCO_2 , PO_2 , pH, HCO_3 and arterial base excess were maintained within normal limits. Mean arterial blood pressure (MABP) was continuously monitored and head and body temperature were maintained at 37.0°C . Rats were mounted on a stereotaxic stage for monitoring of cerebral blood flow (CBF). Before each ischemic insult, blood was gradually withdrawn into a heparinized syringe to reduce systemic blood pressure to 48–50 mm Hg. Cerebral ischemia was then produced by tightening the carotid ligatures bilaterally and maintaining mean blood pressure at 48–50 mm Hg for a period of 10 min. To allow post-ischemic reperfusion, the carotid ligatures were removed, and the shed blood was reinjected to restore MABP to 100–120 mm Hg. The vessels were visually inspected to verify establishment of reperfusion.

Monitoring blood perfusion in the cerebral cortex

A 2 mm^2 burr hole was made over the left frontoparietal cortex approximately 5.0 mm posterior and 3.5 mm lateral to bregma. Under a Zeiss operating microscope, the bone was drilled to a thin layer with a cutting burr under saline irrigation, and a cortical area with blood vessels less than 50 μm diameter was selected by visualization through the thin bone layer, and a fiberoptic

probe (1 mm) was placed thereupon. The fiberoptic probe when coupled to a PeriFlux 4001 Master laser Doppler blood perfusion monitor (Perimed, Inc.) measures cerebral blood perfusion in a 1 mm³ tissue region. The Doppler signals were routed to a polygraphic recording system interfaced to a personal computer, via an A to D converter, utilizing data acquisition software (Perisoft for Windows).

Histopathology

After 7 days of reperfusion following ischemia, rats were anesthetized with isoflurane and perfused with FAM (a mixture of 40% formaldehyde, glacial acetic acid, and methanol, 1:1:8 by volume) as previously described (Perez-Pinzon et al., 1997). The head was removed and immersed in FAM at 4 °C for 1 day. The coronal brain blocks were embedded in paraffin; coronal sections of 10-μm thickness were stained with hematoxylin and eosin. Sections containing the hippocampus at 3.8 mm posterior to bregma were examined by a blinded observer. Normal neuronal counts were made within the hippocampal CA1 (Raval et al., 2005).

Immunohistochemistry

Rats were perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at different stages of the estrous cycle, OvX and 48 h after 17β-estradiol to OvX (sample size was four in all experimental groups). Coronal brain sections that included the hippocampus were cut at 30 μm thickness on a cryostat. Staining was performed on free-floating sections in PBS+0.8% Triton-X 100. Following pre-blocking with 10% goat serum, sections were incubated for 24 h at 4 °C with primary anti-pCREB (rabbit polyclonal, 1:1000) and anti-microtubule-associated protein 2 (MAP-2; mouse monoclonal, 1:1000) or NeuN (mouse monoclonal, 1:400), markers for neurons. Following overnight washing, the sections were then incubated with the fluorescent secondary antibodies, rhodamine-labeled antimouse and FITC-labeled-antirabbit (1:400), for 24 h at 4 °C. The sections were rinsed in PBS, mounted onto slides using Prolong Antifade kit (Molecular Probes) and viewed on a Carl Zeiss LSM-510 confocal microscope (Raval et al., 2005).

Cell fractionation and Western blot analysis

Hippocampi of rats were collected at (1) different stages of the estrous cycle, (2) 7 days after ovariectomy, (3) 48 h after 17β-estradiol treatment to OvX rats and (4) 1 h after induction of ischemia. The hippocampal samples were homogenized in buffer (4 mM ATP, 100 mM KCl, 10 mM imidazole, 2 mM EGTA, 1 mM MgCl₂, 20% glycerol, 0.05% Triton X-100, 17 μg/ml PMSF, 20 μg/ml soybean trypsin inhibitor, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 1 mM Na₃VO₄). The homogenate was then centrifuged at 4 °C at 480×g for 10 min. The resulting (1) pellet and (2) supernatant were treated separately to acquire the nuclear and cytosolic fractions, respectively (1). The pellet was washed twice, resuspended in nuclear lysis buffer (pH 8.0; 20 mM Hepes, 400 mM NaCl, 0.5 mM EDTA, 2 mM MgCl₂, 1 mM DTT, proteinase inhibitor (5 μl proteinase inhibitor cocktail/1 ml lysis buffer), 1 mM Na₃VO₄) and centrifuged at for 15 min. The resulting supernatant was the nuclear fraction (2). The resulting supernatant was recentrifuged at 32,000×g for 20 min, to isolate the cytosolic fraction; the pellet was discarded. The cytosolic or nuclear fractions were analyzed for protein content using the Bio-Rad protein assay kit. The proteins were separated by 12% SDS-PAGE. Equal amounts of protein from each group were run on the same gel and analyzed at the same time. Protein was transferred to Immobilon-P (Millipore) membrane and incubated with the primary antibody anti-β-actin (monoclonal; 1:1000; Sigma), anti-lamin, antiphospho-CREB (pSer133; rabbit polyclonal, 1:1000; Cell Signaling Tech), anti-CREB (rabbit polyclonal; 1:1000; Cell Signaling Tech), anti-MAPK (p42/44; rabbit polyclonal, 1:1000; Cell Signaling Tech), anti-Akt (pSer473, rabbit polyclonal, 1:1000; Cell Signaling Tech), and anti-CaMKII (rabbit

