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Estrogen Receptor α Mediates Estrogen’s Immune Protection in Autoimmune Disease

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Estrogens are known to influence a variety of autoimmune diseases, but it is not known whether their actions are mediated through classic estrogen receptor α (ERα). The presence of a functional ER was demonstrated in secondary lymphoid tissues, then ER expression was shown at both the RNA and protein levels in these tissues. Use of ER knockout mice revealed that both the estrogen-induced disease protection and the estrogen-induced reduction in proinflammatory cytokines were dependent upon ERα in the prototypic Th1-mediated autoimmune disease experimental autoimmune encephalomyelitis. These findings are central to the design of selective ER modifiers which aim to target biologic responses in specific organ systems.

Estrogens have biological activity in numerous organ systems including the immune system, the reproductive system, the cardiovascular system, the CNS, bone, breast, colon, and prostate. An understanding of which estrogen receptor (ER) mediates estrogen’s action in a given organ system is central to the development of selective ER modifiers (SERMs) which aim to target therapeutic effects and minimize toxic side effects.

Gender and hormonal influence play a role in a wide variety of human autoimmune diseases. Multiple sclerosis (MS), rheumatoid arthritis (RA), uveitis, and thyroiditis improve during pregnancy while other diseases such as systemic lupus erythematosus (SLE) tend to worsen (1). An immune shift from Th1 to Th2 that occurs during pregnancy as the maternal immune system attempts to avoid fetal rejection has been proposed as a mechanism underlying the improvement in Th1-mediated autoimmune diseases during pregnancy (1).

Pregnancy is a complex situation characterized by changes in numerous factors including a steady increase in levels of estrogens. Interestingly, increases in estrogens appear to recapitulate the effect of pregnancy on autoimmune diseases, at least in part. Estrogen treatment in mice decreases the severity of experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA), while it worsens SLE (2–5). Immune shifts from Th1 to Th2 have been observed during estrogen treatment in these animal models (2, 3, 6). There is also evidence in humans with MS and RA that estrogen treatment may be beneficial, while estrogen treatment of SLE worsens the disease (7–13).

Estrogens may act through genomic or nongenomic mechanisms. Genomic mechanisms were originally described and mediated through ERα, the “classic” ER (14). However, since characterization of ERα, other ERs have been described including ERβ (15). Most recently, ERγ has been detected, but thus far only in teleosts (16). Nongenomic mechanisms involve membrane-bound estrogens (17) with a putative membrane-associated “ER-X” being recently reported (18). Interestingly, the estrogen-mediated protection in CIA was recently shown to possibly be caused by genomic effects since treatment with ICI 182,780 blocked the protective effect of estrogen on disease when used at a dose known to block ERα and ERβ, but not membrane effects (19). However, it was not determined whether a nuclear receptor was involved in the estrogen-induced immune protection in CIA. In this manuscript, the prototypic Th1-mediated autoimmune disease EAE is used to determine whether the protective effect of estrogen treatment is mediated through classic ERα.

**Materials and Methods**

**Animals and reagents**

Homozygous male ERα knockout mice were generated by backcrossing the knockout (20) onto the C57BL/6 strain for 16 generations. Littermates were used as wild-type (WT) controls. Animals were maintained in accordance with guidelines established by the National Institutes of Health and as mandated by the University of California, Los Angeles Office for the Protection of Research Subjects. Human ovary was purchased from BioChain Institute (Hayward, CA). Myelin oligodendrocyte glycoprotein (MOG) 35–55 peptide was synthesized to >98% purity by Cliron Mimotopes (San Diego, CA).

**In vitro estrogen treatment and cytokine analysis**

C57BL/6 mice were immunized with MOG35–55 peptide (100 μg/mouse) and draining lymph node cells were harvested after 10 days. Cells were stimulated in vitro with autoantigen (25 μg/ml) in the presence or absence of estradiol (28 ng/ml). After 6 h of stimulation, TNF-α was detected by intracellular staining with TNF-α-specific Ab (BD PharMingen, San Diego, CA) and subsequent FACS analysis.

**EMSA**

Cytoplasmic or nuclear proteins from spleen and brain were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents based on the manufacturer’s instruction (Pierce, Rockford, IL). Samples were preincubated
with biotin-conjugated estrogen response element (ERE; Panomics, Redwood City, CA) then subjected to electrophoresis. Recombinant ERα and ERβ receptors (Affinity, Golden, CO) were used as positive controls.

