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NEUROPROTECTIVE AND ANTI-INFLAMMATORY EFFECTS OF ESTROGEN RECEPTOR LIGAND TREATMENT IN MICE

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Abstract

Demyelination and neurodegeneration is a major contributor in the progression of disability in multiple sclerosis (MS). Thus, the development of therapies that are neuroprotective has elicited considerable interest. Estrogens and estrogen receptor (ER) ligand treatments are promising treatments to prevent MS-induced neurodegeneration and a multicenter phase II clinical trial of estriol as beneficial therapy in MS is underway. Here, we discuss studies performed in our laboratory that examined effects of ER ligands in the inflammatory/demyelinating disorder experimental autoimmune encephalomyelitis (EAE), a model of MS. Administration of estriol or 17 β -estradiol reduced clinical severity and this clinical disease improvement was associated with favorable changes in cytokine production. There was a significant decrease of neuronal pathology in gray matter along with myelin and axon preservation in white matter of spinal cords of mice with EAE. In subsequent experiments, we contrasted results of ER α versus ER β ligand treatment. While ER α ligand treatment was anti-inflammatory, ER β ligand treatment was not. ER β ligand treatment nevertheless reduced demyelination and preserved axon numbers in white matter and prevented neuronal abnormalities in gray matter. Clinically, ER α ligand treatment abrogated disease at the onset, while ER β ligand treatment had no effect at disease onset, but promoted recovery. Thus, unlike ER α ligand treatment, ER β ligand treatment was protective at the level of the target organ, independent of anti-inflammatory effects in the peripheral immune system. ER β ligand treatment should be considered as a potential neuroprotective agent for MS and other neurodegenerative diseases, particularly since breast and uterine cancer are mediated through ER α .

Keywords

estrogen receptor ligands; anti-inflammation; neuroprotection; EAE

1. Introduction

Recent introduction of immunomodulatory therapies have considerably improved the therapeutic options for patients with multiple sclerosis (MS). These agents reduce relapse rate,

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and prevent the accumulation of MRI lesion load in clinically definite MS resulting in a modest effect on delaying the progression time to disabling stages caused by neuronal loss(1,2). There are no directly neuroprotective agents available for treatment of MS. During the course of MS the only time patients have encountered alleviation of MS symptoms, is during the late 3rd trimester of pregnancy(3). Pregnancy, by necessity, involves a relative state of immunosuppression as the fetus carries paternally derived antigens, and it is likely that high levels of estrogen associated with pregnancy contribute to this.

Endogenous ER ligands include estrone, 17- β -estradiol, and estriol; 17- β -estradiol is the predominant form of estrogen present in males and non-pregnant females while E3 is present at high levels during late pregnancy. There has been particular interest in the immunosuppressive role of estriol. Estriol is produced by the placenta that peaks during the third trimester, may be responsible for the decrease in MS-related symptoms during late pregnancy(3) (4). High dose E3 treatment has been shown to reduce the incidence and severity of EAE in two model systems (5,6). Estriol levels appear to mirror most closely the reduction in relapse frequency seen during the third trimester of pregnancy, and there has already been a pilot study of estriol as a therapeutic agent in non-pregnant patients with MS that reported an 80% reduction in MRI disease activity over 6 months(7). A follow-on phase II/III clinical trial is currently under way of estriol and Copaxone in female MS patients.

2. Estrogen treatment improves EAE

The disease-modulating effects of estrogens have been demonstrated in CNS injury, ischemia, neurodegeneration and aging(8–10). Over the last 10 years, numerous studies have shown that estrogen treatment (both estriol and estradiol) administered prior to disease onset ameliorates experimental autoimmune encephalomyelitis (EAE) in mice(6,11–15). Estriol treatment when administered after disease onset is also effective in reducing EAE clinical signs(5). Finally, both estradiol and estriol are efficacious in female and male mice with EAE (16).

