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

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A B S T R A C T

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.

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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; MIP-1α, monocyte chemoattractant protein 1; MIP-2α, macrophage inflammatory protein 2α; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium 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-κB 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 (Rx) 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 Rx 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]. Rx decreased the number of microglia and astrocytes in aged mice [16]. Tam and Rx 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 Rx suppressed the expression of interleukin-6 and interferon-γ-inducible protein-10 induced by LPS via attenuating nuclear translocation of NF-κB [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 Rx 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* O26:B6, IC1182,780, 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 polystyrene-coated 75 cm² 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% CO2 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 × 10⁵ cells/cm² 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>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
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<tbody>
<tr>
<td>TNFα</td>
<td>AGCCCTTCATGATGACCCTATGTA</td>
<td>CCGGACTCCCGTGATGCTAACT</td>
</tr>
<tr>
<td>IL-1β</td>
<td>CACCTCTCAAGCCACAGAA</td>
<td>ACGGTTCATGCTGAACT</td>
</tr>
<tr>
<td>MCP-1</td>
<td>TGCTCAGGCTACGATGATT</td>
<td>CGGCCGACTCTGGGGAATC</td>
</tr>
<tr>
<td>MIP-2α</td>
<td>CTCCTCTGCTCAAGACGTC</td>
<td>CACAAAGAACCCGTGACCCC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ACGACCCCTTTCACTTTGCT</td>
<td>CTTGACTGTCCGGTGAACCT</td>
</tr>
</tbody>
</table>
2.5. Enzyme-linked immunosorbent assay (ELISA)

The levels of TNFα and IL-1β present in the cultured supernatants of microglia were evaluated with the TNFα and IL-1β Mini ELISA Development Kit (PeproTech, Rocky Hill, NJ, USA) according to the manufacturer’s instructions.

2.6. Microglia-conditioned media

The culture medium of the microglia was replaced with new medium immediately before the LPS treatment. SERMs or other inhibitors were added again in the culture. The conditioned media were collected 24 h after administration of LPS, centrifuged to remove cell debris, and stored at –80 °C until use.

2.7. SH-SY5Y cell culture

Human neuroblastoma SH-SY5Y cells (CRL-2266, American Type Culture Collection, Manassas, VA, USA) were placed into culture dishes and cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. The media were replaced every 3–4 days. Cells were sub-cultured when they reached 80–90% confluence.

2.8. Measurement of cell viability

Twenty-four hours after treatment of SH-SY5Y cells with conditioned media, cell viability was measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. MTT was added to the cell-culture medium at a final concentration of 0.5 mg/mL. After incubating the plates at 37 °C for 2 h in a 5% CO₂ and 95% air atmosphere, the resulting purple pellets were dissolved in a 40 mM HCl/isopropanol solution. The absorbance was read at 570 nm with a microplate reader (Bio-Rad Hercules, CA, USA). The percentage of cell survival was calculated with the value of the untreated cells taken as 100%.

2.9. Immunocytochemistry

Microglia cultured on glass cover slips were fixed with 4% paraformaldehyde at room temperature for 30 min. After excess paraformaldehyde was removed by 50 mM glycine, cells were permeabilized with 0.2% Triton X-100 for 15 min and then blocked using 1% bovine serum albumin (BSA). Rabbit polyclonal antibodies directed against ERMα (1:50, Abcam, Tokyo, Japan) and ERMβ (1:50, Abcom) in PBS with 1% BSA and 0.1% Triton X-100 were then applied for 3 h, followed by incubation for 1 h in Alexa488 anti-rabbit IgG (1:250, Molecular Probes, Eugene, OR, USA). Nuclei were stained with 1 μg/ml Hoechst 33342 for 5 min. Images were obtained with an inverted fluorescent microscope (BZ-9000, Keyence, Osaka, Japan).