**RT-PCR**

Total RNA (2 μg) was reverse-transcribed to cDNA using the Reverse Transcription System (Applied Bioscience, Branchburg, NJ). For amplification, the outer primer set corresponded to nucleotides 461–480 (sense) and 1918–1937 (anti-sense) of the ERα gene, while with the inner primer set corresponded to 659–679 (sense) and 981–1003 (anti-sense) as previously numbered (21). The primer sequences are as follows: mERα outer set sense 659–480 5′-GCC GCC TTC AGT GCC AAC AG-3′; mERα inner set sense 659–679 5′-AAT TCT GAC AAT CGA GCC CAG-3′; mERα inner set anti-sense 1003–981 5′-GTG CTT CAA CAT TCT CCC TCC TC-3′; mERβ outer set anti-sense 1937–1918 5′-AGG AAT GTG CTG AAG TGG AG-3′.

**Western blots**

Intracellular proteins were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (as above) and Western blots run as described previously (22). To detect ERα (67 kDa) a rabbit ERα-specific primary Ab (Santa Cruz Biotechnology, Santa Cruz, CA) was used with a biotin-labeled goat anti-rabbit IgG secondary Ab. Alternatively, westerns were probed with E2-BSA (Sigma-Aldrich, St. Louis, MO) labeled with biotin using the EZ-link Sulfo-NHS-LC-Biotinylation kit (Pierce).

**In vivo hormone treatment, induction of active EAE, and cytokine analysis**

C57BL/6 male mice were treated with estriol (5 mg s.c. 90-day release pellets) or with placebo pellets (Innovative Research of America, Sarasota, FL) as described (2). One week later, active EAE was induced by immunizing with MOG35–55 peptide in complete Freund’s adjuvant as described (23). Mice were monitored daily for signs of EAE on a standard EAE grading scale of 0–5 (0, unaffected; 5, moribund). Mice in each group were sacrificed at days 16–24 during EAE, splenocytes were harvested and stimulated with autoantigen (MOG35–55 peptide at 25 μg/ml) as described (2). Supernatants were collected after 48 h and levels of TNF-α, IFN-γ, IL-2, and IL-5 were determined by cytometric bead array (BD PharMingen).

**Results**

**Functional ER are present in peripheral immune tissues**

We first examined immune cells from secondary lymphoid tissues of mice for the presence of a functional ER. Lymph node cells from MOG peptide 35–55-immunized C57BL/6 mice were stimulated with the autoantigen in the presence or absence of estriol in vitro, and a significant decrease in TNF-α (TNF-α) was observed, p = 0.036 (Fig. 1A). This reduction in TNF-α production during an autoantigen-specific immune response had been previously reported during in vivo estrogen treatment of mice with EAE (3). These in vitro data indicated that estrogen’s effect can be mediated by a direct effect of an estrogen on murine immune cells during an autoantigen-specific immune response. Next, we assessed murine splenic tissue proteins for their ability to bind to the ERE. In the nuclear fraction, binding was evidenced by retardation of migration of bands in EMSA (Fig. 1B). The lack of well delineated bands in lanes containing tissue proteins, with clear single bands in lanes containing recombinant proteins, is likely due to heterogeneity in ER types within tissues. This is consistent with the fact that either ERα or ERβ could bind the ERE and that variants of ERs have been demonstrated in nonimmune tissues previously (24, 25). Together the alteration in cytokine production during in vitro estriol treatment of immune cells and the binding of splenic protein to ERE indicated that functional ERs are expressed in murine secondary lymphoid tissues.

**ERα expression at the RNA and protein levels**

ERα expression has been reported in bone marrow and thymus (26–28), but direct evidence of expression in murine peripheral immune tissues is lacking. While RT-PCR data detecting ERα transcripts in peripheral immune tissues have been reported in mice, there has been no sequencing of these products (29, 30). Furthermore, expression of ERα at the protein level in the peripheral immune system has remained poorly characterized since Western blot data were negative for specific bands of the correct size (31) while flow cytometry of immune cells was positive (29, 32, 33). Together these previous reports raised the question of whether the classic ERα, vs another ER variant (24, 25), was detected in immune cells by flow cytometry. Thus, we characterized ERα expression in secondary lymphoid tissues of mice at the RNA level by both nested RT-PCR and sequencing and at the protein level by Western blot using both Ab and ligand binding. Nested RT-PCR using ERα-specific primers revealed bands of the expected size in both spleen (Fig. 2A) and lymph node (data not shown). The product from the inner primer set was then sequenced and confirmed to be ERα. Western blots of splenic tissue using an ERα-specific Ab detected expression at the protein level at the expected size of 67 kDa (Fig. 2B). ERα expression at the protein
ERα MEDIATES ESTROGEN’S IMMUNE PROTECTION IN AUTOIMMUNE DISEASE

