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Age and 17β-estradiol effects on blood–brain barrier tight junction and estrogen receptor proteins in ovariectomized rats

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ABSTRACT

Age and estrogen levels alter blood–brain barrier (BBB) tight junction (TJ) regulation, impacting brain homeostasis and pathological outcomes. This examination evaluated BBB TJ and estrogen receptor (ER) protein expression changes in young (8–10 week) and middle-aged (10–12 month) ovariectomized female Fisher-344 rats with chronic 17β-estradiol or placebo treatment. Middle-aged rats showed decreased protein expression of occludin with 17β-estradiol (55 kDa band) or placebo (45, 55, 60 kDa bands) treatment compared to respective young. In young animals, 17β-estradiol treatment increased expression of the occludin 55 kDa band over placebo; however, this effect was lost in the middle-aged animals. In both young and middle-aged animals, expression of claudin-5 (23, 32 kDa bands) and ERα (66 kDa) increased with 17β-estradiol treatment, while junctional adhesion molecule-A showed no change across all groups. However, ERα expression (66 kDa) was significantly reduced in the middle-aged animals compared to young placebo treated animals. Measurement of BBB TJ permeability via in situ perfusion of ^{14}C-sucrose showed no change with age or treatment. Our results show that increasing age and 17β-estradiol treatment alters the expression of ERα and distinct BBB TJ protein isoforms without altering functional paracellular permeability.

Introduction

The blood–brain barrier (BBB) exists as a physical and metabolic barrier between the peripheral circulation and the central nervous system to maintain brain homeostasis. A primary component of BBB is the existence of highly specialized tight junctions (TJs). These TJs regulate the paracellular flux of hydrophilic molecules across the BBB. In aging populations, significant structural and functional changes in the BBB can contribute to negative pathological outcomes (Zeevi et al., 2010). Shifts in estrogen levels affiliated with the onset of menopause, as well as with hormone therapies, may further contribute to alterations in BBB function. To date, little is known as to the impact of aging and hormone treatment with regards to BBB TJ protein alterations.

The integrity of the BBB TJs is maintained by three key transmembrane proteins: occludin, claudins, and junction adhesion molecules (JAMs). Occludin is a tetraspanning membrane protein capable of regulating the TJs to changes in acute vascular dynamics (Sandoval and Witt, 2008). Claudins are a family of proteins, with claudins-5 identified as a primary regulator of TJ permeability (Piontek et al., 2008). Increased claudin-5 expression has been shown to decrease BBB paracellular permeability (Honda et al., 2006). In contrast, claudin-5-deficient mice show increased small molecule paracellular permeability (Nitta et al., 2003). Lastly, the JAMs are a family of immunoglobulin proteins. JAMs are involved in maintaining TJ integrity, signaling of cytoskeletal-associated proteins, and are also involved in leukocyte diapedesis (Mandell and Parkos, 2005; Weber et al., 2007). Homophilic JAM-A (a.k.a. JAM-1, JAM) interactions have been shown to stabilize cellular junctions (Mandell et al., 2004), while decreased JAM-A expression has been shown to correlate with loss of BBB TJ integrity (Yeung et al., 2008). It has become increasingly evident that changes/shifts in function and regulation of these respective TJ proteins contribute to BBB paracellular regulation and altered microvascular function.

There is strong evidence that the processes of aging and hormonal changes alter BBB TJ integrity, which may predispose individuals to enhanced negative outcomes subsequent to pathological stress (Bake et al., 2009; Bake and Sohrabji, 2004; Chi et al., 2006; DiNapoli et al., 2008; Selvamani and Sohrabji, 2010). In this regard, age dependent alterations in BBB integrity and use of estrogen based treatments have significant implications with regards to stroke outcomes. Stroke modeling has typically been evaluated in young ovariectomized (OVX) females, where estrogen treatments have been shown to reduce infarct volumes (Dubal et al., 1998; Simpkins et al., 1997). Yet, the impact of estrogen replacement in aging animals remains controversial, with some studies identifying reductions in infarct volumes (Dubal and Wise, 2001; Toung et al., 2004) and others identifying increased infarct volumes (Selvamani and Sohrabji, 2010). Thus, understanding alterations in BBB TJ proteins is critical in

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delineating why these paradoxical effects are occurring. Specifically, how might changes in estrogen levels and/or estrogen receptor activity contribute to TJ protein alterations correlate with age? The actions of estrogen are mediated by two principle receptor subtypes: ERα and ERβ; both of which are found within the cerebral microvasculature (Jesmin et al., 2003; Stirone et al., 2005a; Stirone et al., 2003). Both ERα and ERβ are capable of mediating transcriptional activity (genomic) and rapid-signaling responses (non-genomic). The non-genomic actions of estrogen have been shown to mediate numerous second messenger pathways (Levin, 2009), many of which have been identified to be involved in the regulation of TJ protein function, localization, and expression (Sandoval and Witt, 2008). Similar to TJ proteins, evaluation of ERα banding expression identifies changes with age and hormonal treatment (Kim and Bender, 2009; Stirone et al., 2003). While functional significance of these bands has not been fully elucidated, changes in ERs with age and estrogen treatment may impact downstream TJ protein expression and regulation.

The goal of this study was to delineate changes in banding expression in key TJ proteins (occludin, claudin-5, JAM-A) and estrogen receptors (ERα, ERβ) with age and 17β-estradiol treatment. Secondly, to determine how observed changes in expression of the TJ proteins and ERs correlated with changes in paracellular permeability. Within this examination we used young (8–10 week) and middle-aged (10–12 month) female Fisher-344 rats, which were OVX to mimic menopause induction. Each group was evaluated with and without chronic 17β-estradiol treatment. Middle-aged animals within this context are consistent with early menopause, a period associated with initial hormone replacement in humans. Delineating the expression changes of the respective proteins over changes in age and treatments may provide critical insight into in both physiological and pathological regulation of the TJs.

