The Protective Effect of 17β-Estradiol on Experimental Autoimmune Encephalomyelitis Is Mediated through Estrogen Receptor-α

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Low-dose estrogen (E2) treatment significantly inhibits the clinical signs and histopathological lesions of experimental autoimmune encephalomyelitis (EAE), and is being used in clinical trials to treat multiple sclerosis. To assess the role of intracytoplasmic estrogen receptors in mediating suppression of EAE, we studied mice with disrupted estrogen receptor-α (Esr1) and -β (Esr2) genes. We demonstrate that the protective effect of E2 is abrogated in B6.129-Esr1tm1Unc mice (Esr1−/−) but not in B6.129-Esr2tm1Unc mice (Esr2−/−). The loss of E2-mediated protection from EAE in Esr1−/− mice immunized with the encephalitogenic MOG-35–55 peptide was manifested phenotypically by the development of severe acute clinical signs and histopathological lesions even in the presence of moderately high serum E2 levels. This is in contrast to C57BL/6 wild-type (WT) mice and Esr2−/− mice in which E2 treatment resulted in comparable serum levels and markedly suppressed clinical signs of EAE and abolished inflammatory lesions in the CNS. This pattern showing a lack of E2-dependent inhibition of EAE in Esr1−/− mice was mirrored by an enhanced rather than a reduced secretion of TNF-α, IFN-γ, and interleukin (IL)-6 in MOG-specific splenocytes and a lack of inhibition of message for inflammatory cytokines, chemokines and chemokine receptors in CNS tissue. These results indicate that the immunomodulatory effects of E2 in EAE are dependent on Esr1 and not Esr2 signaling. (Am J Pathol 2003, 163:1599–1605)

Autoimmune diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA), Grave’s disease, systemic lupus erythematosus (SLE), and myasthenia gravis, are sexually dimorphic in that they occur more frequently in women than in men.1 This dimorphism may be due to multiple factors, but gender-related differences in immune responsiveness and sex hormones are likely to play a significant role.2 It is well documented that the first clinical symptoms of MS develop post-puberty.3 Additionally, increased levels of sex hormones produced during pregnancy are associated with a significant reduction in the severity of MS, while clinical symptoms are often exacerbated postpartum, a time characterized by significant alternations in sex hormone levels.3–5 We demonstrated previously that administration of low doses of 17β-estradiol reduced the severity of experimental autoimmune encephalomyelitis (EAE) in several different inbred strains of female mice.6–8 The exact E2-sensitive immune-related target genes have not been fully characterized. Our data from DNA microarray studies in BV8S2 transgenic mice suggests that E2 down-regulates several members of the TNF gene family including TNF-α and Pgrp, and the chemokine RANTES, and up-regulates the expression of several cytokines, chemokines/receptors, adhesion molecules, and activation markers.9 The effects of E2 on gene expression in pathogenic, recruited, and regulatory cells in EAE are likely to be the result of estrogen receptor-mediated signaling.

Although E2 can have a protective effect on susceptibility to EAE, much remains to be learned about the

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molecular mechanisms underlying its immunomodulatory activity and regulation of the sexual dimorphism and pathogenesis of autoimmune diseases. A major question is the nature of the signaling pathways underlying these effects. There is considerable controversy as to whether or not E2 exerts its biological effects directly or indirectly on immune cells and whether or not they are the result of genomic responses. To elicit a classical genomic response, and to be a direct target for E2, the cells of the immune system must express the cognate receptors. The first E2 receptor to be cloned was the E2 genomic response, and to be a direct target for E2, the question is the nature of the signaling pathways underlying these effects. There is considerable controversy as to whether or not E2 exerts its biological effects directly or indirectly on immune cells and whether or not they are the result of genomic responses. To elicit a classical genomic response, and to be a direct target for E2, the cells of the immune system must express the cognate receptors. The first E2 receptor to be cloned was the E2 receptor-α gene (Esr1). Among cells relevant to the functioning of the immune system, Esr1 is expressed by thymocytes, thymic epithelial cells, T cells, B lymphocytes and their precursors, and non-hematopoietic bone marrow cells. The existence of a second iER, estrogen receptor-β (Esr2), was identified and has been shown to be expressed by non-hematopoietic bone marrow cells and in the human thymus and spleen. The precise roles of these two receptors in regulating cell-specific responses are still not well defined. However, it is becoming evident that Esr1 and Esr2 may be responsible for regulating different biological functions based on their expression patterns, localization profiles, and protein structures. For example, Esr1 and Esr2 differ in their C-terminal ligand-binding domains and in their N-terminal transactivator domains. In addition to these two cytoplasmic receptors, there is also a membrane-associated E2 receptor (mER) that may play a role in mediating the non-genomic actions of E2. However, mER regulation of gene expression cannot be fully excluded since some phenotypes elicited by steroid hormones appear to be the result of membrane receptor activation of G-protein-coupled second messenger pathways. In the current study, we use B6.129-ESR1 mice lacking Esr1 and B6.129-ESR2tm1Unc mice lacking Esr2 to address for the first time which ER mediates the protective effect of E2 on EAE.