FIGURE 2. ERα is expressed at the RNA and protein levels in mouse spleen. A, ERα gene expression was detected in murine spleen by nested RT-PCR. RNA from murine uterus tissue served as a positive control. No RNA served as a negative control. The inner primer PCR product was confirmed by sequencing. B, ERα protein expression was demonstrated in spleen. Western blots of tissue proteins were probed with ERα-specific primary Ab. A protein of the expected size for ERα (67 kDa) was detected in spleen. Human ovary and murine brain served as positive control tissues. Rabbit IgG served as a primary Ab negative control. The amounts of protein loaded per lane were 100 μg for spleen and brain and 60 μg for ovary. C, ERα expression at the protein level is confirmed by probing Western blots with estradiol-BSA. Western blots of tissue proteins were probed with E2-BSA-biotin instead of ERα-specific Ab. A protein of the expected size for ERα (67 kDa) was detected in spleen. Human ovary and murine brain served as positive control tissues. Probing with BSA-biotin alone served as a negative control.

level was then confirmed by probing Western blots with 17β estradiol-BSA whereby a band was identified in spleen which colocalized to the size of the band detected with ERα-specific Ab probing (Fig. 2C). Together these data are conclusive evidence of the expression of classic ERα in murine peripheral immune tissues.

The estrogen-mediated protection in EAE is dependent upon ERα

While an estrogen-mediated protective effect had been previously reported in EAE and in CIA, the mechanism of the immune protection has remained unknown. Therefore, we used the EAE model to determine whether the estrogen induced immune protection in this prototypic Th1 autoimmune disease was mediated through ERα. Since mechanisms of action of estrogens have been shown to differ depending upon whether physiologic or pharmacologic doses are used (34), we used a dose of estriol for EAE treatment which had previously been shown to induce estriol levels in sera of mice which were physiologic with natural murine pregnancy (2). Homozygous ERα KO mice were not protected from disease during estriol treatment, while WT mice were protected as evidenced by a significant decrease in disease severity in estriol vs placebo treated, p < 0.0001 (Fig. 3). These data demonstrate that the protective effect of estriol in EAE is dependent upon the presence of an intact ERα.

The estrogen-mediated immune modulation in EAE is dependent upon ERα

Significant reductions in proinflammatory cytokine production (TNF-α, p < 0.005; IFN-γ, p < 0.005; and IL-2, p < 0.05) were observed during autoantigen-specific immune responses of WT mice treated with estriol as compared with placebo (Fig. 4). Importantly, these reductions did not occur in ERα knockout mice treated with estriol. Since not all cytokines were reduced by estriol treatment, apoptosis was an unlikely explanation for the decrease in the above cytokines in estriol-treated WT mice. Levels of the Th2 cytokine IL-5 were increased in estriol-treated WT mice as compared with placebo-treated WT mice. This estriol-induced increase in IL-5 production was less dramatic but still present in ERα KO mice. Together these data demonstrate that both the disease protection and the reduction in proinflammatory cytokine production induced by estriol treatment in EAE are mediated through ERα.

Discussion

Recently ERα was shown to be critical in estrogen-induced protection against brain injury during stroke (34). Direct estrogen-induced, ERα-mediated protective effects on the CNS remain possible in EAE as well. However, the fact that estrogen treatment influences a wide variety of autoimmune diseases which do not have the CNS as a target organ indicates that the protective effects of estrogen are clearly not limited to direct effects on the CNS. This conclusion is consistent with the finding in this report that the
FIGURE 4. The estrogen-mediated immune modulation in EAE is dependent upon ERα. A reduction in proinflammatory cytokine production occurred in estriol-treated WT, but not ERα KO, mice. EAE mice in each group were sacrificed at days 16–24 during EAE, and splenocytes were harvested and stimulated with MOG35-55 peptide. Supernatants were collected and levels of TNF-α, IFN-γ, IL-2, and IL-5 determined by cytometric bead array. Data are expressed as percent change in two to six pooled experiments for indicated cytokine in supernatants from estriol or placebo-treated EAE mice, each as compared with the placebo-treated EAE. Homozygous ERα knockout = KO. Error bars indicate variation between experiments. *, p < 0.05; **, p < 0.005 when comparing cytokine levels in estriol vs placebo treated. Cytokine values in picograms per milliliter from a representative experiment are as follows: TNF-α: WT Placebo = 862.7 ± 198.5, WT Estriol = 71.1 ± 387.4, KO Placebo = 497.7 ± 142.0, KO Estriol = 497.7 ± 32.9; IFN-γ: WT Placebo = 1545.9 ± 92.0, WT Estriol = 152.5 ± 160.4, KO Placebo = 1320.4 ± 16.3, KO Estriol = 1118.8 ± 265.2; IL-2: WT Placebo = 137.8 ± 74.5, WT Estriol = 10.9 ± 1.7, KO Placebo = 94.1 ± 23.7, KO Estriol = 95.7 ± 12.7; IL-5: WT Estriol = 970.0 ± 12.2, WT Placebo = 29.4 ± 5.5, KO Estriol = 39.8 ± 18.8, KO Placebo = 18.2 ± 2.9.