polyclonal, 1:5000; Promega) for the detection of β -actin, lamin, pCREB, pMAPK, CaMKII, and pAkt, respectively. β -Actin and lamin were used as loading controls. Immunoreactivity was detected using enhanced chemiluminescence (Amersham-Pharmacia Biotech). Western images were digitized at eight-bit precision by means of a charge-coupled device-based camera (Xillix Technologies Corp.) equipped with a 55 mm Micro-Nikkor lens (Nikon, Japan). The camera was interfaced to an advanced image-analysis system (MCID Model M2, Imaging Research, Inc.). The digitized immunoblots were subjected to densitometric analysis using MCID software.

Real time PCR assay

Total RNA was isolated from rat hippocampus using QIAGEN miRNeasy mini kit (Valencia, CA, USA). One microgram of total RNA was reverse-transcribed using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions. Real-time TaqMan® PCR was performed to evaluate the mRNA levels of CREB in normal cycling (diestrus stage), OvX and 17 β -estradiol treated groups. Glyceral-dehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize the samples. The means of the ΔC_T for the diestrus samples at each time point were used as the "calibrator" for the calculation of $\Delta\Delta C_T$. The mean $\Delta\Delta C_T$ for each group was used to calculate the relative expression of CREB (fold change = $2^{-\Delta\Delta C_T} = [(C_T \text{ gene of interest} - C_T \text{ internal control}) \text{ sample A} - (C_T \text{ gene of interest} - C_T \text{ internal control}) \text{ sample B}]$) (Schmittgen and Livak, 2008). Thus, the relative expression levels reported here are calibrated by the diestrus group and normalized by the GAPDH internal control (fold change = $2^{-\Delta\Delta C_T} = [(C_T \text{ of CREB} - C_T \text{ of GAPDH}) \text{ OvX/estradiol} + \text{OvX A} - (C_T \text{ of CREB} - C_T \text{ of GAPDH}) \text{ diestrus}]$). For further details on these calculations please see reference (Schmittgen and Livak, 2008). The gene-specific TaqMan primers for GAPDH and CREB were purchased from Applied Biosystems. Quantitative PCR was performed on 15 ng cDNA template on a 7300 Real Time PCR Sequence Detection System (Applied Biosystems). Cycling conditions were performed according to the manufacturer's instructions. Each sample was assayed in triplicate.

Statistical analysis

The data are presented as mean value $\pm$ SEM. An analysis of variance (ANOVA) followed by a multiple comparison procedure (Bonferroni's test) was used to analyze the statistical differences among groups. To investigate existence of correlation between increasing circulatory 17 β -estradiol level during the transition from diestrus to proestrus, and protein levels of various kinases under investigation, we performed correlation coefficient r , a measure of the stochastic dependence of the two variables. $P < 0.05$ was considered significant.

RESULTS

Our first hypothesis was that the endogenous surge of 17 β -estradiol activates the CREB-mediated signaling cascade in the CA1 region of hippocampus and protects neurons against ischemia in normal rats. To test this hypothesis we sacrificed rats at each stage of the estrous cycle. During the transition from the diestrus to proestrus the rising levels of estradiol were associated with the observed increased immunoreactivity for pCREB (Fig. 2A, B). In the proestrus to estrus transition, the time of peak transient 17 β -estradiol, hippocampus showed sustained pCREB protein levels relatively higher than that of diestrus levels ($P < 0.05$ against diestrus). The transition from estrus to metestrus was associated with reduction in pCREB immunoreactivity and protein level. At diestrus, when circulating gonadal hormone concentrations were lowest, the pCREB immunoreactivity and protein content fell to minimal levels (Fig. 2A–C, Table 1 presents estradiol plasma levels). Fig. 2C showed significant correlation between the higher level of 17 β -estradiol at proestrus stage and pCREB protein levels as compared to diestrus.