While a variety of anti-inflammatory mechanisms of estrogen treatment in EAE have been described, these are not mutually exclusive of more direct neuroprotective mechanisms, since estrogens are lipophilic, readily traversing the blood brain barrier(17). Some of the estrogen effects could be genomically mediated, due to interaction of ligand with the estrogen receptor isoforms α or β (ER α , ER β). To determine whether effect of 17 β estradiol and estriol mediated protection in EAE was due to stimulation of ER α alone, we treated mice with a highly selective ER α ligand, 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT)(18). ER specific ligands provide a pharmacological approach to study each ER implication. PPT displays 400-fold more binding affinity for ER α than ER β and is inactive on ER β transcriptional activity (19). Whereas 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) displays 100-fold more affinity for ER β than ER α and has a 170-fold greater relative potency in transcriptional assays for ER β than for ER α (20). In a subsequent publication, we then showed differential effects of ER α and ER β ligand treatment in EAE(21).

2.1 Differential effects of ER α and ER β ligands on clinical severity of EAE

EAE was induced in wild type, ER α or ER β deficient mice, each treated with 17- β estradiol, the highly selective ER α (PPT) or ER β (DPN) ligand. 17 β -estradiol treatment ameliorated clinical disease in wild type (Figure 1A) and in ER β knock(18). ER α ligand treatment ameliorated clinical disease in both wild type and ER β knock out mice(Figure 1A, C), but not in ER α knock out mice(18, 21). In contrast, ER β ligand treatment had no significant effect early in disease (up to day 20 after disease induction), but then demonstrated a significant protective effect later in disease (after day 20) (Figure 1B(21)). Our data showing a protective effect using the ER β ligand DPN in active EAE in C57BL/6 mice were surprising given that another ER β ligand (WAY-202041) was shown to have no effect in adoptive EAE in SJL mice

(22). Since WAY-202041 was shown to have a 200 fold selectivity for ER β as compared to ER α , while DPN has a 70 fold selectivity(20), it was possible that DPN was not sufficiently selective for ER β *in vivo*. The *in vivo* selectivity of DPN during EAE was confirmed by administration of DPN to ER β KO mice (Figure 1C(21)). DPN-treatment was no longer protective to ER β KO mice with active EAE. These data demonstrated the *in vivo* selectivity of PPT for ER α and DPN selectivity for ER β during EAE at the dose used in our studies.

2.2 Differential effects of ER α and ER β ligand on Autoantigen-Specific Cytokine production

Analysis of autoantigen-specific proinflammatory cytokine production during both early and later stages of EAE indicated that ER α ligand treatment significantly reduced TNF α , IFN γ , and IL6, while increasing IL5, during both early and later stages of EAE. In contrast, ER β ligand treatment was not statistically different from vehicle treatment at either the early or later time points (18,21). These results indicated that while ER α ligand treatment induced favorable changes in cytokine production during the autoantigen specific immune response, ER β ligand treatment did not.

2.3 Differential effects of ER α and ER β ligand on CNS inflammation

The onset and progression of EAE is associated with infiltration of immune cells into the CNS. Spinal cord sections from various groups were assessed for inflammation by histology (Hematoxylin and Eosin, H&E) and immunohistochemistry (CD3⁺ T cells and Mac3⁺ macrophages)(21). On H&E staining, vehicle treated EAE mice had extensive white matter inflammation at both the early and later (Figure 2A(21)) time points as compared to normal controls. As compared to vehicle treated EAE, this inflammation was significantly reduced by treatment with the ER α ligand(18,21). In contrast, extensive white matter inflammation was present in the ER β ligand treated group at both the early and later time points(21). Treatment with the ER α ligand but not the ER β ligand reduced CD3⁺ T lymphocytes, and Mac 3⁺ macrophage lineage cells at both the early(21) and later (Figure 2B,C(21)) time points. Together, these data indicated that ER α but not ER β ligand treatment reduced inflammation in the CNS of mice with EAE.

2.4 Preservation of neurons, axons, and myelin

Extensive demyelination and axon loss occurs at the sites of inflammatory cell infiltrates in EAE mice during early and late in disease (Figure 2(18)). Quantification of number of axons and myelin density within the dorsal column and lateral funniculus of EAE spinal cords showed significant decreases (Figure 2C(18,21)). There was a significant loss of myelin and axons in vehicle-treated late EAE mice compared to normal control mice. In contrast to vehicle treated mice with EAE, treatment with ER α and ER β ligand significantly attenuated the loss of axons in mice with EAE (Figure 2D). We have confirmed that estrogen treatment spared GM neuronal pathology in the spinal cord of mice with EAE (18). Similarly in the presence of ER α and ER β ligand treatment, EAE mice showed significant improvement in neuronal numbers over vehicle treated mice during early(21) and late in disease (Figure 2E(21)).

