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Inhibition of hippocampal estrogen synthesis by reactive microglia leads to down-regulation of synaptic protein expression

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ABSTRACT

Activation of microglia may facilitate age-related impairment in cognitive functions including hippocampal-dependent memory. Considerable evidence indicates that hippocampal-derived estrogen improves hippocampal-dependent learning and memory. We hypothesize that activated microglia may inhibit *de novo* hippocampal estrogen synthesis and in turn suppress hippocampal synaptic protein expression. The present study aimed to elucidate the role of lipopolysaccharide (LPS)-activated microglial HAPI cells on estrogen synthesis and expression of synaptic proteins using H19-7 hippocampal neurons with a neuron–microglia co-culture system. LPS induced expression of the microglial activation markers major histocompatibility complex II (MHC II), CD11b, and ionized calcium-binding adapter molecule 1 (Iba1). Prolonged LPS exposure also enhanced the secretion of interleukin (IL)-6 and nitric oxide (NO) from microglial HAPI cells. Exposure to either LPS-activated microglia or IL-6, significantly suppressed the expression of synaptic proteins and the secretion of *de novo* hippocampal estrogen in H19-7 hippocampal neurons. In addition, LPS-activated microglia also decreased the expression of estrogen receptors (ERα and ERβ) in H19-7 hippocampal neurons. Our findings demonstrate a potential mechanism of microglia activation underlying the reduction in estrogen-mediated signaling on synaptic proteins in hippocampal neurons, which may be involved in hippocampal-dependent memory formation.

1. Introduction

Microglial cells are the resident macrophages present in the central nervous system. In aged and neurodegenerated brains, activation of microglia is accompanied by a dramatic morphological change from the ramified resting state to an amoeboid appearance. Activated microglia exhibit increased expression of specific markers, including major histocompatibility complex II (MHC II), CD11b, and ionized calcium-binding adapter molecule 1 (Iba1). In addition, chemokine receptors, such as C-C chemokine receptor 5 (CCR5) and C-X-C chemokine receptor 3 (CXCR3) are also induced (Frank et al., 2006; Block et al., 2007; Miller and Streit, 2007; Luo et al., 2010). Activated microglia-derived inflammatory mediators including interleukin (IL)-1β, tumor necrosis factor-α (TNF-α), nitric oxide (NO), and reactive oxygen intermediates inhibit synaptic plasticity and hippocampal-dependent memory formation (Frank et al., 2006; Tanaka et al., 2006; Terrando et al., 2010; Liu et al., 2012; Lee et al., 2013). Suppression of these factors, as exemplified by reduction of IL-1β, can completely prevent memory deficits (Barrientos et al., 2002; Bilbo et al., 2005). Although previous studies have suggested that activated microglia-derived inflammatory mediators are involved in the impairment of hippocampal-dependent memory (Bilbo et al., 2005; Tanaka et al., 2006; Terrando et al., 2010), how these factors contribute to memory deficits remains largely unknown.

Menopause is believed to be an important factor for memory deficits in age-related neurodegenerative disorders (McEwen and Alves, 1999; Adams et al., 2001; Benedusi et al., 2011). Consequently, estrogen replacement therapy (ERT) has become an alternative treatment to reduce the risk of neurodegenerative disorders and prevent memory impairment in postmenopausal women (McEwen and Alves, 1999). However, due to its detrimental side effects, the use of ERT has been limited (Gorenstein et al., 2011). In addition to gonads, estrogen is also synthesized *de novo* in

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hippocampal neurons, which is a source of rapid estrogen signaling in hippocampal neuronal circuitry (Prange-Kiel et al., 2006; Fester et al., 2011; Srivastava et al., 2011; Chamniansawat and Chongthammakun, 2012). Hippocampal-derived estrogen has been shown to be essential for synaptic plasticity and memory formation (Prange-Kiel et al., 2006; Zhou et al., 2010; Srivastava et al., 2011; Chamniansawat and Chongthammakun, 2012). There is a growing body of clinical evidence indicating that postmenopausal women who are treated with aromatase inhibitors exhibit deficits in learning and memory (Shilling et al., 2001). Recently, long-term potentiation (LTP), number of mushroom spines, and number of spine synapses were reported to be significantly decreased in aromatase inhibitor-treated ovariectomized (OVX) mice, compared with untreated OVX mice (Shilling et al., 2001; Zhou et al., 2010; Vierk et al., 2012). Moreover, our previous study has demonstrated the priming effect of hippocampal-derived estrogen to organize hippocampal neurons upon stimulation by exogenous estrogen (Chamniansawat and Chongthammakun, 2012). However, exogenous estrogen treatment could not restore the number or spine density in aged female rats (Adams et al., 2002). Aged OVX animal showed significant reduction in synaptic density and working memory compared to those of young OVX animals (Adams et al., 2002; Bailey et al., 2011). It seems probable that gonadal estrogen is not a primary factor for the determination of age-related memory impairment. Because microglia activation is involved in memory deficits and hippocampal-derived estrogen is essential for memory formation (Frank et al., 2006; Tanaka et al., 2006; Terrando et al., 2010; Liu et al., 2012; Lee et al., 2013), we hypothesized that activated microglia may inhibit the *de novo* synthesis of hippocampal estrogen, which in turn inhibits synaptic protein expression. The present study aimed to determine the effect of microglia activation on the hippocampal estrogen synthesis and synaptic protein expression using a neuron–microglia co-culture model.