Estrogen-induced alterations in cytokine production during autoantigen-specific immune responses were mediated through ERα.

Regardless of the effects of estrogen treatment on cytokine production, it is interesting to contrast our findings with those in a previous report in which estradiol was given in vivo and primary T cell responses to OVA were shown to be enhanced for Th1 cytokine production, with this effect dependent upon ERα (35). While both studies agree that ERα is important for immune effects, the studies differ in that Th1 cytokine production was decreased by estrogen treatment in our paper while being increased in the previous paper. The differences between the two papers could be that estriol was used in our studies while estradiol was used in the previous study. Estradiol and estradiol are clearly not the same. Estradiol has been shown to lead to hypertension, increased coagulation and to protect from osteoporosis more than estriol (36–41). They can even have opposing effects. For example, estradiol can protect from estradiol-mediated breast cancer (42, 43). However, since reports by three groups have shown that both estradiol and estriol are protective in both EAE and CIA (2, 3, 44, 45), there do not appear to be major differences between the two estrogen types in immune effects on disease. Indeed, the finding that estradiol treatment increases Th1 cytokine production during OVA-specific immune responses (35) is in direct contrast to previous reports by several groups that estradiol treatment ameliorates the prototypic Th1-mediated disease EAE. Perhaps differences between the effect estradiol treatment on OVA-specific responses in healthy mice and autoantigen-specific responses in EAE mice relates to either the strain or the age of the mice treated. F1 generation C57BL/6 mice were used in the former study while F1 C57BL/6 mice were used in our study. However, such strain differences also appear to be an unlikely explanation since estradiol has been shown to ameliorate EAE in numerous strains (C57BL/6, SJL, B10.PL, B10RHI). The age of the mice treated remains a consideration since the former report involved treatment of 4-wk-old mice, while all the EAE studies involved treatment of adult mice at least 8 wk of age. Differences in the nature of the immune response to OVA vs autoantigen is also a consideration.

While both estradiol and estriol have been shown to ameliorate EAE, we have focused considerable attention on estriol treatment of EAE for two reasons. First, unlike estradiol which is present during the menstrual cycle of the nonpregnant state, estradiol is not present in appreciable amounts in the nonpregnant state. The nonpregnant female state is not a time of protection from autoimmune diseases such as MS and RA, since nonpregnant females are known to be highly susceptible. Estriol becomes detectable only during the first trimester of pregnancy and increases progressively throughout gestation, and pregnancy is a state of relative protection from MS and RA. Thus, there a correlation between disease protection and the presence of estradiol, while the relationship between disease protection and the presence of estradiol appears more complex. Second, we focused on estriol treatment of EAE as a translational research strategy for MS in light of the fact that estriol is known to have less toxicity in humans than estradiol (36–38). The ultimate goal of the findings in this manuscript are to define the precise mechanism of action of the therapeutic efficacy of estriol treatment in EAE. These findings will then be contrasted to findings of the precise mechanism of action of estrogen related toxicities such as increased risk of breast cancer, endometrial cancer, and vascular disease. Our findings suggest that therapeutic efficacy may be achieved through stimulation of ERα. If it is ultimately shown that some or all toxicities are mediated through ERβ or nongenomic effects, then a window of selectivity exists whereby one could treat disease with an ERα agonist or other SERM to achieve efficacy and minimize toxicity. In contrast, if it is ultimately shown that all toxicities are mediated through ERα, then selectivity will not be possible at the level of the ER. However, selectivity may still be achievable through modulation of tissue-specific cofactors involved in ERα action. To pursue the latter, one must first define the ER used in a given tissue for a given biologic effect. Then one may begin to identify tissue-specific differences in cofactors for each of the biologic effects.

In conclusion, the protective effect of estrogen treatment on autoantigen-specific immune responses in a prototypic Th1-mediated autoimmune disease is mediated through ERα. This does not however rule out additional nongenomic (membrane, ER-X) or

estriol specific cofactors for each of the biologic effects.
genomic (ERβ, ERγ) effects on estrogen-mediated immune protection. The finding of an important role for ERα carries far reaching implications for the design of SERMs for the treatment of a wide variety of autoimmune diseases which aim toward optimizing efficacy and minimizing toxicity. They are equally as relevant to the design of SERMs for treatment of nonautoimmune diseases with different target organs (the reproductive system, the cardiovascular system, bone, breast, colon, and prostate) which aim to avoid effects on the immune system.

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