2.5 Recovery of motor performance due to ER β ligand treatment

To assess the clinical significance of the neuroprotective effect of ER β ligand treatment on EAE we used motor rotarod performance test (Figure 3(21)). Vehicle treated EAE mice were unable to remain on the rotarod, beginning at day 12 after disease induction. Similar to vehicle-treated mice, ER β ligand treated mice were also unable to remain on the rotarod apparatus, beginning at day 12, but in contrast to vehicle treated mice, ER β treated mice later during EAE had significant recovery of their ability to remain on the rotarod(Figure 3(21)). Further, this improvement in rotarod performance late during EAE with ER β ligand treatment was no longer

observed in the ER β KO. These data demonstrated that the DPN treatment induced recovery in motor performance later in disease was mediated through ER β .

Together these data demonstrated that ER β ligand treatment in the presence of inflammation was neuroprotective and induced functional clinical recovery in motor performance at later time points of disease during EAE.

3. Importance of specific ER ligand treatments

Estrogen treatment has been effective in numerous neurodegenerative disease models including Parkinson's disease, spinal cord injury, cerebellar ataxia, Down's Syndrome, epilepsy, and some models of stroke and Alzheimer's disease(23–26), and translational work using estrogen treatment for human neurodegenerative diseases has begun. Estrogens in the form of hormone replacement therapy have been associated with side effects and therefore are not recommended for use in healthy menopausal women(27). However, a more recent analysis of the WHI data revealed that estrogen had beneficial effects when therapy was started soon after menopause, but not when hormone therapy was initiated years after menopause(28). While the risk:benefit ratio in debilitating neurodegenerative diseases is clearly different than the risk:benefit ratio in healthy individuals, optimizing efficacy and minimizing toxicity, remains the goal. Hence, determining which estrogen receptor mediates the neuroprotective effect of estrogen treatment is of central importance. The only previously described neuroprotective agents for EAE are glutamate receptor blockers(29–31) and Na⁺ channel blockers(32,33). Glutamate and Na⁺ channel blocker treatments result in a modest reduction in neurologic impairment and the effect is lost after cessation of treatment(30). In the case of Na⁺ channel blockers, symptoms get worse and lead to death(34). Because Na⁺ channel and glutamate activity are needed for normal neuronal plasticity and memory(35), treatments with these blockers may be associated with significant toxicity(34–36).

Our data demonstrate that while treatment with an ER β ligand is not anti-inflammatory, it is neuroprotective. Similar neuroprotective effects of ER β ligand has been observed in acoustic trauma, memory and depression(37–39). Differential modulation of intracellular calcium, up regulation of growth factors and activation of interacting second messenger pathways by ER β activation are most likely involved in initiating cell survival and/or prevention of cell apoptosis during disease(40–43). The neuroprotective actions of ER β ligand could be directly or indirectly on estrogen receptor containing neurons, astrocytes and oligodendrocytes. The selective neuroprotective effects of ER β ligands are of clinical relevance since both breast and uterine endometrial cancer are mediated through ER α , not ER β .

In a phase I pilot trial, as compared with pretreatment baseline, relapsing remitting patients treated with oral estriol (8 mg/day) demonstrated significant decreases in delayed type hypersensitivity responses to tetanus, interferon-gamma levels in peripheral blood mononuclear cells, and gadolinium enhancing lesion numbers and volumes on monthly cerebral magnetic resonance images. When estriol treatment was stopped, enhancing lesions increased to pretreatment levels. When estriol treatment was reinstituted, enhancing lesions again were significantly decreased(7,44). It is interesting to note that estriol has a more than 5-fold preference for the activation of human ER β over ER α , and it is a quantitatively predominant estrogen metabolite produced during pregnancy. The very high levels of estriol present during pregnancy may produce a differential activation of the ER β signaling system in the pregnant woman and fetus for fulfilling various unique physiological functions(45). For neurodegenerative diseases with only a minimum inflammatory component, treatment with an ER β ligand that possesses only neuroprotective properties may be sufficient. For diseases, such as MS, with a significant inflammatory component, a standard anti-inflammatory treatment could be used in combination with ER β ligand treatment.

Understanding the mechanisms leading to cumulative neurological disability in patients with MS and further developing effective therapeutic strategies aimed at reducing disease progression is a major goal in MS research. Estrogens and estrogen receptor (ER) ligands are promising treatments to prevent neurodegeneration in the CNS(7,46).