Materials and Methods

Animals

Female B6.129-Esr1tm1Unc (Esr1+/−) and B6.129-Esr2tm1Unc (Esr2+/−) mice were purchased from Taconic Laboratory (Germantown, NY). The mice were housed and cared for in the Animal Resource Facility at the Portland VA Medical Center according to institutional guidelines.

Hormone Treatment

For treatment of mice with 17β-estradiol, 3-mm pellets containing 2.5 mg of E2 (Innovative Research of America, Sarasota, FL), providing a constant continuous controlled release of hormone over a period of 60 days, were implanted subcutaneously on the dorsal back of each mouse 7 days before immunization for induction of EAE. Control animals were sham-operated and implanted with pellets containing saline.

Induction of Active EAE

Mice were immunized with 400 μg of MOG-35–55 peptide in complete Freund’s adjuvant (CFA) containing 200 μg of Mycobacterium tuberculosis H37Ra by subcutaneous injection over four sites on the flank, with additional injections of Pertussis toxin (Ptx) on days 0 and 2. Mice were examined daily for clinical signs of EAE according to the following scale: 0, no signs; 1, limp tail; 1.5, moderate hind limb weakness with difficulty in righting; 2, moderate hind limb weakness without ability to right itself; 2.5, moderate hind limb weakness (waddling gait) without ability to right itself; 3, moderately severe hind limb weakness with paralysis of one limb; 4, severe hind limb weakness; 4.5, severe hind limb weakness with mild forelimb weakness; 5, paraplegia with no more than moderate forelimb weakness; 5.5, paraplegia with severe forelimb weakness (quadriplegia); and 6, moribund condition.

IL-6 Quantification

Splenic cells were cultured at 4 × 10^6/ml and stimulated with the appropriate Ag in 24-well culture plates. Cell supernatants were recovered after 72 hours. Measurement of cytokines was performed by ELISA using cytokine-specific capture and detection Abs (BD Pharmingen, San Diego, CA). A standard curve for IL-6 was generated using recombinant mouse cytokines (BD Pharmingen), and the concentration of cytokine in the cell supernatants was determined by interpolation from the appropriate standard curve.

Cytometric Bead Array (CBA)

TNF-α, IFN-γ, IL-5, IL-4, and IL-2 were detected using the mouse inflammation cytokine CBA kit from BD Biosciences (San Jose, CA). Fifty μl of cell culture supernatant was mixed with 50 μl of the mixed capture beads and 50 μl of the mouse phycoerythrin detection reagent. The tubes were incubated at room temperature for 2 hours in the dark, followed by a wash step. The samples were then resuspended in 400 μl of wash buffer before acquisition on the FACScan. The data were analyzed using CBA software. Standard curves were generated for each cytokine using the mixed bead standard provided in the kit, and the concentration of cytokine in the cell supernatant was determined by interpolation from the appropriate standard curve.

RNase Protection Assay (RPA)