2.10. Immunoblotting

Microglia were collected and lysed with RIPA buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS). Equal amounts of protein were loaded and separated using SDS-PAGE with a 10% or 12% (w/v) polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. The blocked membranes were incubated with the primary antibodies: anti-ERMα (1:1000, Abcam), anti-ERMβ (1:1000, Abcam), and anti-α-tubulin (1:2000, Sigma–Aldrich, St. Louis, MO, USA). The membranes were incubated with peroxide-conjugated secondary antibodies (Thermo Fisher Scientific, Waltham, MA, USA) and then visualized using peroxide substrates (SuperSignal West Pico, Thermo Fisher Scientific). The band intensity was quantified using Image J software (NIH, MD, USA).

2.11. Plasmid construction and luciferase assay

The sequence of the estrogen-responsive element (ERE) was taken from the report of Ciana et al. [23]. The 2 oligonucleotides including 3 canonical estrogen-responsive elements (3 × ERE) spaced by 8 bp, 5’-AGGTCACTAGTACCCAGGCTCAGT-GACCTATCCGCGAGGTGACCT-3’ and 5’-AGGTCACTGTCGACCTGAGTCACTGTGACCCTGCGATCTAGTGACCTG-GACCT-3’, were annealed and the resulting double-strand oligo was ligated into the EcoRV site of the pGL4.24 vector (Promega, Madison, WI, USA), and the resulting construct was named pGL4.24-C3 × ERE. The pGL4.24 and pGL4.24-C3 × ERE constructs were transfected into rat primary microglia using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Twenty-four hours after transfection, cells were treated with SERMs or E2. Luciferase reporter activity was measured using the Luciferase Assay System (Promega) and the GloMax 20/20 Luminometer (Promega). Luciferase activity was normalized to total protein levels.

2.12. Statistical analyses

All data are expressed as the mean ± standard error of the mean (S.E.M.). The statistical analyses were performed using one-way analysis of variance (ANOVA), followed by the Student’s t-test or Dunnett’s test. Multiple comparisons were made using the Holm’s or Bonferroni correction methods. Probability (P) values of <0.05 were considered to be statistically significant.

3. Results

3.1. SERMs Tam and Rlx suppressed the expression of proinflammatory molecules induced by LPS in rat primary microglia

When rat primary microglia were treated with various concentrations of Tam and Rlx for 24 h, decreases in the viability of primary microglia were observed by treatment with 10 μM of Tam or Rlx (Fig. 1). Therefore, we have used SERMs at the concentration of 0.3, 1, and 3 μM in this study.

Fig. 1. Toxicity of SERMs, Tam and Rlx on rat primary microglia. Rat primary microglia were pretreated with 0.3, 1, 3, or 10 μM of Tam or Rlx for 24 h, and then cell viability was evaluated by MTT assay. The values are the means ± S.E.M. of five separate experiments. **P < 0.01 vs. the untreated group.
After stimulation of rat primary microglia with LPS for 6 h, mRNA levels of proinflammatory cytokines TNFα and IL-1β, chemokines, monocyte chemoattractant protein 1 (MCP-1), and macrophage inflammatory protein 2α (MIP-2α) were increased (Fig. 2A–D). In addition, levels of TNFα and IL-1β released from the microglia into the culture medium were also elevated, indicating that microglia were activated by treatment with LPS (Fig. 3A and B). Treatment of microglia with Tam (0.3, 1, and 3 μM) or Rlx (0.3, 1, and 3 μM) suppressed the increase in mRNA expression of TNFα, IL-1β, MCP-1, and MIP-2α that were elicited by treatment with LPS (Fig. 2A–D). The action of Tam on the attenuation of microglial activation showed dose-dependency and was strongest at 3 μM, while the suppressive effects of Rlx on microglial activation were strongest at the 1 μM concentration. Treatment with Tam or Rlx also attenuated the secretion of TNFα and IL-1β into the medium (Fig. 3A and B). The suppressive effects of Rlx on microglial activation tended to be greater than those of Tam but there were no significant differences between the action of Tam and Rlx (Figs. 2 and 3). Treatment with Tam or Rlx alone did not affect mRNA levels of inflammatory molecules (Fig. 2A–D) and the amounts of inflammatory cytokines in the culture medium (Fig. 3A and B). These results indicate that SERMs Tam and Rlx suppressed the activation of rat primary microglia.

