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Estrogen Protects Against Cellular Infiltration by Reducing the Expressions of E-Selectin and IL-6 in Endotoxin-Induced Uveitis

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Anterior uveitis associated with Behcet’s disease and ankylosing spondylitis preferentially occurs in adult men, which may suggest the effects of sex hormones on acute anterior uveitis. Recently, estrogen receptors in the vascular endothelium have been reported to be involved in several pathological conditions. In the present study, we examined the gender differences in susceptibility to endotoxin-induced uveitis (EIU) and the effects of estrogen on anterior inflammation. EIU was induced in adult male, female, and ovariectomized female Lewis rats (200 g) by hind footpad injection of 200 μg of LPS. In EIU, cellular infiltration was more marked in male than in female rats, and ovariectomy increased cellular infiltration. Treatment with 10 μg of 17β-estradiol significantly reduced the cell number in male and ovariectomized female rats with EIU. Estrogen receptor immunoreactivity was found in the nucleus of vascular endothelium and in some stromal cells of the iris-ciliary body. Semiquantitative PCR revealed that E-selectin and IL-6 gene expressions were increased in rats following LPS injection, and an overdose of tamoxifen, an estrogen receptor antagonist, reversed the effect of 17β-estradiol on E-selectin, but not its effect on IL-6. These observations suggested that the down-modulation of these inflammatory genes by estrogen may contribute to the reduction in cellular infiltration in acute anterior uveitis. The Journal of Immunology, 1999, 163: 374–379.

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terior uveitis presenting with marked cellular infiltration is often associated with Behcet’s disease, ankylosing spondylitis, Reiter’s syndrome, and HLA Ag B27-associated uveitis. These forms of uveitis are more commonly observed in middle-aged male patients than in females (1, 2). The mechanism of uveitis in these diseases has not been clarified, but the gender preference in these acute types of anterior uveitis suggests that sex hormones may be involved in the inflammatory reaction. Recently, estrogen receptors were reported to be present in the vascular endothelium (3, 4), and the roles of estrogen have been investigated under the various physiological and pathological conditions; estrogen induces the constitutive type of NO synthase (NOS)3 in the endothelium, which is known to play an important role in maintenance of the vascular system (4), and estrogen receptors in human smooth muscle cells play a functional role in coronary atheroprotection, which may explain the increase in frequency of cardiac disease after menopause in female patients (5). Because the endothelium could be a source of various key factors for pathological events such as growth factors, cytokines, and adhesion molecules (6–8), estrogen receptors in the vascular endothelium may play a number of roles in these processes (9, 10).

Endotoxin-induced uveitis (EIU) is an animal model of acute anterior inflammation that is caused by an injection of LPS (11). In this model, vascular endothelium, macrophages, and other cells may be directly activated by LPS in principal. Although this model is not an actual simulation of clinical uveitis, such as Behcet’s disease or Reiter’s syndrome, subsequent cytokine activation with inflammatory reactions may more or less mimic an acute phase of anterior uveitis. In this model, cellular infiltration and the increase in protein level in the aqueous humor reach maximum levels at ~24 h after LPS treatment (12, 13). We reported previously that selectins play key roles in the initiation and retention of cellular infiltration in EIU; P-selectin is expressed on the vascular endothelium in the iris-ciliary body 15 min after LPS treatment and may contribute to the initial phase of cellular infiltration (14); E-selectin may contribute to the maintenance of cellular infiltration into the inflammatory site during inflammation; its inhibition resulted in the early resolution of uveitis (15). In cultured HUVEC, estrogen has been reported to reduce the expression of E-selectin (3). Considering the higher frequency of male patients with anterior uveitis and the importance of E-selectin in EIU, we postulated that estrogen may modulate cellular infiltration in EIU, and that E-selectin may be one of the target molecules in this process. Also, modulation of proinflammatory cytokines such as IL-1, IL-6, and TNF by estrogens in monocytes and macrophages was reported by several authors (16–18). Because increased expressions of these cytokine genes were reported in EIU (13, 19, 20), we speculated that estrogen could also modulate the expression of some of these genes in EIU. Thus, in the present study, we first examined whether there is a gender difference in the susceptibility to anterior uveitis following LPS injection. We subsequently examined whether estrogen modulates cellular infiltration and, if so, whether estrogen has some effect on the expression of E-selectin in EIU. We also screened for several proinflammatory cytokines to see whether estrogen could modulate their gene expressions in vivo in EIU.

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3 Abbreviations used in this paper: NOS, NO synthase; EIU, endotoxin-induced uveitis; E2, 17β-estradiol; iNOS, inducible NOS.
Materials and Methods

Animal model
EIU was induced in adult male and female Lewis rats (200 g) by injection of 200 μg of LPS (Salmonella minnesota, Sigma, St. Louis, MO) diluted in 0.1 ml of sterile saline into one hind footpad. In ovariectomized female rats, EIU was induced 7 days after ovariectomy.