Estrogen-mediated phosphorylation of CREB occurs via activation of various protein kinases involving CaMKII, MAPK and protein kinase B (Akt) (Lee et al., 2004; Wu et al., 2005). Therefore, we further tested whether CaMKII, MAPK or Akt was involved in this endogenous estrogen-mediated cyclic pattern of pCREB. Western blot analysis demonstrated that during the diestrus to proestrus transition, we observed a 56% (156 ± 17 ; $n=5$) and 71% (171 ± 27 ; $n=5$) increase in phosphorylated MAPK and CaMKII, respectively ($P<0.05$ against diestrus) (Fig. 3B, E). Fig. 3C and 3F showed significant correlation between increasing levels of 17β -estradiol from diestrus to proestrous stage and pMAPK/CaMKII protein levels. The transition from proestrus to estrus and metestrus was associated with sustained CaMKII and MAPK protein levels. The cyclic variation in ovarian hormones did not reflect phosphorylation of Akt in hippocampus (Fig. 3G, H, I).

Based on these results, the question arose whether or not there was any difference in histopathological outcome after global cerebral ischemia induced at various stages of the estrous cycle. To answer this question we subjected female rats to cerebral ischemia at all four stages of the estrous cycle. All physiological variables (pH, PCO_2 , PO_2 and plasma glucose concentration) remained unchanged throughout the surgical procedure of cerebral ischemia in all the experimental groups. These data are given in Table 1. Typical arterial blood pressure recording trace is presented as Fig. 4. Histopathology was carried out 7 days following ischemia. The number of normal neurons per slice in the CA1 hippocampal region in control rats (without ischemic episode) was 1100 ± 68 and an ischemic insult to diestrus females decreased number of normal neurons by 82% (189 ± 30 ; $n=5$; $P<0.05$) (Fig. 5A, B). Interestingly, the number of normal neurons was significantly increased by 17% (377 ± 59 ; $n=5$; $P<0.05$ against diestrus) and 26% (470 ± 50 ; $n=5$; $P<0.05$ against diestrus) when ischemia was induced during the proestrus and estrus stages, respectively. These results suggested that an endogenous increase in 17β -estradiol activates the CREB-mediated signaling cascade in the CA1 region of hippocampus, and thus induced resistance against ischemia.

Our second hypothesis was that a single bolus of acute 17β -estradiol pretreatment provided protection against ischemia in OvX rats. To test this hypothesis, OvX rats were treated with 17β -estradiol or vehicle alone and 48 h later ischemia was induced. We performed histological assessment of neuronal death in the CA1 region of the hippocampus 7 days after induction of cerebral ischemia. Ovarian hormone deprivation decreased the number of normal neurons by 89% (11% normal neurons; 144 ± 13 ; $n=4$) as compared to the control group (1100 ± 68) after cerebral ischemia. Interestingly, an estradiol bolus to OvX rats 48 h prior to ischemic insult increased the number of normal neurons by 43% (601 ± 77 ; $n=5$; dose $5\text{ }\mu\text{g/kg}$); 47% (663 ± 84 ; $n=5$; dose $10\text{ }\mu\text{g/kg}$) and 32% (453 ± 24 ; $n=5$; dose $50\text{ }\mu\text{g/kg}$) as compared to the OvX group ($P<0.05$, Fig. 5). Based on these results, for subsequent studies we carried out 17β -estradiol pretreatment at $5\text{ }\mu\text{g/kg}$ dose and demonstrated that the time window for neuroprotection induction afforded by 17β -estradiol lasted for 72 h (Fig. 5). These results suggested that an estradiol bolus to OvX rats mimicked endogenous estradiol-mediated tolerance and protected the hippocampus against ischemic damage.

