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Selective estrogen-receptor modulators suppress microglial activation and neuronal cell death via an estrogen receptor-dependent pathway

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ABSTRACT

Growing evidence shows that steroid hormones, especially 17β-estradiol (E2), protect neuronal cells by attenuating excess activation of microglia. However, the use of E2 in the clinic is controversial because of its peripheral actions in reproductive organs and its potential to increase risk for endometrial cancer and breast cancer. Selective estrogen-receptor modulators (SERMs) bind to estrogen receptors (ERs), but their effects as ER agonists or antagonists are dependent on the target tissue. SERMs pose very little cancer risk as a result of their anti-estrogen action in reproductive organs, but their action in the brain is not well understood. In this study, we investigated the effects of SERMs tamoxifen (Tam) and raloxifene (Rlx) on microglial activation and subsequent neuronal injury. Tam and Rlx suppressed the increases in proinflammatory cytokines and chemokine expression that were induced by lipopolysaccharide (LPS) in rat primary microglia cultures. The microglial-conditioned media pretreated with Tam or Rlx significantly attenuated cellular injury in SH-SY5Y cells elicited by microglial-conditioned media treated with LPS alone. Rat primary microglia expressed ERα and ERβ primarily in the nucleus, and thus we examined the involvement of ERs in the suppressive action of Tam and Rlx on microglial activation using a pure ER antagonist, ICI182,780. Pretreatment with ICI182,780 abolished the suppressive effects of SERMs on microglial activation, as well as their protective action on SH-SY5Y cells. A luciferase assay using a vector with three estrogen response elements (EREs) revealed that Tam and Rlx activated ERE-mediated transcription in rat primary microglia. Taken together, these results suggest that Tam and Rlx suppress microglial activation and subsequent neuronal cell death via an ER-mediated transcription pathway. SERMs could represent a novel therapeutic strategy for disorders of the central nervous system based on their ability to suppress neuroinflammation.

1. Introduction

Microglia are the primary immune cells of the central nervous system (CNS) and are activated quickly in response to external pathogens or cell debris, after which they act by releasing inflammatory factors or engulfing foreign bodies to mediate the inflammatory response. However, excessive activation of microglia may be harmful for host cells; microglia can promote the development of some neuronal diseases by producing large amounts of cytokines and other inflammatory molecules such as tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), nitric oxide, and reactive oxygen species. Indeed, activated microglia are reported to be associated with the pathogenesis of Parkinson's disease [1] and Alzheimer's disease [2]. In these diseases, a large number of activated microglial cells, which have the potential to release inflammatory cytokines, gather around lesions, indicating that microglia-mediated inflammatory responses could be a mechanism in a variety of neurodegenerative diseases. In addition, microglia with abnormal activity are reportedly involved in brain ischemia–reperfusion injury, trauma, epilepsy, depression, and schizophrenia [3–5]. Therefore, the regulation of microglial activity is crucial to maintain physiological function in the brain and to prevent the onset and development of CNS disorders.

Abbreviations: CNS, central nervous system; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL1β, interleukin-1β; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein 1; MIP-2α, macrophage inflammatory protein 2α; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; Rlx, raloxifene; SERM, selective estrogen-receptor modulator; Tam, tamoxifen; TNFα, tumor necrosis factor α.

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17β-Estradiol (E2),which is synthesized in, and secreted from, peripheral endocrine glands such as the ovary, the placenta, and the adrenal cortex, passes through the blood–brain barrier to perform diverse functions in the CNS. In addition, the brain possesses an inherent endocrine system and de novo synthesizes E2. Recently, increasing evidence has shown that E2 protect neurons from excess or prolonged inflammation in the brain. Treatment with E2 suppresses inflammatory cytokine expression and nitric oxide production induced by lipopolysaccharide (LPS) in microglia [6,7]. These suppressive effects are mediated via the estrogen receptor (ER) and act by blocking DNA binding and transcriptional activity of NF-kB p65 by preventing its nuclear translocation [8]. E2 has also been reported to inhibit neuroinflammation in an ER-dependent manner in studies using in vivo models of CNS diseases [9,10]. However, although the neuroprotective and anti-inflammatory effects of E2 in the brain are well documented in animal models of neurodegenerative disorders and other diseases, the use of E2 in the clinic is controversial because of its peripheral actions in reproductive organs and its potential to increase risk of endometrial cancer and breast cancer. Therefore, alternative compounds that share some mechanisms of action with E2 might represent treatments for CNS disorders with a better safety profile than E2.