Drug treatment
17β-Estradiol (E2, Sigma; 1, 10, or 100 μg/rat) was resolved in ethanol, diluted in 0.2 ml/rat of PBS, and administered i.p. at the time of LPS treatment. Tamoxifen was used as an estrogen receptor antagonist and was administered i.p. 1 h before LPS treatment at a dose of 150 μg/rat in 0.2 ml of PBS over 10 mg/rat of E2.

Evaluation of inflammatory response in EIU
The number of infiltrating cells into the aqueous humor and the aqueous humor protein concentration were used as indicators of the degree of anterior inflammation. Inflammation was evaluated at 24 h after LPS treatment, when cellular infiltration into the aqueous humor reached a maximum level. The rats were sacrificed, and the aqueous humor was collected at 24 h after LPS injection as described previously (21). Briefly, the aqueous humor was collected from both eyes by anterior chamber puncture (∼15 μl/rat) using a 30-gauge needle under a surgical microscope; the samples were used for either cell counting or protein measurement. For cell counting, the aqueous humor sample was suspended with an equal amount of 0.4% trypan blue solution, and the cells were counted using a hemocytometer under a light microscope; the number of cells per field (an equivalent of 0.1 μl) was manually counted, and the number of cells per milliliter was obtained by averaging the results of four fields from each sample. For protein measurement, each sample was centrifuged to remove the cell pellet; the protein concentration in the supernatant was measured using bicinchoninic acid protein assay reagent (Pierce, Rockford, IL) with BSA as a standard.

Immunohistochemistry
The tissue fixation and immunohistochemical techniques used for examination of paraffin-embedded sections have been described previously (14, 15). Animals were anesthetized (pentobarbital sodium, 100 mg/kg) at 7 h after LPS injection (n = 3) and subsequently killed by cardiac perfusion of 4% paraformaldehyde in PBS. Whole enucleated eyes were fixed in 4% paraformaldehyde solution overnight at 4°C before paraffin embedding and were sectioned at 5 μm for immunohistochemical analysis. Sections were deparaffinized, and endogenous peroxidase was blocked with 0.3% hydrogen peroxide-methanol. Each section was incubated for 30 min with blocking serum. The sections were incubated overnight at 4°C in a humidified chamber with mouse antiserotonin reagent (ABR, Golden, CO) or polyclonal anti-E-selectin Ab (R&D Systems, Minneapolis, MN) and subsequently washed for 10 min with PBS. Immunostaining was performed by the avidin-biotin peroxidase complex method (ABC Elite kit; Vector Laboratories, Burlingame, CA) with 3,3′-diaminobenzidine tetrahydrochloride (Dako, Glostrup, Denmark) as the substrate. The tissue sections were counterstained for methyl green. As a negative control, normal mouse IgG was used instead of the primary Ab. Other staining procedures were the same as described above.

Quantification of gene expressions of E-selectin and proinflammatory cytokines in the iris-ciliary body
Gene expressions of E-selectin, IL-1α, IL-1β, IL-6, TNF, and inducible NOS (iNOS) in the iris-ciliary body were studied using semiquantitative PCR. Eyes were enucleated at 6 h after LPS treatment. Each enucleated eye was cut into two pieces along the limbus, and the iris-ciliary body and retina were separately collected with fine forceps. RNA was extracted from the iris-ciliary body using the acid guanidinium thiocyanate-phenol-chloroform extraction method (22). The extracted RNA was quantified, and 5 μg of each sample was used to make cDNA with a first strand DNA synthesis kit (Amersham Pharmacia Biotech AB, Uppsala, Sweden). PCR was conducted by the method of Saiki et al. with slight modifications (23). The PCR conditions were: denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s. The reaction was initiated by adding 2 U of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT); at total of 30–35 cycles were performed for E-selectin, IL-1α, IL-1β, IL-6, TNF, iNOS, and GAPDH, respectively, using the primers described in Table 1. The PCR products were derived from the target cDNA sequences. PCR was conducted in a semiquantitative manner as described previously (13). Briefly, 2 μl of radiolabeled dCTP was added to the PCR mixture. Following electrophoresis of the PCR products, the bands were excised; the radioactivity incorporated into the DNA was measured by Cerenkov scintillation counting. A standard curve was drawn from the radioactivities by serial dilution of the template cDNA. The relative quantity of the expressed gene in the cDNA was calculated from this standard curve. The cDNA concentration was first normalized by PCR with the primers for GAPDH, and the relative expression of the target gene was subsequently determined.

Statistical analysis
Data are expressed as means ± SD. Statistical analysis was performed using the Bonferroni method. p values of <0.05 were regarded as significant.