3.2. SERMs inhibit neuronal injury by attenuating microglial activation by LPS

The conditioned medium from rat primary microglia cultures treated with LPS for 24-h induced cell death in SH-SY5Y human neuroblastoma cells 24 h after incubation (Fig. 4B). Because treatment of SH-SY5Y cells with LPS produced no cytotoxicity (Fig. 4A), factor(s) that were released from microglia in response to LPS mediated cell death in SH-SY5Y cells. When the microglial culture from which the conditioned medium was collected was pretreated with Tam or Rlx before the LPS addition, cell death induced by this conditioned medium was significantly lower than that induced by the conditioned medium with LPS alone (Fig. 4B). The inhibitory effects of Tam and Rlx on cell death were highly correlated with their suppression of microglial activation (Figs. 2 and 4B). Therefore, these results suggest that Tam and Rlx suppressed SH-SY5Y cell death by inhibiting microglial activation elicited by LPS.

3.3. ERα and ERβ were primarily expressed in the nuclei of rat primary microglia

Immunocytochemistry showed that ERα immunoreactivity was detected primarily in the nucleus in untreated and
LPS-treated microglia, indicating that ERα is largely expressed in microglial nuclei (Fig. 5A). ERα was considered to be expressed at low levels in the cytosol because a weak ERα signal was detected (Fig. 5A). ERβ was also detected primarily in the nucleus and modestly in the cytosol of rat primary microglia cultured cells (Fig. 5B). There was no change in the localization of ERα and ERβ during LPS stimulation (Fig. 5A and B). Immunoblotting to measure expression levels of ERs showed that treatment with LPS did not affect the expression of ERα and ERβ in microglia (Fig. 5C and D). Together, these data indicate that rat primary microglia express ERα and ERβ primarily in the nucleus, and the abundance of these receptors is not influenced by LPS treatment.

3.4. The suppressive effects of Tam and Rlx on microglial activation were mediated by an ER-dependent transcription pathway

When rat primary microglia were pretreated with ER antagonistICI182,780 and then treated with Tam or Rlx for 24 h, and subsequently stimulated with LPS for 6 h, ICI182,780 nearly completely abolished the suppressive effects of Tam and Rlx on increased TNFα and IL-1β mRNA expression by LPS in microglia (Fig. 6A), indicating that ER antagonism counteracted microglial inactivation by Tam or Rlx. Furthermore, the conditioned media from cells pretreated with ICI182,780 before treatment with Tam or Rlx and the addition of LPS also abolished the protective action of Tam and Rlx on SH-SY5Y cell death induced by LPS-treated cell conditioned medium (Fig. 6B). Treatment with ICI182,780 alone did not affect inflammatory molecule expression (Fig. 6A) as well as cell viability (Fig. 6B).

We next examined whether Tam and Rlx activate ER-dependent transcription. ERs interact with EREs in gene promoters to activate downstream gene transcription [24]. Thus, we performed a luciferase assay using a luciferase-expressing vector cloned with 3 EREs (pGL4.24-3 × ERE) at the upstream of the luciferase gene. Treatment of rat primary microglia transfected with pGL4.24-3 × ERE with 10 nM E2 increased luciferase activity compared with untreated cells, indicating that pGL4.24-3 × ERE activates microglial ER-ERE-dependent transcription of the luciferase gene (Fig. 7). Treatment of the microglia with Tam or Rlx induced significantly higher luciferase activity than that observed in untreated cells. Rlx tended to induce stronger luciferase activity than Tam at the same concentration, but no significant difference was detected between the treatments. Taken together, these results suggest that Tam and Rlx suppress microglial activation elicited by LPS via activating ER-dependent transcription.
4. Discussion

SERMs, Tam and Rlx attenuated microglial expression of proinflammatory cytokines and chemokines induced by LPS in rat primary microglia, indicating that Tam and Rlx suppressed the activation of rat primary microglia. Suuronen et al. reported that Tam attenuated the release of interleukin-6 and nitric oxide from activated primary microglia [25]. Thus, our results support their
findings. In addition, we demonstrated for the first time that Tam and Rlx inhibited neuronal cell death induced by LPS-treated microglial conditioned media, suggesting that the neuroprotective effects of Tam and Rlx are mediated by the suppression of microglial activation. Because activated microglia are known to release harmful factors such as reactive oxygen species and nitric oxide in addition to proinflammatory cytokines and chemokines [26], Tam and Rlx are thought to protect neuronal cells by suppressing the production and/or release of these harmful molecules.