Total RNA was extracted from spinal cords (SC) using the STAT-60 reagent (Tel-Test, Friendswood, TX). Chemokine expression was determined using the RiboQuant RPA kit to assess the expression of chemokines and chemokines receptors (Pharmingen) according to manufacturer’s instructions.
Table 1. Susceptibility to MOG 35–55 Induced EAE in Placebo- and E2-Treated C57BL/6, B6.129-Esr1−/−, and B6.129-Esr2−/− Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence</th>
<th>Onset</th>
<th>Peak</th>
<th>CDI</th>
<th>E2 (pg/ml)</th>
<th>T4 (pg/ml)</th>
<th>Thymic Index (mg/g)</th>
<th>Uterine Index (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>10/10</td>
<td>9.9 ± 1.8</td>
<td>4.8 ± 1.0</td>
<td>61.3 ± 20.8</td>
<td>20 ± 18</td>
<td>36.2 ± 34.6</td>
<td>3.7 ± 0.4</td>
<td>4.5 ± 1.7</td>
</tr>
<tr>
<td>E2</td>
<td>3/10**†‡§</td>
<td>21.2 ± 3.8**†‡§</td>
<td>0.8 ± 1.3**†‡§</td>
<td>1.1 ± 2.5**†‡§</td>
<td>1,795 ± 60.3**†‡§</td>
<td>131.2 ± 120.5**†‡§</td>
<td>1.8 ± 0.2**†‡§</td>
<td>13.3 ± 6.1**†‡§</td>
</tr>
<tr>
<td>B6.129-Esr1−/−</td>
<td>Placebo</td>
<td>8/8</td>
<td>12.4 ± 2.7</td>
<td>4.6 ± 0.8</td>
<td>58.5 ± 14.7</td>
<td>12 ± 20.5†</td>
<td>259/6 ± 200.8*†§</td>
<td>2.4 ± 0.5</td>
</tr>
<tr>
<td>E2</td>
<td>8/8</td>
<td>16.1 ± 2.7**†‡§</td>
<td>5.3 ± 0.7**†‡§</td>
<td>60.2 ± 2.7**†‡§</td>
<td>1,432 ± 238*†‡§</td>
<td>1635 ± 1306.9*†‡§</td>
<td>1.3 ± 0.2**†‡§</td>
<td>4.9 ± 0.9</td>
</tr>
<tr>
<td>B6.129-Esr2−/−</td>
<td>Placebo</td>
<td>5/5</td>
<td>12.0 ± 1.0</td>
<td>5.5 ± 0.0</td>
<td>77.1 ± 9.2</td>
<td>36.8 ± 10.3</td>
<td>29.6 ± 18.4</td>
<td>2.8 ± 0.9</td>
</tr>
<tr>
<td>E2</td>
<td>1/4*†‡§</td>
<td>30.0</td>
<td>0.5 ± 1.0**†‡§</td>
<td>0.5 ± 1.0**†‡§</td>
<td>1171.5 ± 378.8**†‡§</td>
<td>75.0 ± 32.4**†‡§</td>
<td>1.3 ± 0.3**†‡§</td>
<td>12.7 ± 2.6**†‡§</td>
</tr>
</tbody>
</table>

*P values ≤ 0.05, as compared to C57BL/6 treated with placebo pellet.
**P values ≤ 0.05, as compared to B6.129-Esr1−/− treated with placebo pellet.
†P values ≤ 0.05, as compared to B6.129-Esr1−/− treated with E2 pellet.
‡P values ≤ 0.05, as compared to B6.129-Esr2−/− treated with placebo pellet.
§P values ≤ 0.05, as compared to B6.129-Esr2−/− treated with E2 pellet.
¶P values ≤ 0.05, as compared to C57BL/6 treated with E2 pellet.

Histology

Representative mice were selected from the E2 and placebo-treated control group. Spinal cords were isolated and fixed in 10% paraformaldehyde. Transverse sections of the spinal cords were stained with Luxol Fast Blue-Periodic acid or with hematoxylin and eosin (H&E) and analyzed by light microscopy.

E2 and Testosterone Serum Levels

Serum E2 and testosterone levels were measured using a radioimmunoassay as described previously.6

Statistical Analysis

Differences in the incidence of EAE in the respective mouse strains was tested using χ2. A one-way analysis of variance and Student-Newman-Keuls multiple comparisons test was used to evaluate significant differences in quantitative EAE trait values (day of onset, E2 levels, T4 levels, thymic index and uterine index). The Kruskal-Wallis test was used to evaluate non-parametric EAE trait values (peak score and CDI score). In all tests, P values ≤0.05 were used as the significance threshold.

Results

EAE in Esr1−/− and Esr2−/− Mice

We sought to determine whether there were differences between Esr1 and Esr2 in mediating the inhibitory effects of E2 on EAE. Thus, B6.129-Esr1tm1Unc (Esr1−/−) mice, B6.129-Esr2tm1Unc (Esr2−/−) mice, and their C57BL/6 WT littermate controls were immunized with MOG-35–55 peptide + CFA + Ptx in the presence of implanted E2 or placebo pellets, and monitored for clinical and histological signs EAE (Table 1 and Figures 1 and 2). All of the WT C57BL/6 mice treated with placebo pellets developed severe EAE, and as expected from our previous studies,6–9 treatment of the WT mice with E2 pellets markedly reduced the incidence and severity of clinical and histological disease. Interestingly, both Esr1−/− and Esr2−/− mice treated with placebo pellets developed EAE that was nearly indistinguishable from WT controls. Of critical importance, however, treatment with E2 pellets had no inhibitory effect on the severity of clinical or histological disease in Esr1−/− mice, although onset of EAE was delayed by 4 days. In contrast, the identical treatment with E2 almost completely inhibited clinical and histological EAE in Esr2−/− mice (Table 1 and Figure 1).