Vehicle treatment did not show any significant differences in the number of normal neurons as compared to the OvX groups. We used isoflurane anesthesia during the surgical procedure of ovariectomy. Isoflurane is known to induce neuronal tolerance (Kapinya et al., 2002). To address this, we carried out a control experiment by exposing female rats to sham-OvX. During this sham procedure rats were exposed to anesthesia for a period similar to that of the OvX group. Ischemia was induced 7 days later in sham-operated females (at diestrus stage of estrous cycle). The number of normal neurons in the CA1 hippocampal region in the sham-OvX group did not vary from that in the OvX group. These results suggested that isoflurane exposure 7 days prior to ischemia showed no neuroprotective effect. This might be due to insufficient

exposure time or an inappropriate time window between exposure to anesthesia and a subsequent ischemic insult.

It has been demonstrated that the higher dose of estradiol could activate progesterone receptors. To rule out the possibility of progesterone-mediated neuroprotective effect against ischemic injury in current study we treated OvX rats with progesterone receptor antagonist immediately after 17 β -estradiol treatment and rats were exposed to ischemia 48 h later. The number of normal neurons in the CA1 hippocampal region in the estradiol plus progesterone receptor inhibitor-treated OvX group (517 ± 22 ; $n=7$; dose 5 $\mu\text{g/kg}$ estradiol and 5 mg/rat progesterone inhibitor-mifepristone; McCullers et al., 2002; Ghomari et al., 2003; Auger and Forbes-Lorman, 2008) did not significantly vary from that in the estradiol-treated OvX group (601 ± 77 ; $n=5$; dose 5 $\mu\text{g/kg}$). After global ischemia, our control group treated with progesterone inhibitor alone demonstrated no difference in cell loss compared to vehicle-treated animals (data not shown). Since OVX plus progesterone receptor inhibitor treatment had no effect on the number of normal neurons, these results rule out the possibility of progesterone receptor activation following bolus estradiol treatment (dose 5 $\mu\text{g/kg}$) and its involvement in observed neuroprotection.

One of estrogen's putative effects in mediating neuroprotection involves changes in CBF (Pelligrino et al., 1998; McNeill et al., 2002; Duckles et al., 2006). To test whether a 17 β -estradiol bolus alters CBF during induction of cerebral ischemia, we measured CBF using laser-Doppler flowmetry. The recordings did not show any significant difference in cerebral perfusion during or immediately after an ischemic episode between the following groups: diestrus, estrus, OvX plus 17 β -estradiol (5 $\mu\text{g/kg}$) or vehicle-treated female rats (Fig. 6).

Additionally, to characterize the mechanism by which an estradiol bolus protects the hippocampal CA1 against ischemia, we treated OvX (7 days) females with 17 β -estradiol and sacrificed them 48 h later (without ischemic episode). To confirm that either ovariectomy or estradiol treatment did not cause neuronal death, we performed histology and counted normal neurons in CA1 region. We found no difference in number of normal neurons between control rats (without ischemic episode; 1100 ± 68 ; $n=4$) and OvX (1138 ± 16 ; $n=4$) or OvX plus estradiol-treated rats (1129 ± 51 ; $n=4$). Deprivation of ovarian hormones after ovariectomy significantly reduced levels of pMAPK, CaMKII and pCREB (Fig. 7). Semi-quantitative analysis of Western blot demonstrated 55% (46 ± 9 ; $n=4$), 17% (83 ± 10 ; $n=4$) and 60% (40 ± 7 ; $n=4$) reduction in pCREB, CaMKII and pMAPK levels as compared to diestrus ($P<0.05$), respectively. Interestingly, an estradiol bolus to OvX rats demonstrated 132% (177 ± 10 ; $n=4$), 94% (176 ± 29 ; $n=4$) and 58% (98 ± 16 ; $n=4$) increase in phosphorylated MAPK, CaMKII and CREB, respectively, as compared to the OvX group. This increased phosphorylation of CREB, CaMKII and MAPK showed correlation with increase 17 β -estradiol level after a bolus of 17 β -estradiol to OvX rats (Fig. 7C, F, I). To confirm our results that the 17 β -estradiol-induced increased phosphorylation of CREB in live hippocampal neurons we performed immunohistochemistry. Results demonstrated increased immunoreactivity for pCREB in NeuN-positive CA1 neurons at 48 h after estradiol treatment to OvX rats (Fig. 8).