Selective estrogen receptor modulators (SERMs) include compounds with mixed agonist/antagonist action at the ER. SERMs bind to ERs, but their action as an ER agonist or antagonist is dependent on the target tissue and cell types, and the nature of this relationship varies with SERM compounds [11]. Tamoxifen (Tam), which was the first SERM compound to be used clinically, has been widely applied in the treatment of breast cancer, in which it functions as an ER antagonist. In contrast, Tam has estrogen-like characteristics in skeletal tissue [12]. Raloxifene (Rlx) is a second-generation SERM that was developed to function as an ER agonist in bone and as an ER antagonist in reproductive tissues, and is prescribed for the prevention and treatment of postmenopausal osteoporosis [13].

Some groups have reported neuroprotective effects of Tam and Rlx using in vivo experimental models. Treatment with Tam suppressed experimental spinal cord injury through attenuation of TNFα and IL-1β levels [14], and induced regeneration of the rat sensory cortex after a penetrating brain injury [15]. Rlx decreased the number of microglia and astrocytes in aged mice [16]. Tam and Rlx reduced the number of microglia in rats with intraperitoneal administration of LPS [17] as well as brain trauma [18]. Liu et al. demonstrated that Tam attenuated microglial activation and brain injury elicited by irradiation [19]. Furthermore, in astrocytes, SERMs including Tam and Rlx suppressed the expression of interleukin-6 and interferon-γ-inducible protein-10 induced by LPS via attenuating nuclear translocation of NF-kB [20]. However, the effects of SERMs in the brain, especially in microglia, are still not well understood. In this study, we examined the action of SERMs Tam and Rlx on microglial activation induced by LPS, with a focus on ER in rat primary microglia.

2. Materials and methods

2.1. Materials

LPS from *Escherichia coli* 026:B6, ICI182,780, and E2 were obtained from Sigma–Aldrich (St. Louis, MO, USA). Tamoxifen citrate was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Raloxifene hydrochloride was purchased from Cayman Chemical (Ann Arbor, MI, USA). All other chemicals were obtained from Wako Pure Chemical Industries, Nacalai Tesque (Kyoto, Japan), or Sigma–Aldrich and were of reagent grade.

2.2. Animals

All animal procedures were performed in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology (Japan), and the Animal Care and Use Committee of Hiroshima University (Hiroshima, Japan). Pregnant Wistar rats were obtained from Kyudo (Kumamoto, Japan) and were maintained in a temperature-controlled animal facility with 12 h light–dark cycles.

2.3. Primary microglia culture

Primary microglia cultures were prepared from 1-2-day-old Wistar rats (both males and females were used for microglia preparation) according to the well-established "shaking off" method [21]. Brains were excised and the meninges were carefully removed. The tissue was dissociated by passing it through a 250-μm nylon mesh with the aid of a rubber policeman. After washing with Hanks' balanced salt solution, the cell suspension was triturated with a Pasteur pipette and plated in a poly-L-lysine-coated 75 cm^2 plastic culture flask at a density of 1 brain per flask in 10 mL tissue culture medium, which consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 5 μg/mL insulin, and 0.5 ng/mL granulocyte-macrophage colony-stimulating factor. Cultures were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The medium was changed 2 or 3 times per week. After 9–12 days, microglia were harvested by shaking the flask at 150 rpm for 15 min and seeded at a density of 1×10^5 cells/cm^2 in microglia culture medium (DMEM with 10% charcoal-stripped FBS and 5 μg/mL insulin). Culture medium was changed to remove non-adhering cells 30 min after seeding. After culturing for 24 h, reagents were added to the microglia. The cultures of isolated microglia were uniformly immunopositive for CD11b and contained greater than 95% microglial cells.

2.4. Total RNA extraction and real-time PCR

Determination of mRNA levels was performed as previously described [22]. Briefly, total RNA was extracted from microglia using a High Pure RNA Isolation Kit (Roche Diagnostics K.K., Tokyo, Japan). Single-stranded cDNA was synthesized from 0.5 μg of total RNA following the ReverTra Ace protocol (Toyobo, Osaka, Japan) with a random primer (9-mer; Takara Bio Shiga, Japan). Real-time PCR was performed using a LightCycler instrument (Roche Diagnostics) with Sybr Green Real-time PCR master mix (Toyobo). The primer sequences used in this study are listed in Table 1. The levels of mRNA were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level, and the values of treated cells were divided by those of untreated cells to give relative mRNA levels.

Table 1
Primer sequences used in this study.

Gene	Forward primer	Reverse primer
TNFα	AGCCCTGGTATGAGCCCATGTA	CCGGACTCCGTGATGTCTAAGT
IL-1β	CACCTCTCAAGCAGAGCACAGA	ACGGGTTCCATGGTGAAGTC
MCP-1	TGTCTCAGCCAGATGCAGTT	CAGCCGACTCATTGGGATCA
MIP-2α	CCCTCCTGTGCTCAAGACTC	CCACAACAACCCCTGTACCC
GAPDH	AACGACCCCTTCATTGACCT	CCTTGACTGTGCCGTTGAACT