We also evaluated the lack of Esr1 and Esr2 on other E2 functions in placebo- or E2-treated mice with EAE. Quantification of serum E2 levels revealed a low but measurable concentration of endogenous E2 in Esr1−/− placebo-treated mice that was similar to that present in C57BL/6 WT littermate controls, but lower than in Esr2−/− mice (Table 1). Moreover, treatment with E2 pellets produced comparable levels of serum E2 in all three mouse strains, indicating that the lack of functional estrogen receptors did not effect E2 degradation. As

Figure 1. Wild-type C57BL/6, Esr1−/−, and Esr2−/− female mice were implanted with 2.5 mg 17β-estradiol (E2) or placebo pellets, respectively, 1 week before the induction of EAE. E2 treatment suppressed actively induced EAE in C57BL/6 and Esr2−/− mice, while in Esr1−/− mice this protective effect was lost. The data show mean clinical EAE scores for each day for C57BL/6 female mice treated with saline or E2 pellets, B6.129-Esr1−/− females treated with saline or E2 pellets, and B6.129-Esr2−/− females treated with saline or E2 pellets.
Taken together, these results are consistent with either \( \text{Esr2}^{-/-} \) or mice but not \( \text{Esr1}^{-/-} \) mice. In contrast, lack of either \( \text{Esr1} \) or \( \text{Esr2} \) resulted in a moderate decrease in thymic index compared to WT controls, and treatment with E2 strongly diminished thymus weight in all three mouse strains. Testosterone levels were low but detectable in WT and \( \text{Esr2}^{-/-} \) mice, but were markedly increased in \( \text{Esr1}^{-/-} \) mice, consistent with a previous report.\(^{17}\) Taken together, these results are consistent with previous findings regarding the role of \( \text{Esr1} \) and \( \text{Esr2} \) signaling, and serve as internal controls for the observed effects on EAE.\(^{18-22}\)

Cytokine, Chemokine, and Chemokine Receptor Production by \( \text{Esr1}^{-/-} \) and \( \text{Esr2}^{-/-} \) Mice

In accordance with our previous studies,\(^{6-9}\) we observed that E2 treatment significantly reduced the secretion of TNF-\( \alpha \) and IFN-\( \gamma \) by MOG-35–55 peptide-stimulated spleen cells recovered from WT mice at the peak of EAE (Figure 3). A similar result occurred in \( \text{Esr2}^{-/-} \) mice, although levels of both cytokines were reduced compared to WT placebo-treated mice. In contrast, E2 treatment significantly enhanced secretion of TNF-\( \alpha \) in \( \text{Esr1}^{-/-} \) mice with EAE compared to treatment with placebo (Figure 3). IL-2, IL-4, and IL-5 levels were very low and were not affected by E2 treatment in any mice. Since E2 has been purported to up-regulate serum IL-6 levels through \( \text{Esr1} \) and not \( \text{Esr2} \),\(^{23}\) and elevated IL-6 levels have been implicated in mediating suppression of delayed-type hypersensitivity responses in post-burn patients and experimental animals,\(^{24}\) we compared the levels of IL-6 secretion by splenic cells in E2- versus placebo-treated \( \text{Esr1}^{-/-} \) and \( \text{Esr2}^{-/-} \) mice. As is shown in Figure 3, IL-6 production was significantly reduced in E2-treated WT mice without EAE, but was significantly increased in E2-treated versus placebo-treated \( \text{Esr1}^{-/-} \) mice with EAE. These data indicate that the up-regulation of IL-6 is not restricted to \( \text{Esr1} \) signaling and that increased levels of IL-6 do not account for suppression of clinical and histological EAE.

We previously demonstrated that E2 treatment influenced the expression of cytokine, chemokine and chemokine receptor mRNA in the spinal cords of C57BL/6 mice treated with 2.5-mg E2 pellet.
mice with EAE mRNA levels for many of the cytokines (Lymphotixin, TNF-α, and IFN-γ), chemokines (RANTES, MIP-1α, MIP-2, IP-10, and MCP-1) and chemokine receptors (CCR1, CCR2, and CCR5) were detectable at similar levels in the spinal cords of placebo-treated WT, Esr1−/−, and Esr2−/− mice with clinical signs of EAE (Figure 4). Treatment of EAE with E2 significantly reduced the expression of all detected cytokines, chemokines, and chemokine receptors in WT C57BL/6 mice and Esr2−/− mice, but did not significantly change the level or pattern of expression in CNS tissue from E2-treated Esr1−/− mice that developed EAE (Figures 4, A to C). Thus, the inhibitory effect of E2 on inflammatory factors in CNS that was highly significant in C57BL/6 and Esr2−/− mice was lost in Esr1−/− mice.