Finally, we confirmed the hypothesis that a bolus 17 β -estradiol treatment to OvX rat induced CREB gene expression in hippocampus. Real-time PCR results demonstrated increased CREB mRNA expression at 24 h after estradiol treatment to OvX rats (Fig. 9). The relative expression of CREB was significantly higher in estradiol-treated OvX (2.2 ± 0.4 , $n=5$; $P<0.01$) compared to untreated OvX rats (0.74 ± 0.3 ; $n=4$). This result suggested that single-bolus 17 β -estradiol pretreatment to an OvX rat induced CREB protein synthesis, phosphorylation and protect hippocampal neurons against ischemia (Fig. 10).

DISCUSSION

The role of estrogen supplement in women's cerebrovascular health after menopause remains controversial (Gibson et al., 2006). The failure of the Women's Estrogen for Stroke Trial raises concerns regarding the safety of chronic estrogen treatment in women (Viscoli et al., 2001). Thus, it is important to determine the safety of estrogen therapy for the prevention of cerebrovascular disorders in peri- and post-menopausal women. This underscores a need for additional studies to provide an understanding of the balance between the deleterious and beneficial effects of estrogens. In this context, we demonstrated that the increasing titers of endogenous 17 β -estradiol during the transition from diestrus to the proestrus of the estrous cycle conferred protection to the hippocampus against global cerebral ischemia. This neuroprotection was linked to activation of the CREB-mediated signaling cascade. Further, a single bolus of exogenous 17 β -estradiol injection in OvX (ovarian hormone deprived) rats induced similar degree of neuroprotection and activated CREB pathway (Fig. 5 and Fig. 7). This estradiol-mediated neuroprotection in OvX rats lasted 72 h after a bolus injection and was lost over the period of 96 h (Fig. 5). Based on these results we propose that intermittent 17 β -estradiol replacement at every 48/72 h for longer duration might render same benefits of chronic 17 β -estradiol and improve cerebrovascular health while avoiding side effects. Further studies are required to establish a regimen of intermittent injection at low dose of 17 β -estradiol. In context, a recent study simulated the pre-ovulatory estrogen surge in OvX rats after sequential low doses of estradiol, and confirmed that physiological estradiol levels were sufficient to profoundly affect hippocampal function (Scharfman et al., 2007).

Our study showed that increased levels of circulating 17 β -estradiol during the diestrus to estrus transition correlated with increased phosphorylation of MAPK, CaMKII, CREB and the degree of CA1 neuroprotection against cerebral ischemia. Interestingly, estrogen mediates phosphorylation of CREB within an hour and is sustained for at least 24 h in neurons *in vivo* and *in vitro* (Lee et al., 2004). According to Abraham and Herbison this rapid phosphorylation of CREB involves estrogen receptor activation (Abraham et al., 2004; Abraham and Herbison, 2005). Estrogen has been shown to upregulate phosphorylation of CREB via Akt augmentation and prevent ischemic damage in brain (Choi et al., 2004; Lee et al., 2004; Zhao et al., 2006). However, Akt protein did not correlate to changes in 17 β -estradiol levels. A recent study demonstrated that estrogen's neuroprotective effect was maintained in Akt1 knockout mice after stroke (Li et al., 2008). Thus, we conjectured that the observed neuroprotective effect of 17 β -estradiol during the proestrus to metestrus transition was independent of Akt. Further, we showed that reduction or deprivation of ovarian hormones as in diestrus stage or due to ovariectomy, respectively, resulted in loss of estradiol-conferred protective effects in neurons. This loss of estradiol-mediated resistance was reflected by a significantly reduced number of normal neurons in the CA1 region after cerebral ischemia. Overall, these results suggested that endogenous intermittent increase in levels of 17 β -estradiol induced CREB phosphorylation in hippocampus and conferred resistance to CA1 neurons against ischemia/stress (Fig. 10). Similarly, a bolus exogenous administration of 17 β -estradiol in OvX rats 48/72 h prior to global cerebral ischemia initiated the pCREB pathway in hippocampus and conferred resistance to the hippocampus against cerebral ischemia.