Acknowledgments

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Figure 1.

Differential effects with ER ligands on chronic EAE

Ovariectomized C57BL/6 female mice were given daily subcutaneous injections of an ER ligand (ER α ligand-PPT and ER β -DPN) during active EAE and graded using the standard EAE grading scale.

(A) Mean clinical scores of ER α ligand treated mice (blue circle) as compared to vehicle treated mice were significantly reduced during the entire disease course.

(B) Mean clinical scores of ER β ligand treated mice (blue circle) as compared to vehicle treated mice, were not significantly different early in disease (up to day 20 after disease induction), but they became significantly improved later during EAE, (following day 30 after disease induction).

(C) DPN treatment *in vivo* during EAE remains highly selective for ER β . Clinical scores in ovariectomized ER β KO C57BL/6 mice with active EAE were no different when comparing DPN treated with vehicle treated(21).

Figure 2.

Even without anti-inflammatory effect $ER\beta$ ligand treatment is neuroprotective. (A–C) Treatment with an $ER\alpha$ ligand, not an $ER\beta$ ligand, reduced inflammation in spinal cords of mice with EAE. Representative H&E (A), anti-CD3 antibody (B), and anti-Mac 3 antibody (C) stained thoracic spinal cord sections (4X magnification) from healthy control, as well as vehicle, $ER\alpha$ ligand (PPT), and $ER\beta$ ligand (DPN) treated EAE mice, all killed at day 40 (late) after disease induction. Compared to controls, vehicle treated EAE spinal cords showed multifocal to coalescing areas of inflammation in the leptomeninges and white matter, around blood vessels, and in the parenchyma of the white matter. $ER\alpha$ ligand-treated spinal cords had reduced inflammation as compared to vehicle treated EAE, whereas $ER\beta$ ligand-treated did

not have reduced levels of inflammation. Anti-CD3 antibody and anti-Mac 3 antibody staining revealed that the inflammation was composed of both T cells and macrophage lineage cells, respectively. Both T cells and macrophage lineage cell staining was reduced with ER α ligand, but not ER β ligand treatment(21).

(D) Treatment with an ER α ligand and an ER β ligand each preserved myelin basic protein immunoreactivity and spared axonal pathology in white matter of spinal cords of mice with EAE. Part of the anterior funniculus of thoracic spinal cord sections was imaged at 40X co-immunostained with anti-NF200 (green) and anti-MBP (red). Distinct green axonal centers surrounded by red myelin sheaths can be seen in normal controls, PPT and DPN treated EAE mice from 40 day after disease induction. Vehicle treated mice show reduced axonal numbers and myelin, along with focal demyelination (white stars) and loss of axons(21).

(E) Treatment with an ER α ligand and an ER β ligand each preserved neuronal staining in gray matter of spinal cords of mice with EAE. Split images of thoracic spinal cord sections stained with NeuN (red) in (i) and Nissl in (ii) at 4X magnification, derived from normal healthy control mice, vehicle treated EAE, ER α ligand (PPT) treated EAE and ER β ligand (DPN) treated EAE mice, each sacrificed at day 40 after disease induction. Panel (iii) is a merged confocal scan at 40X of NeuN⁺ (red) and β 3-tubulin⁺ (green) co-labeled neurons from an area represented by dotted white square area in (i). Panel (iv) is a 40X magnification of Nissl stained area in solid black square in (ii). A decrease in NeuN⁺ immunostaining and Nissl staining was observed in the dorsal horn, intermediate zone and ventral horn of vehicle treated EAE mice as compared to normal control. White arrows in panel (iii) denote loss of NeuN⁺ staining. In contrast, EAE mice treated with either PPT or DPN had preserved NeuN and Nissl staining(21).

Figure 3.

ER β ligand treatment results in recovery of motor function in EAE.

(a) Mean time on rotarod decreased abruptly at day 12 after disease induction in both the vehicle and DPN treated EAE mice. But after day 30 the DPN treated group demonstrated significant recovery of motor function, while the vehicle treated did not improve. Estradiol treatment served as a positive control for a treatment effect. (b) In contrast to the improvement observed with DPN treatment of wild type mice, no improvement was observed at the later phase of disease in DPN treated ER β KO mice(21).