Discussion

We previously demonstrated that low-dose E2 therapy is beneficial in the treatment of EAE in two different inbred mouse strains. We now demonstrate for the first time that the protective effect of E2 is abrogated in Esr1−/− mice, but not in Esr2−/− mice. The loss of the protective effect of E2 in Esr1−/− mice immunized with the encephalitogenic MOG-35–55 peptide was manifested by the development of severe clinical and histological EAE even in the presence of moderately high serum E2 levels. This is in contrast to C57BL/6 WT mice and Esr2−/− mice, in which E2 treatment resulted in comparable serum E2 levels and markedly suppressed clinical signs of EAE and abolished inflammatory lesions in the CNS. This pattern showing a lack of E2-dependent inhibition of EAE in Esr1−/− mice was mirrored by an enhanced rather than a reduced secretion of TNF-α, IFN-γ, and IL-6 in MOG-specific splenocytes and a lack of inhibition of message for inflammatory cytokines, chemokines, and chemokine receptors in CNS tissue.

Although the severity of clinical and histological EAE was not diminished in E2-treated Esr1−/− mice, these mice did exhibit a 4-day delay in disease onset compared to C57BL/6 controls or Esr2−/− mice. This may be the result of residual Esr2 signaling, which leads to less efficient E2 protection. MOG-specific splenocytes from placebo-treated Esr1−/− and Esr2−/− mice had strongly reduced levels of secreted TNF-α and IFN-γ compared to WT controls, suggesting that lack of Esr1 or Esr2 might limit production of these cytokines. However, treatment with E2 strongly increased the secretion of these cytokines to WT levels in Esr1−/− mice, suggesting a possible enhancing effect of E2 mediated through the residual Esr2 gene. E2 treatment also significantly enhanced secretion of IL-6 in MOG peptide-stimulated splenocytes from Esr1−/− mice, indicating that E2 up-regulation of IL-6 is not restricted to Esr1 signaling and that increased levels of IL-6 do not account for suppression of EAE, as proposed previously. In addition, E2 treatment of Esr1−/− mice did not change the expression profile in the spinal cord of the cytokines lymphotixin, TNF-α, and IFN-γ; the chemokines RANTES, MIP-1α, MIP-2, IP-10, and MCP-1; and the chemokine receptors CCR1, CCR2, and CCR5, in contrast to C57BL/6 WT and Esr2−/− mice in which E2 treatment reduced the levels of all of these inflammatory factors. In this regard, it is pertinent that Wang et al reported that the two-ligand/receptor pairs, CCL5/CCR5 and CXCL10/CXCR3, may play a role during active MS and serve as predictors of future disease activity. Also Eikelenboom et al showed an increase in CCR5 and CXCR3+ T cells in both the blood and cerebrospinal fluid of MS patients.

Based on incomplete development of the mouse reproductively tract (ovaries) in B6.129-Esr1−/− mice, it has been suggested that the action of Esr2 is directly dependent on the presence of a functional Esr1 gene. On the other hand, E2 continues to provide protection against vascular injury in mice in which either Esr1 or Esr2 has been disrupted, suggesting that each of the two known E2 receptors is sufficient to mediate vascular protection. In ischemic injury within the brain, E2 can use either ER subtype, or alternatively, protection may be receptor-independent. Similarly, in our study, lack of Esr1 or Esr2 resulted in a moderate decrease in thymus weight compared to WT controls, and treatment with E2 strongly diminished thymus weight in all three mouse strains. E2 and estrogen receptor are known to effect thymic development and function, and our results support a role for either Esr1 or Esr2. The spectrum of biological activities mediated by E2 receptor subtypes in diverse disease settings may be explained by: 1) the differences in tissue distribution of the subtypes, 2) the complexity of E2 receptor co-activators which may be different for each cell type, or 3) the existing complexities in the nature of E2 protective mechanisms, which include classical (genomic) and nonclassical (nongenomic) action.

Our data strongly implicate the classical (genomic) action of E2 in protection against EAE, because we observed a pronounced E2-dependent regulation of cytokine secretion in spleen and inhibition of message for cytokines, chemokines, and chemokine receptors in the spinal cord of immunized mice. Our study demonstrates for the first time that these E2-regulated immunomodulatory effects are mediated by selective signaling through Esr1. Preliminary studies indicate that iERs are present on CD4+ T cells and monocytes, and it remains plausible that Esr1 on immunocytes may directly mediate E2 effects on EAE.

References

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