Materials and methods

Antibodies and chemicals

Anti-occludin, and anti-claudin-5 antibodies were purchased from Zymed Laboratories (San Francisco, CA). Anti-occludin (1:500, 0.5 mg/ml) is a polyclonal rabbit antibody specific for the occludin protein directed against the N-terminal region of rat, mouse or human occludin proteins. Anti-claudin-5 (1:1000, 0.25 mg/ml) is a monoclonal mouse antibody. ERα is a rabbit polyclonal antibody (H-184, Santa Cruz Biotechnology, Santa Cruz, CA 1:500) that reacts with amino acid residues 2–185, N-terminus. ERβ is a mouse monoclonal antibody (1:1000, Abcam; Cambridge, MA) that reacts with the recombinant full length protein (53–59 kDa). Anti-actin (0.2 mg/ml) mouse monoclonal antibody (Sigma-Aldrich, St. Louis, MO) is directed against the C-terminus of the actin protein (1:2000, 42 kDa; clone AC-40). Anti-mouse IgG and anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham (Buckinghamshire, UK). Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich.

Animals

Female Fischer-344 rats of two age groups, young (8–10 weeks, 120–190 g) and middle-aged (10–12 months, 220–290 g, retired breeders), were used for the examinations (Harlan Laboratories, IN Rats were housed in rooms with a 12-h light/dark cycle (20–22 °C) with water and food available *ad libitum*. All experiments were conducted in accordance with the institutional approval of the animal use subcommittee, which subscribes to the NIH Guide for Care and Use of Laboratory Animals. All rats were allowed to acclimate to the facility prior to any procedure being performed. Additionally, as middle-aged animals may present a heterogenous profile over the chronic treatment period, all animals were ovariectomized for measurement across a consistent based line.

Ovariectomy

Animals were anesthetized with intraperitoneal injections of (1 ml/kg) consisting of ketamine/xylazine (80/6 mg/ml). Bilateral removal of the ovaries was performed using a dorsal midline incision. Rats were given 1 week to recover from surgery in order to ensure depletion of endogenous estrogen prior to any kind of hormonal treatment. Ovariectomizing middle-aged animals is consistent with early menopause, a period associated with initial hormone replacement in humans.

Drugs and hormonal treatment

Sustained release (21 days) placebo and 17β-estradiol pellets (2.5 mg/pellet) were purchased from Innovative Research of America (Sarasota, FL). Following OVX recovery period of 1 week, rats were briefly anesthetized with subcutaneously implanted via trochar with either placebo or 17β-estradiol ($n = 5$–7/group/set). All subsequent evaluations were conducted following the full 21-day treatment course.

Measurement of serum 17β-estradiol

Prior to harvesting brain microvessels, or *in situ* brain perfusion, serum samples were obtained to measure 17β-estradiol. Peripheral blood samples were withdrawn from the descending renal artery immediately prior to harvesting microvessels via Vacutainer (Beckmen Dickson, Franklin Lakes, NJ). Blood was allowed to clot on ice, centrifuged at 1500 × *g*, 4 °C for 10 min, and the supernatant collected as serum. The serum was immediately frozen in liquid nitrogen and stored at −80 °C until analysis. From the serum, estradiol levels were measured by enzyme immunoassay (Cayman Chemical, Ann Arbor, MI). Estradiol levels (pg/ml ± SEM) for young and middle-aged OVX placebos were 13.971(±) 1.982 and 17.006(±) 1.979 pg/ml, respectively. Estradiol levels for young and middle-aged OVX 17β-estradiol-treated rats were 123.506 (±) 17.520 and 155.095 (±) 25.279 respectively. No statistically significant differences were found in estradiol levels between young and middle-aged placebos or between young and middle-aged estrogen treated rats.

Harvesting of brain microvessels

Following treatment period, brain microvessels were isolated from rat cerebral gray matter as previously described (Huber et al., 2006; Witt et al., 2005). This methodology produces and enriched microvascular capillary fraction, and as such potential pericyte contribution may occur. Rats received 1.0 ml/kg im of ketamine/xylazine and were subsequently decapitated. Meninges and choroid plexus were excised from the cerebral hemispheres, which were then homogenized in a Dounce tissue grinder with 6 ml of 4 °C microvessel isolation buffer (103 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 15 mM HEPES, 2.5 mM $NaHCO_3$, 10 mM D-glucose, 1 mM sodium pyruvate, dextran [MW 64,000, 10 g/l]; pH 7.4) with a protease inhibitor cocktail (Complete, Roche Diagnostics, Indianapolis, IN). Subsequently, 6 ml of 26% dextran (4 °C) were added, the mixture vortexed, and then centrifuged at 5600 × *g* for 10 min. The supernatant was aspirated, and the pellets resuspended in 10 ml of microisolation buffer and passed through a 70-μm filter (Falcon, Becton-Dickinson; Franklin, NJ). The filtered homogenates were centrifuged at 3000 × *g* for 10 min at 4 °C and protein was extracted from the pellets with 6 M urea lysis buffer (6 M urea, 0.1% Triton X-100, 10 mM Tris, pH 8.0, 1 mM dithiothreitol, 5 mM $MgCl_2$, 5 mM EGTA, 150 mM NaCl) containing protease inhibitor cocktail