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Brain-derived estrogen exerts anti-inflammatory and neuroprotective actions in the rat hippocampus

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ABSTRACT

17β-estradiol (E2) has been implicated to play a critical role in neuroprotection, synaptic plasticity, and cognitive function. Classically, the role of gonadal-derived E2 in these events is well established, but the role of brain-derived E2 is less clear. To address this issue, we investigated the expression, localization, and modulation of aromatase and local E2 levels in the hippocampus following global cerebral ischemia (GCI) in adult ovariectomized rats. Immunohistochemistry (IHC) revealed that the hippocampal regions CA1, CA3 and dentate gyrus (DG) exhibited high levels of immunoreactive aromatase staining, with aromatase being co-localized primarily in neurons in non-ischemic animals. Following GCI, aromatase became highly expressed in GFAP-positive astrocytes in the hippocampal CA1 region at 2–3 days post GCI reperfusion. An ELISA for E2 and IHC for E2 confirmed the GCI-induced elevation of local E2 in the CA1 region and that the increase in local E2 occurred in astrocytes. Furthermore, central administration of aromatase antisense (AS) oligonucleotides, but not missense (MS) oligonucleotides, blocked the increase in aromatase and local E2 in astrocytes after GCI, and resulted in a significant increase in GCI-induced hippocampal CA1 region neuronal cell death and neuroinflammation. As a whole, these results suggest that brain-derived E2 exerts important neuroprotective and anti-inflammatory actions in the hippocampal CA1 region following GCI.

1. Introduction

17β-Estradiol (E2, estrogen) is a steroid hormone that has been implicated to be neuroprotective against a variety of neurodegenerative disorders, including stroke, Alzheimer's disease (AD) and Parkinson's disease, although controversy exists (Brann et al., 2007; Yao and Brinton, 2012; Simpkins et al., 2012; Bourque et al., 2012). With respect to stroke, studies in rats, mice and gerbils found a sex difference in brain injury following cerebral ischemia, with young adult female animals having smaller infarct volume as compared to young adult males (Brann et al., 2007; Alkayed et al., 1998; Park et al., 2006). Similarly, a number of studies have documented sex differences in stroke risk and outcome in humans, with women generally protected against stroke, at least until menopause (Murphy et al., 2004; Di Carlo et al., 2003). Many groups, including our own, have shown that *exogenous* administration of E2 dramatically reduces infarct volume in cortex and hippocampus following focal or global cerebral ischemia (GCI) in ovariectomized female mice, rats and gerbils, and in male rats and gerbils (Brann et al., 2007; Zhang et al., 2008; Simpkins et al., 1997; Dubal et al., 1998; Shughrue and Merchenthaler, 2003; Zhang et al., 1998).

It has been generally assumed that the neuroprotective effects of E2 are primarily due to ovarian-derived E2. However, work by a number of laboratories has shown that certain areas of the brain exhibit high expression of the E2 generating enzyme, aromatase, which has raised the possibility that brain-derived E2 may have important roles in the CNS. For instance, work within the last decade in rodents, birds, monkeys, and humans has shown that forebrain structures, in particular the hippocampus CA1–CA3 regions, exhibits high expression of aromatase as indicated by *in situ* hybridization, RT-PCR and immunohistochemical analysis, and can produce significant levels of E2 levels that are equivalent to or even higher than that observed in the circulation (Veiga et al.,

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2005; Hojo et al., 2004; Azcoitia et al., 2011; Higaki et al., 2012; Yague et al., 2008; Fester et al., 2011; Garcia-Segura, 2008; Mukai et al., 2010; Shen et al., 1994). It should be noted that the cerebral cortex has also been reported to express aromatase (Azcoitia et al., 2011; Stoffel-Wagner et al., 1999; Srivastava et al., 2010), and thus brain-derived E2 may also regulate cortical functions. In support of this possibility, global aromatase knockout mice have been reported to have greater cortical damage following focal cerebral ischemia than wild type ovariectomized mice, suggesting that brain-derived E2 may have neuroprotective actions in the cerebral cortex (McCullough et al., 2003).

With respect to the hippocampus, treatment of cultured mouse hippocampal neurons with an aromatase inhibitor has been reported to result in a significant decrease in axon outgrowth and dendritic spines in the CA1 region (Fester et al., 2011; Mukai et al., 2010; Kretz et al., 2004; Rune and Frotscher, 2005; von Schassen et al., 2006), as well as a significant decrease of long-term potentiation (LTP) amplitude, dendritic spines and synapses in hippocampal slices *in vitro* (Grassi et al., 2011; Vierk et al., 2012). These results suggest that local E2 in the hippocampus may modulate synaptic function. Interestingly, studies in songbirds have also shown that inhibiting aromatase by intracerebral administration of aromatase inhibitors results in increased damage and apoptosis in the brain after a penetrating injury (Wynne and Saldanha, 2004; Wynne et al., 2008). Aromatase inhibition has also been reported to result in increased hippocampal damage in male rats following excitotoxic injury (Azcoitia et al., 2001).