2. Materials and methods

2.1. Cell culture

2.1.1. Microglial HAPI cells

Microglial HAPI cells were generously provided by Dr. James R. Connor (Hershey Medical Center, Hershey, PA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY) supplemented with 5% fetal bovine serum (PAA, Labolatories, Morningside, Queensland) in a 5% CO_2 humidified atmosphere at 37 °C. In microglia activation experiments, cells were plated onto 6-well plates at density of 1×10^6 cells/well (Corning, NY, USA). After 2 days of subculture, cells were treated with 100 ng/ml of lipopolysaccharide (LPS). In neuron–microglia co-culture, microglial HAPI cells were plated onto the inserted microporous membrane (0.4 μm) of a transwell apparatus (Corning) at density of 1×10^6 cells/well.

2.1.2. H19-7 hippocampal neurons

H19-7 cells of hippocampal origin were grown on poly-L-lysine (PLL)-coated well plates (or coverslips), in DMEM at 33 °C as described previously (Chamniansawat and Chongthammakun, 2012). H19-7 hippocampal cells were transformed with a temperature-sensitive mutant of the simian virus 40 T antigen. During the differentiation process, cells were incubated at 39 °C in DMEM with N_2 supplements (Invitrogen, Carlsbad, CA, USA), and 10 ng/ml basic fibroblast growth factor (Sigma–Aldrich, St. Louis, MO, USA) was added to accelerate the formation of neuronal processes. Recombinant mouse IL-6 (R&D system Inc., Minneapolis, MN) was added at a final concentration of 450 pg/ml. On the other hand, aromatase inhibitor (anastrozole; Sigma) was added to cultures at a final concentration of 25 nM.

2.1.3. Neuron–microglia co-culture and cell treatments

Co-cultures were performed following the method previously described by Xie et al. (2004). After 2 days of subculture, neurons on the bottom of 6-well plate at density of 1×10^6 cells/well (or coverslips) were co-cultured with microglial HAPI cells (1×10^6 cells/membrane) by placing the microglia-containing Transwell® insert into the neuron-containing well. In this co-culture system, the microglia and neurons are in close proximity and shared the same culture medium. However, microglia and neurons are separated by the microporous membrane and do not have direct cell–cell contact. After 24 h of co-culturing, microglial HAPI cells were activated by adding 100 ng/ml LPS, followed by incubation at 37 °C for 5 days.

2.2. Quantitative real time-PCR (qRT-PCR)

Cells were plated onto 6-well plate at a density of 1×10^6 cells/well. Total RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany) and 2 μg of RNA was converted to cDNA with the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). qRT-PCR was performed with the Applied Biosystems PRISM 7700 Sequence Detection System according to the manufacturer's protocol. TaqMan-Gene Expression Assays with FAM™ dye-labeled TaqMan® MGB probes, which contained customized primers, were utilized for qRT-PCR analysis. Two sets of target genes were analyzed in this study: (1) microglial mRNA (MHC II, CD11b, and Iba1) and (2) neuronal mRNA (activity-regulated cytoskeleton associated protein (Arc), post-synaptic density-95 (PSD-95), and synaptophysin). GAPDH was utilized as a positive endogenous control with water as a negative control. The relative expression of mRNA was calculated using the comparative Ct method with SDS software v.1.3.1 (Applied Biosystems).

2.3. Western blot analysis

Microglial HAPI cells or H19-7 hippocampal cells were plated into 6-well plates at a density of 1×10^6 cells/well and left to adhere overnight. At the end of the experiment, supernatants were removed and estrogen, IL-6, and IL-10 concentrations were determined using the ELISA assays. Adherent cells were lysed using Pierce® Ripa Buffer (Thermo Fisher Scientific Inc., Rockford, IL, USA), and equal amounts of protein (25 μg) were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with the following primary antibodies at a dilution of 1:1000: mouse anti-estrogen receptor (ER) α, mouse anti-ERβ, mouse anti-IL-6, mouse anti-inducible nitric oxide synthase (iNOS), mouse anti-IL-10, mouse anti-Arc, mouse anti-synaptophysin, mouse anti-β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA), rabbit anti-PSD-95, or rabbit anti-Bcl2 (Abcam, Cambridge, UK). Membranes were probed with HRP-conjugated secondary antibodies (Zymed, San Francisco, CA) and detection utilized Thermo Scientific SuperSignal® West Pico Substrate (Thermo Fisher Scientific Inc.) captured on CL-XPosure Film (Thermo Fisher Scientific Inc.).

2.4. Enzyme-linked immunosorbent assay (ELISA)

The presence of either cytokines (IL-6 and IL-10) in the supernatant of microglial HAPI cell culture or estrogen in the supernatant of H19-7 hippocampal neuronal culture was detected using ELISA kit (R&D Systems Inc.). The culture medium was pipetted into micro-assay wells, pre-coated with monoclonal antibody, followed by incubation at 37 °C for 30 min. Washing