Estradiol Protects White Matter of Male C57BL6J Mice against Experimental Chronic Cerebral Hypoperfusion

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Background and Purpose: Estradiol is a sex steroid hormone known to protect the brain against damage related to transient and global cerebral ischemia. In the present study, we leverage an experimental murine model of bilateral carotid artery stenosis (BCAS) to examine the putative effects of estradiol therapy on chronic cerebral hypoperfusion. We hypothesize that long-term estradiol therapy protects against white matter injury and declarative memory deficits associated with chronic cerebral hypoperfusion. *Methods:* Adult male C57BL/6J mice underwent either surgical BCAS or sham procedures. Two days after surgery, the mice were given oral estradiol (Sham+E, BCAS+E) or placebo (Sham+P, BCAS+P) treatments daily for 31-34 days. All mice underwent Novel Object Recognition (NOR) testing 31-34 days after the start of oral treatments. Following sacrifice, blood was collected and brains fixed, sliced, and prepared for histological examination of white matter injury and extracellular signal-regulated kinase (ERK) expression. *Results:* Animals receiving long-term oral estradiol therapy (BCAS-E2 and Sham-E2) had higher plasma estradiol levels than those receiving placebo treatment (BCAS-P and Sham-P). BCAS-E2 mice demonstrated less white matter injury (Klüver–Barrera staining) and performed better on the NOR task compared to BCAS-P mice. ERK expression in the brain was increased in the BCAS compared to sham cohorts. Among the BCAS mice, the BCAS-E2 cohort had a greater number of ERK + cells. *Conclusion:* This study demonstrates a potentially protective role for oral estradiol therapy in the setting of white matter injury and declarative memory deficits secondary to murine chronic cerebral hypoperfusion. **Key Words:** Estrogen—neuroprotection—white matter disease—chronic hypoperfusion—ischemia.
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Introduction

Vascular cognitive impairment is a leading cause of dementia in older adults.[1] Chronic cerebral hypoperfusion (CCH) is implicated in the pathogenesis of subcortical white matter injury and resultant loss of neurons that contributes to neurocognitive decline.[2] Studies support a critical role for cerebral vascular dysfunction in the onset and progression of both sporadic and familial forms of Alzheimer's disease.[3,4] To date, therapeutic efforts to target vascular cognitive impairment have been unsuccessful.

Mechanisms regulating cerebral protection are not well understood.[5-7] Clinical studies demonstrate that the sex steroid estradiol (17β-estradiol; E2) can protect the brain by activating prosurvival pathways in neurons and regulating cerebral blood flow (CBF).[8,9] Nonhuman, experimental

models show that activation of estrogen receptors (ER) is a critical step in the hormone's ability to protect neurons.[2,10,11] It is known that activation of the ERα isoform in neurons enhances the genomic expression of antiapoptotic genes. However, research suggests that activation of this genomic mechanism alone cannot fully account for the steroid hormone's neuroprotective actions.[12]

Understanding mechanisms involved in cerebral protection is increasingly important as the prevalence of age-related brain disease rises. In this study, we leverage the murine bilateral carotid artery stenosis (BCAS) model to study protective effects of long-term oral estradiol administration on white matter injury in the setting of chronic cerebral hypoperfusion. We evaluate white matter ischemic injury in the corpus callosum and neurocognitive outcome. Further, we explore activation of the extracellular signal-regulated kinase (ERK) pathway.

Methods and Materials

Animals

All procedures utilized in this study were approved by the Institutional Animal Care and Use Committee (IACUC; protocol # 20036) of the University of Southern California and carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). All mice were male C57BL/6J (12 weeks of age) and housed in a barrier facility with free access to food and water on a 12-hour light–dark cycle. There were 42 mice (25-27 g; The Jackson Laboratory, Bar Harbor, Maine, USA) chosen and assigned random numbers for use in this study. Control mice (n = 14) did not undergo any surgical procedure. Fourteen mice underwent the BCAS surgery and 14 mice underwent sham surgery (see below). Within each group (control, sham, BCAS), mice were treated with either oral estradiol therapy or placebo. This yielded 6 groups: [Placebo (P); Estradiol (E2); Sham + P; Sham + E2; BCAS + P; and BCAS + E2]. Each cohort of mice underwent behavioral testing (Novel Object Recognition [NOR] paradigm). Following sacrifice, brains were harvested for quantification of white matter injury and immunohistochemical analysis. At the time of sacrifice, blood was drawn for measurement of estradiol levels.

Bilateral Carotid Artery Stenosis Procedure

Fourteen male mice were subjected to the BCAS procedure using external microcoils (Sawane Spring Co., Ltd., Hamamatsu City, Japan) as previously described.[13,14] Briefly, after a 7-day quarantine period, mice were anesthetized (4% isoflurane and maintained with 2% isoflurane in 30%-50% oxygen and 70%-50% nitrogen) and placed in the prone position. A Laser Doppler Flowmetry microtip fiber probe was fixed to the skull (Bregma point: posterior +1 mm/right +5 mm). The mouse was then placed in the supine position. Through a midline cervical incision, both common carotid arteries were exposed and an external microcoil (.18-mm diameter, 2.5-mm length) was applied to each. Sham-operated animals (n = 14) underwent the same procedure, except the microcoils were not placed. CBF values were recorded in the supine position just prior to surgery, following application of the first microcoil, and following application of the second microcoil using a Probe 418-1 master probe/PF 5010 laser Doppler Perfusion Monitoring Unit (Perimed AB, Jarfalla, Sweden). Unless otherwise stated, mice were humanely euthanized 35-38 days after the BCAS/sham procedure (including 4 days of behavioral testing). One BCAS-operated animal (BCAS + Placebo) was euthanized due to surgical procedure complications.

Oral Estradiol Preparation and Treatments

Estradiol dosage and method of preparation was performed according to Strom et al.[15] To prepare experimental oral steroid hormone treatments, a 1-mg 17β-estradiol tablet (Watson Labs, Allergan, Dublin, Republic of Ireland) was pulverized using a mortar and pestle. Then 1.12 µg of crushed 17β-estradiol tablet was added to .312 µL of sesame oil and allowed to dissolve for 2 days while rocking at 4°C. The total volume of treated oil was then added to 60 mg Nutella (hazelnut cocoa spread, Ferrero SpA, Alba, Italy). For each mouse, 17β-estradiol-or placebo-treated Nutella (2 mg) was given per 1-g animal weight.[15] Placebo and estradiol tablets contain the following inactive ingredients: anhydrous lactose, magnesium stearate, microcrystalline cellulose, and polacrilin potassium. Mice were handled and fed with treated Nutella daily between 8 am and 10 am as follows: each mouse was removed from its home cage, weighed, and then individually placed into a separate clean cage containing the Nutella treatment. Treated Nutella was placed onto the centers of white glass tiles (4 cm^2) and placed into the center of the cage. Mice were given no more than 5 minutes to consume treatments. Animals were given oral estradiol or placebo treatments for 31-34 days; oral treatments started 2 days following BCAS or sham surgical procedures to allow for recovery. During task phases mice were given oral treatments post behavior training throughout (see below). Plasma estradiol was measured (see below).

Novel Object Recognition (NOR) Task

Declarative memory was tested in experimental and control mice (n = 41) using a NOR task.[16] Testing commenced 31-34 days after the start of oral estradiol and placebo treatments and continued for 4 days. The object recognition task was conducted using a black plexiglass open field box with a white colored bottom. Three objects were used for the task: 2 red wood cubes (familiar objects) and 1 yellow wood triangle (novel object). Immediately before testing, mice were taken from the home cage and

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