Mitochondrial dysfunction has been implicated as a cause of ischemic neuronal injury (see review; Lipton, 1999). Recent studies demonstrated the presence of estrogen receptors in brain mitochondria and that estrogen promoted mitochondrial respiratory efficiency via decreased oxidative stress (Razmara et al., 2007; Simpkins and Dykens, 2007). On the other hand, we demonstrated that 17 β -estradiol pretreatment induced neuroprotection in the CA1 region of hippocampus via CREB activation (Raval et al., 2006). We demonstrated that a high circulating 17 β -estradiol level correlates with increased pCREB in hippocampus. CREB phosphorylation has been demonstrated to increase B-cell leukemia/lymphoma 2 (Bcl-2) expression following

estrogen treatment (Lee et al., 2004). Bcl-2 is known to stabilize the mitochondrial membrane potential and prevent the release of cytochrome *c* and APAF-1 into the cytoplasm, thus blocking activation of the caspase cascade and subsequent cell death (Shimizu et al., 1999; Desagher and Martinou, 2000). Chronic estrogen pretreatment has been shown to attenuate ischemia-induced caspase-3 activation (Jover et al., 2002), cytochrome *c* release from hippocampal mitochondria (Bagetta et al., 2004), and to correlate with neuroprotection (Simpkins and Dykens, 2007). In parallel, the present findings demonstrated that the natural higher level of 17 β -estradiol or a bolus of 17 β -estradiol in OvX rats maintained higher levels of pCREB which might upregulate Bcl-2 and reduced post-ischemic cytochrome *c* release. Briefly, the post-ischemic early release of cytochrome *c* initiates a vicious cycle of caspase 3 and delta-protein kinase C (delta-PKC) activation resulting in secondary mitochondrial dysfunction and delayed hippocampal neuronal death in a rat model of cerebral ischemia (Zhao et al., 2003; Bright et al., 2004, 2007; Raval et al., 2005).

A number of mechanisms likely contribute to estrogen-mediated neuroprotection (Bagetta et al., 2004; Lee et al., 2004; Connell et al., 2007; Crosby et al., 2007; Jover-Mengual et al., 2007). One important mechanism appears to be the ability of estrogen to increase the vasodilatory capacity of the cerebral vasculature during cerebral ischemia (Alkayed et al., 1998; McNeill et al., 2002; Duckles et al., 2006). In this report we tested the hypothesis that an exogenous 17 β -estradiol bolus provides protection against ischemia by altering intra-ischemic CBF. Overall, our results suggested that the neuroprotective effect of 17 β -estradiol could not be caused solely by its effect on CBF alone in a global cerebral ischemia model. Consistent with our results, Wang et al. suggested that estrogen provides ischemic neuroprotection through mechanisms unrelated to improvement of intra-ischemic cerebral perfusion (Wang et al., 1999). This finding has been supported by our recent study, in which we demonstrated that 17 β -estradiol pretreatment was protective in an *in vitro* model, where there is no vascular effect (Raval et al., 2006). However, in our study, blood flow measurements were obtained before, during and immediately after ischemia at short intervals of 5 min. Estradiol-17 β -induced changes in blood flow hours and days after lethal ischemia may contribute to neuronal survival but could not be detected in our experimental paradigm. Such phenomena have been reported in a middle cerebral artery occlusion model where 17 β -estradiol treatment to OvX female rats decreased infarct volume and increased CBF after 1–2 days (Yang et al., 2000). These studies clearly suggested a need to monitor CBF over a period of days following 17 β -estradiol bolus to observe changes in CBF and its possible role in neuroprotection against ischemia.

CONCLUSION

Finally, our results suggest that both endogenously cycling 17 β -estradiol and an exogenous bolus of 17 β -estradiol protects hippocampus against ischemia via activation of CREB pathway. This study also suggests that a new single-dose 17 β -estradiol regimen may provide pharmacological access to this protective state, especially in different surgical procedures where it is essential to provoke ischemic episodes in brain. This study also emphasizes a need to investigate bolus intermittent estrogen hormone replacement regimen, which mimics the endogenous estrogen surge-to promote improved cardio- and cerebro-vascular health and reduce the impact of stroke/cerebral ischemia incidents in post-menopausal women while avoiding the known side effects of chronic estradiol treatment.

Abbreviations

Akt, protein kinase B
Bcl-2, B-cell leukemia/lymphoma 2
CaMKII, calcium-calmodulin-dependent protein kinase

CBF, cerebral blood flow
 CREB, cyclic-AMP response element binding protein
 FAM, 40% formaldehyde, glacial acetic acid, and methanol, 1:1:8 by volume
 GAPDH, glyceraldehyde-3-phosphate dehydrogenase
 MABP, mean arterial blood pressure
 MAPK, mitogen-activated protein kinase
 MAP-2, microtubule-associated protein 2
 OvX, ovariectomized
 PBS, phosphate-buffered saline
 WHI, Women's Health Initiative

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Fig. 1.

