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 17β-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 ERα versus ERβ ligand treatment. While ERα ligand treatment
was anti-inflammatory, ERβ ligand treatment was not. ERβ ligand treatment nevertheless reduced
demyelination and preserved axon numbers in white matter and prevented neuronal abnormalities in
gray matter. Clinically, ERα ligand treatment abrogated disease at the onset, while ERβ ligand
treatment had no effect at disease onset, but promoted recovery. Thus, unlike ERα ligand treatment,
ERβ ligand treatment was protective at the level of the target organ, independent of anti-inflammatory
effects in the peripheral immune system. ERβ 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 ERα.

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,
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 (ERα, ERβ). To determine whether effect of 17β estradiol and estriol mediated protection in EAE was due to stimulation of ERα alone, we treated mice with a highly selective ERα 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 ERα than ERβ and is inactive on ERβ transcriptional activity (19). Whereas 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) displays 100-fold more affinity for ERβ than ERα and has a 170-fold greater relative potency in transcriptional assays for ERβ than for ERα (20). In a subsequent publication, we then showed differential effects of ERα and ERβ ligand treatment in EAE(21).

2.1 Differential effects of ERα and ERβ ligands on clinical severity of EAE

EAE was induced in wild type, ERα or ERβ deficient mice, each treated with 17-β estradiol, the highly selective ERα (PPT) or ERβ (DPN) ligand. 17β-estradiol treatment ameliorated clinical disease in wild type (Figure 1A) and in ERβ knock(18). ERα ligand treatment ameliorated clinical disease in both wild type and ERβ knock out mice(Figure 1A, C), but not in ERα knock out mice (18, 21). In contrast, ERβ 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 ERβ ligand DPN in active EAE in C57BL/6 mice were surprising given that another ERβ ligand (WAY-202041) was shown to have no effect in adoptive EAE in SJL mice.
Since WAY-202041 was shown to have a 200 fold selectivity for ERβ as compared to ERα, while DPN has a 70 fold selectivity(20), it was possible that DPN was not sufficiently selective for ERβ in vivo. The in vivo selectivity of DPN during EAE was confirmed by administration of DPN to ERβ KO mice (Figure 1C(21)). DPN-treatment was no longer protective to ERβ KO mice with active EAE. These data demonstrated the in vivo selectivity of PPT for ERα and DPN selectivity for ERβ during EAE at the dose used in our studies.

2.2 Differential effects of ERα and ERβ ligand on Autoantigen-Specific Cytokine production

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

2.3 Differential effects of ERα and ERβ 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 (Hemotoxylin 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 ERα ligand(18,21). In contrast, extensive white matter inflammation was present in the ERβ ligand treated group at both the early and later time points(21). Treatment with the ERα 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 ERα but not ERβ 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 funnicipus 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 ERα and ERβ 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 ERα and ERβ 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 ERβ ligand treatment

To assess the clinical significance of the neuroprotective effect of ERβ 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, ERβ ligand treated mice were also unable to remain on the rotarod apparatus, beginning at day 12, but in contrast to vehicle treated mice, ERβ 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 ERβ ligand treatment was no longer
observed in the ERβ KO. These data demonstrated that the DPN treatment induced recovery in motor performance later in disease was mediated through ERβ.

Together these data demonstrated that ERβ 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 ERβ ligand is not anti-inflammatory, it is neuroprotective. Similar neuroprotective effects of ERβ 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 ERβ activation are most likely involved in initiating cell survival and/or prevention of cell apoptosis during disease(40–43) The neuroprotective actions of ERβ ligand could be directly or indirectly on estrogen receptor containing neurons, astrocytes and oligodendrocytes. The selective neuroprotective effects of ERβ ligands are of clinical relevance since both breast and uterine endometrial cancer are mediated through ERα, not ERβ.

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 ERβ over ERα, 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 ERβ 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 ERβ 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 ERβ 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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References


Differential effects with ER ligands on chronic EAE
Ovariectomized C57BL/6 female mice were given daily subcutaneous injections of an ER ligand (ERα ligand-PPT and ERβ-DPN) during active EAE and graded using the standard EAE grading scale.

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

(B) Mean clinical scores of ERβ 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 then became significantly improved later during EAE, (following day 30 after disease induction).

(C) DPN treatment in vivo during EAE remains highly selective for ERβ. Clinical scores in ovariectomized ERβ KO C57BL/6 mice with active EAE were no different when comparing DPN treated with vehicle treated(21).
Even without anti-inflammatory effect ERβ ligand treatment is neuroprotective. (A–C) Treatment with an ERα ligand, not an ERβ 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, ERα ligand (PPT), and ERβ 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. ERα ligand-treated spinal cords had reduced inflammation as compared to vehicle treated EAE, whereas ERβ 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 ERα ligand, but not ERβ ligand treatment (21).

(D) Treatment with an ERα ligand and an ERβ 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 ERα ligand and an ERβ 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, ERα ligand (PPT) treated EAE and ERβ 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 β3-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.
ERβ 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 ERβ KO mice(21).