It is well known that the hippocampal CA1 region is highly vulnerable to GCI, which can occur after cardiac arrest, asphyxiation, and hypotensive shock (Neumann et al., 2013; Harukuni and Bhardwaj, 2006), and can lead to significant neuronal damage, cognitive defect and mortality. It is currently unknown whether brain-derived E2 in the hippocampal CA1 region has a neuroprotective role against GCI, and whether it can modulate neuroinflammation that occurs after GCI. To address these deficits in our knowledge, the goals of the current study were: (1) to access whether aromatase and local E2 levels change in the hippocampus following GCI, (2) to determine the cell types containing aromatase and local E2 expression in ischemic and non-ischemic animals, and (3) to assess whether antisense oligonucleotide knockdown of aromatase and local E2 levels in the hippocampus affects GCI-induced neurodegeneration and inflammation in ovariectomized rats.

2. Materials and methods

2.1. Animal model of global cerebral ischemia

All procedures were approved by the Georgia Regents University Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health guidelines for animal research. Adult Sprague Dawley female SD rats were obtained from Harlan Inc., and studied at 3 months of age. All the rats were bilaterally ovariectomized one week before the induction of GCI. GCI was performed by four-vessel occlusion (4-VO) as described previously (Pulsinelli and Brierley, 1979; Zhang et al., 2008; 2013). In brief, both common carotid arteries (CCAs) of the rat were separated under anesthesia and a silastic ligature was placed loosely around each artery before the incision was closed. Immediately after this procedure, both vertebral arteries at the level of the alar foramina were permanently electrocauterized with bipolar cauterization. After 24 h recovery, both CCAs were exposed under light anesthesia with isoflurane and occluded with aneurysm clips to induce 8 min transient forebrain ischemia. Successful forebrain ischemia was ensured by monitoring the pupils for dilation and being unresponsive to light, and loss of righting reflex of each animal during cerebral ischemia. The clips were then removed, and blood flow through the carotid arteries was inspected and confirmed before the wound was sutured. Normal rectal temperature was maintained with a heat lamp and thermal blanket during surgical procedures. Animals in the sham group underwent identical procedures except that the CCAs were exposed, but there were no occlusion.

2.2. Intracerebroventricular (icv) antisense administration

Alzet osmotic mini-pumps (model 1007D, 7 day release; Durect Corporation, Cupertino, CA) were filled with 20 nmol of HPLC-purified aromatase antisense oligodeoxynucleotide (AS-ODN, 5′-ATCAGCAAGTCCTCGAGCAT-3′, synthesized by Integrated DNA Technologies, Inc.,) or scrambled missense (MS, 5′-CCGCGAAAA TCGCTTTAGCA-3′) in sterile 0.9% saline. The last 3 bases on both the 5′ and 3′ were end-phosphorothioated to limit ODN degradation. For the cannula and osmotic pump implantation, the animals were fixed in a stereotaxic frame under anesthesia and the skull was exposed following a midline incision. The mini-pumps connected to the Alzet infusion cannula (Brain Infusion Kit 2; Durect Corporation) were implanted subcutaneously under the upper back skin three days before ischemia. The cannula was implanted into the lateral cerebral ventricle based on the following stereotaxic coordinates: anterior/posterior −0.8 mm, medial/lateral −1.5 mm, dorsal/ventral −3.5 mm.

2.3. Hippocampal sample preparation

As described previously (Zhang et al., 2008), animals were sacrificed under anesthesia at the specific time points to process brain tissue and homogenates. Whole brains were removed quickly and the hippocampal CA1 tissues were microdissected from both sides of the hippocampal fissure and immediately frozen in dry ice. Tissues were homogenized using a glass homogenizer in ice-cold homogenization medium consisting of 50 mM HEPES, pH 7.4, 150 mM NaCl, 12 mM β-glycerophosphate, 3 mM dithiotheitol (DTT), 2 mM sodium orthovanadate (Na_3VO_4), 1 mM EGTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, and Halt Protease/phosphatase inhibitor Cocktail (Pierce, Thermo Scientific). The homogenates were centrifuged at 15,000*g* for 30 min at 4 °C and supernatants were collected. Protein concentrations were determined by the Modified Lowry Protein Assay (Pierce, Rockford, ILL), and the samples were aliquoted and stored at −80 °C until use.

2.4. Western blotting

For Western blot analysis, protein samples were boiled with Laemmli loading buffer for 5 min. Equal amounts of protein samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 4–20% Tris–glycine gel, and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). The membranes were blocked in Odyssey Blocking Buffer for 1 h and incubated with primary antibodies at 4 °C overnight. The antibodies against aromatase (sc-14245) and β-actin (sc-130656) were from Santa Cruz Biotechnology, Inc. using a 1:200 dilution. The membrane was then washed with PBS containing 0.1% Tween 20 to remove unbound antibody, followed by incubation with Alexa Fluor 680 goat anti-rabbit IgG or donkey anti-goat IgG for 1 h at room temperature. Bound proteins were visualized using the Odyssey Imaging System (Li-COR Bioscience, Lincoln, NB) and semi-quantitative analyses of the signals were performed with the Image J analysis software (Version 1.30v; NIH, USA). A mean ± SE was calculated from the data for graphical presentation and statistical comparison.