Experimental design. (A) Control: untreated rats used for histopathology to count total neurons in CA1 hippocampus, (B) stages of estrous cycle: depicts rats at diestrus, proestrus, estrus, and metestrus; (C) sham-ovariectomy. In this control experiment rats were exposed to anesthesia for similar periods of ovariectomy. (D) Ovariectomy: rats underwent ovariectomy during the diestrus stage and 7 days later were used for experiment. (E, F) Vehicle or estrogen pretreatment: 7 days after ovariectomy rats were treated with vehicle or 17 β -estradiol (5, 10 or 50 μ g) and 48 h later either sacrificed or exposed to 10 min of ischemic insult produced by bilateral carotid occlusion and systemic hypotension.

Fig. 2.

Endogenous variations of estradiol-induced cyclic pattern of phosphorylation of CREB in CA1 neurons. (A) Confocal images of hippocampal CA1 region depicting co-localization of immunoreactivities for neuronal marker MAP-2 (red) and pCREB (green) (40 $\times$ magnification, scale bar=50 μ M) at various stages of the estrous cycle. Note the increase in pCREB immunoreactivity at proestrus and estrus stages. (B) Representative immunoblots showing pCREB content in the nuclear fraction of rat hippocampus at different stages of the estrous cycle. (C) Densitometric analysis of scanned Western blots showed a cyclic pattern of pCREB protein content. (D) The graph showing statistically significant ($P < 0.003$) correlation between estradiol level and protein content of pCREB during transition from diestrus to proestrus. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

Fig. 3.

Cyclic variations of phosphorylation CREB correlate with activation of MAPK and CaMKII. (A, D, G) Representative immunoblots showing the levels of phosphorylated kinases pMAPK, CaMKII and pAkt in the cytosolic fraction of rat hippocampus at different stages of the estrous cycle. (B, E, H) Densitometric analysis of immunoblots demonstrated a cyclic pattern of pMAPK, CaMKII and pAkt content at different stages of the estrous cycle. (C, F, I) Graphs of correlation between estradiol level and protein content of pMAPK (C), CaMKII (F) and Akt (I) during transition from diestrus to proestrus. Note the presence of statistically significant ($P<0.05$) correlation for pMAPK and CaMKII with changing estradiol levels.

Fig. 4.

Typical arterial blood pressure tracing during cerebral ischemia Representative blood pressure tracing pattern before, during, and after 10 min of ischemic insult produced by produced by bilateral carotid occlusion and systemic hypotension (50 mm Hg).

A **B** **Fig. 5.**

Endogenous higher levels of 17β-estradiol or exogenous 17β-estradiol pretreatment to OvX rats protected the hippocampal CA1 region against cerebral ischemia. (A) Representative histological images in the hippocampal CA1 region; (a) control (without ischemic episode), (b) diestrus, (c) estrus, (d) OvX plus vehicle-treated and (e) 17β-estradiol-pretreated (5 μg/kg) female rats, 7 days after 10 min of cerebral ischemia. Arrow shows normal neurons (scale bar=20 μm). (B) Normal neurons remaining in the CA1 region (which includes the middle, medial and lateral sub-region) of rat hippocampus 7 days after induction of cerebral ischemia in different experimental groups.

Fig. 6.

Estradiol-17 β bolus 48 h prior to cerebral ischemia showed no change in intra-ischemic CBF. CBF (% of baseline) curves of laser-Doppler flowmetry tracing before, during, and after 5 min of ischemic insult produced by bilateral carotid occlusions and systemic hypotension.

Fig. 7.

Estradiol-17β bolus 48 h prior to cerebral ischemia increased phosphorylation of MAPK, CaMKII and CREB. (A) Representative immunoblots showing the levels of the phosphokinase proteins: pMAPK, CaMKII and pCREB in the hippocampus at 48 h after bolus 17β-estradiol injection to OvX rats. (B) Densitometric analysis of scanned Western blots revealed a relative abundance of these phosphorylated kinases in rat hippocampus 48 h after bolus estrogen to OvX rats. (A, D, G) Representative immunoblots showing the levels of phosphorylated kinases pMAPK, CaMKII and pCREB in the hippocampus at 48 h after bolus 17β-estradiol injection to OvX rats. (B, E, H) Densitometric analysis of immunoblots revealed a relative abundance of these phosphorylated kinases in rat hippocampus 48 h after bolus estrogen to OvX rats. (C, F, I) Graphs of correlation between estradiol level and protein content of pMAPK (C), CaMKII (F) and pCREB (I) after ovariectomy and 17β-estradiol treatment to OvX rats. Note the presence of statistically significant ($P < 0.05$) correlation for pMAPK, CaMKII and CREB with changing estradiol levels.