Expression of ERs has been studied in primary microglia, microglial cell lines, and in vivo models. However, ER expression in microglia remains controversial. ERα and ERβ mRNA have been detected in rat primary microglia [6], but there are no reports on ER protein expression in primary microglia. The expression of ERβ was upregulated in monkey microglia during ischemia [27], suggesting that the stress response regulated ER expression. Sierra et al. reported that mouse microglia isolated based on c-fms promoter activity expressed ERβ, but not ERα [28]. They also demonstrated that expression of ERs in microglia was decreased by LPS stimulation. It has also been demonstrated that the mouse microglia cell line BV-2 expresses only ERβ [29]. Thus, the expression of ERs in microglia is thought to depend on species and culture conditions, and is thought to be regulated by various stressful stimuli. In this study, ERα and ERβ were expressed primarily in the nuclei and modestly in the cytosol of rat primary microglia, and the addition of LPS did not affect ER expression. Therefore, rat primary microglia are considered to express ERs constitutively.

The pure ER antagonist ICI182,780 abolished the suppression of microglial activation and neuroprotection mediated by Tam and Rlx indicating that Tam and Rlx bind to ER to exert their effects. Tam has also been reported to block chloride channels, and this pharmacological action is exerted at concentrations on the order of tens of μM [30]. In this study, 1 μM of Tam attenuated microglial activation, supporting that the action of Tam is mediated by ER activation. ER regulates physiological functions primarily by not only regulating the transcription of various genes in the nucleus (genomic action), but also inducing signal transduction in the cytosol independent of its transcriptional effects (nongenomic action) [31]. Because the majority of ER expression in rat primary microglia is in the nucleus, we speculated that the effects of ERs were mediated by genomic action in our experiments. Indeed, we showed that Tam and Rlx activated transcription via ER-ERE interaction. The degree of ERE-dependent transcription induced by 1 μM Tam or Rlx was similar to that produced by 10 nM E2. Therefore, Tam and Rlx attenuated microglial activation and subsequently protected neuronal cells through an ER-mediated transcriptional pathway. Suuronen et al. suggested that SERM-induced anti-inflammmatory responses were not ER-mediated, but attributable to SERM-induced modulation of LPS-activated proinflammatory signaling cascades [25]. Thus, SERMs could attenuate microglial activation via multiple mechanisms.

There are some reports on the mechanisms by which E2 suppresses microglial activation. Transcription factor NF-κB mediates the expression of proinflammatory molecules in microglia, and inflammatory mediators initiate an NF-κB signaling cascade that leads to phosphorylation and activation of IKKβ (a subunit of the inhibitor of κB kinase (IKK) complex). Activated IKKβ phosphorylates the IκB inhibitory protein IκBα, targeting it for ubiquitination and proteosomal degradation, which allows the release of NF-κB into the nucleus, where it binds to its cognate DNA sequences to induce gene expression [32]. Chisletti et al. showed that E2 inhibited the nuclear translocation of NF-κB and attenuated microglial activation [8], demonstrating an interaction between the ER pathway and the NF-κB signaling cascade. Baker et al.