Fig. 8.

Estradiol-17 β bolus increased phosphorylation of CREB in CA1 pyramidal neurons. Confocal images of hippocampal CA1 region depicting co-localization of immunoreactivities for neuronal marker NeuN (red) and pCREB (green) (40 $\times$ magnification, scale bar=50 μ M) at 48 h after 17 β -estradiol treatment to OvX rats. Note (E, F) the increase in pCREB immunoreactivity in 17 β -estradiol-treated OvX rats as compared to untreated OvX rat. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

Fig. 9. Estradiol-17 β bolus to OvX rats increased CREB gene expression in hippocampus. Real-time-PCR of CREB. Data represent fold increase over diestrus. Note the significant increase in hippocampal CREB mRNA levels by 24 h after 17 β -estradiol bolus to OvX rats. $P<0.05$ versus diestrus.

Fig. 10.

Independent of its source (endogenous/exogenous), 17β-estradiol protects the hippocampus against cerebral ischemia via activation of CREB pathway. Simplified scheme depicting the basic hypothesis proposed in the present study. The outer circle represents different phases of the rat estrous cycle. The rising levels of estradiol observed during the transition from diestrus to proestrus or after bolus injection to OvX female rats, induce phosphorylation of MAPK and CaMKII in hippocampal neurons. This phosphorylation of MAPK and CaMKII could be mediated via estrogen receptor activation and/or calcium influx through L-type calcium channels (Lee et al., 2004; Wu et al., 2005). Activated MAPK and CaMKII induced phosphorylation of CREB. This estradiol-mediated CREB signaling pathway in hippocampus induced ischemic tolerance for the duration of that particular cycle or 72 h after a bolus of 17β-estradiol replacement to OvX rats. This estradiol-mediated neuroprotection was lost at the diestrus stage.

Table 1

Physiological variable

Groups	Estradiol (pg/ml)	Glucose (mg/ml)	pH	PCO ₂ (mm Hg)	PO ₂ (mm Hg)	MABP (mm Hg)
Diestrus	16.5±3.2	135.3±20.4	7.43±0.02	38.9±2.1	122.9±6.4	118.8±6.3
Proestrus	38.7±4.4*	147.5±4.2	7.39±0.04	35.4±1.9	108.8±9.7	124.8±7.6
Estrus	26.9±3.3*	145.5±16.4	7.45±0.03	33.1±0.8	107.5±5.1	126.3±7.8
Metestrus	18.0±2.5	145.8±3.5	7.41±0.01	33.9±3.02	113.3±11.4	122.0±8.5
Sham-OvX	16.97±9.4	145.6±2.6	7.47±0.01	37.2±2.1	106.9±12.9	137±5.9
OvX	7.8±2.2	149±19.9	7.44±0.02	36.03±1.9	98.16±25.7	139.4±2.9
OvX + vehicle	5.3±1.5	147±5.8	7.37±0.03	37.3±4.2	114.9±9.7	118.3±1.8
17β-Estradiol treatment (5 μg)+48 h	30.4±3.7*	148.75±8.9	7.38±0.03	37.4±2.56	125.0±6.73	137.5±3.5
17β-Estradiol treatment (10 μg)+48 h	36.2±4.3*	144.8±3.4	7.39±0.02	35.2±2.8	134.0±20.5	133.3±7.3
17β-Estradiol treatment (50 μg)+48 h	52.7±7.5*	136.3±21.4	7.43±0.01	36.0±0.8	109.4±17.5	134.8±5.3
17β-Estradiol treatment (5 μg)+72 h	22.5±2.4	152.6±15	7.38±0.04	37.3±4.3	140.9±8.6	133.8±3.9
17β-Estradiol treatment (5 μg)+96 h	10.4±1.9	141.2±5.7	7.43±0.02	38.7±1.4	133.6±6	124.5±6
			7.47±0.02	37.9±2.5	124.1±9.4	133.3±4.1
			7.43±0.02	36.2±0.9	118.8±7.8	128.4±3.5

Physiological variables were recorded during the period when the animal was under anesthesia. Rows with **bold** background denote values after ischemia.

* $P < 0.05$ as against diestrus value.