**Fig. 6.** Involvement of ER in microglial inactivation and neuroprotection induced by Tam and Rlx. (A) Rat primary microglia were pretreated with 1 μM ICI182,780 (ICI) for 20 min and then treated with 1 μM Tam or Rlx for 24 h, followed by 10 ng/mL LPS stimulation for 6 h. Levels of TNFα and IL-1β mRNA were determined by real-time PCR. Amounts of mRNA are represented as fold-change from those in untreated cells. The values are the means ± S.E.M. of five separate experiments. "**P" < 0.01 vs. the untreated group. "*P" < 0.05 vs. the ICI182,780-treated group. (B) Rat primary microglia were pretreated with 1 μM ICI for 20 min and with 1 μM Tam or Rlx for 24 h, and subsequently stimulated with 10 ng/mL LPS for 24 h. SH-SYSY cells were treated with microglial conditioned media for 24 h and then cell viability was assessed by the MTT assay. The values are the means ± S.E.M. of six separate experiments. "*P" < 0.05 vs. the untreated group. "**P" < 0.01 vs. the LPS-treated group. "##P" < 0.01 vs. the LPS + Tam-treated group. (B) Rat primary microglia were pretreated with 1 μM ICI for 20 min and with 1 μM Tam or Rlx for 24 h, and subsequently stimulated with 10 ng/mL LPS for 24 h. SH-SYSY cells were treated with microglial conditioned media for 24 h and then cell viability was assessed by the MTT assay. The values are the means ± S.E.M. of six separate experiments. "*P" < 0.05 vs. the untreated group. "**P" < 0.01 vs. the LPS + Rlx-treated group.

**Fig. 7.** Induction of ERE-mediated transcription elicited by Tam and Rlx. pGL4.24 or pGL4.24-3 × ERE was transfected into rat primary microglia. Cells were stimulated by 1 μM Tam 1 μM Rlx or 10 nM E2 for 6 h and luciferase activity was measured. The values are the means ± S.E.M. of 5 separate experiments. "*P" < 0.05, "**P" < 0.01 vs. the group of untreated cells with pGL4.24-3 × ERE.
demonstrated that E2 potentiated ERK activation, resulting in the suppression of proinflammatory proteins and inducible nitric oxide synthase expression [29], suggesting that alterations in MAPK pathways may be a potential mechanism by which E2 decreased microglial activity. Further study is needed to identify the events downstream of ER that are elicited by Tam and Rlx.

Interestingly, the suppressive effects of high concentrations of Rlx (3 μM) on microglial activation were weaker than those of low concentrations of Rlx (1 μM) (Fig. 2). We could not study Rlx concentrations greater than 3 μM because of its toxicity to rat primary microglia (Fig. 1). Divergent effects of different doses of E2 have also been reported for macrophage and T cell activation, in addition to microglial activation [6,33]. The sensitive dose-dependence of the effects of E2 on immune cells may be a reflection of the multiple signal transduction pathways that can be regulated by E2 under various circumstances. Likewise, different cell types in the immune system do not respond in the same way to E2 treatment [33]. Because Rlx acts on the ER, Rlx could exhibit divergent effects in a similar manner to E2.

Microglial activation has been recognized as an essential mediator of neuroinflammation and is considered to be involved in various CNS disorders, including neurodegenerative diseases and epilepsy [5,34]. Therefore, therapies that suppress microglial activation could represent treatment strategies for these disorders. Although E2 is a typical steroid hormone that has been reported to attenuate microglial activation [6,7], its effects on carcinogenicity, especially for breast cancer and ovarian cancer, remain a matter of debate [35–37]. SERMs, Tam and Rlx are thought to have less potential to induce carcinogenesis, because Tam and Rlx show anti-estrogen action in the mammary gland and the ovary [13]. In addition, this study, Tam and Rlx at a concentration of 1 μM stimulated ERs expressed in rat primary microglia culture cells. The clinical therapeutic dose of Tam is approximately 1 μM [38,39]. Therefore, SERMs such as Tam and Rlx might be a potential candidate for therapeutic agents for CNS diseases based on their suppressive action of microglial activation.

In conclusion, SERMs such as Tam and Rlx protected neuronal cells, likely by attenuating microglial activation. These suppressive effects of Tam and Rlx on microglial activation were mediated by an ER-dependent transcription pathway. Thus, SERMs might represent a novel strategy to treat CNS disorders via suppression of neuroinflammation.

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