Results
Effects of E2 on cellular infiltration and protein concentration
We first studied the effects of different doses of E2 (1, 10, and 100 μg) on EIU in male rats. In EIU rats, the number of infiltrating cells in the aqueous humor at 24 h after LPS injection was 360 ± 9 cells/μl (mean ± SD, n = 3). Treatment with 10 μg of E2 resulted in the most significant reduction in cell number to 88 ± 6 cells/μl (n = 3, p < 0.0001). At doses of 1 and 100 μg, E2 showed a lesser but significant reduction of cell number to 167 ± 17 and 177 ± 6 cells/μl, respectively (n = 3, p < 0.0001) (Fig. 1A).

The protein concentration in the aqueous humor was 8.9 ± 1.8 mg/ml in EIU rats (n = 3). Treatment with 10 μg of E2 reduced the aqueous humor protein concentration to 7.3 ± 0.9 mg/ml, but this effect was not statistically significant (n = 3). Treatment with 1 or 100 μg of E2 did not reduce the aqueous humor protein concentration (8.6 ± 2.4 and 11.5 ± 0.9 mg/ml, respectively; n = 3) (Fig. 1B).

We subsequently investigated whether there are gender differences in susceptibility to EIU and the effects of E2 on each sex. In EIU, cellular infiltration was more marked in male than in female rats (363 ± 168 cells/μl vs 101 ± 37 cells/μl; n = 5, p < 0.05). E2 further reduced the cell number in female EIU rats to 46 ± 19 cells/μl (n = 5), although this was not statistically significant. In ovariectomized female rats, the cell number increased above that of nonovariectomized female rats to 157 ± 30 cells/μl (n = 5, p < 0.05), which was again markedly reduced to 16 ± 10 cells/μl by treatment with E2 (n = 5, p < 0.005) (Fig. 2A). The aqueous humor protein content was also less in female than in male rats (5.9 ± 1.2 mg/ml vs 13.7 ± 2.5 mg/ml, n = 5, p < 0.001). E2 further reduced the protein level in female EIU rats to 4.4 ± 0.5 mg/ml (n = 5), and ovariectomy increased the protein concentration in EIU to 7.2 ± 3.2 mg/ml (n = 5), although these effects were not statistically significant (n = 5). Treatment with E2 and LPS significantly reduced the protein concentration in ovariectomized female rats to 2.1 ± 0.4 mg/ml (n = 5, p < 0.05) (Fig. 2B).

Next, we investigated whether the effect of estrogen observed here was receptor-mediated. Tamoxifen, an estrogen receptor antagonist, reversed the effect of E2 on cellular infiltration from 112 ± 13 cells/μl to 318 ± 28 cells/μl (n = 3, p < 0.005) in male rats (Fig. 3).
Presence of estrogen receptors in the vascular endothelium

In paraffin-embedded sections, estrogen receptor-like immunoreactivity was consistently observed in the nucleus of vascular endothelium of the iris-ciliary body and in the nucleus of some stromal cells (n = 3; Fig. 4A, arrows). With normal mouse IgG, no specific staining was observed (n = 3; Fig. 4B).

Gene expressions of E-selectin and proinflammatory cytokines in the iris-ciliary body

The levels of mRNA expression of E-selectin and several proinflammatory cytokines were evaluated in the iris-ciliary body using a semiquantitative PCR. PCR products were obtained by PCR using specific primers for E-selectin, IL-1α, IL-1β, IL-6, TNF, iNOS, and GAPDH cDNA in the iris-ciliary body. In this study, control rats were each injected with 100 μl of endotoxin-free saline, and the iris-ciliary bodies were collected 6 h later. The levels of E-selectin mRNA expression are shown as a ratio to the level in the control rats (Fig. 5). E-selectin gene expression was 23-fold higher in male rats receiving LPS injection, and was reduced to...
12.6-fold in rats receiving a simultaneous i.p. injection of E₂ (n = 5, p < 0.05). The expression of E-selectin mRNA was then reversed to 20.9-fold in rats receiving E₂ and tamoxifen with LPS, and tamoxifen added with LPS resulted in a 22.7-fold increase in the E-selectin mRNA level. As for proinflammatory cytokines, the mRNA levels of all of the tested cytokines were significantly increased in the iris-ciliary body following LPS injection, but a significant effect of E₂ was only observed on IL-6 gene expression. In EIU male rats, the level of IL-6 mRNA expression was raised to 3.0-fold of that for control rats; this level was reduced to the control level in rats receiving a simultaneous i.p. injection of E₂ (n = 5, p < 0.05). The decreased expression of IL-6 mRNA by E₂, however, was not reversed by tamoxifen (Fig. 6). The levels of mRNA of IL-1α, IL-1β, TNF, and iNOS were not significantly changed by either E₂ or tamoxifen.

**Discussion**

In the present study, we demonstrated a possible effect of estrogen on cellular infiltration in anterior uveitis. To our knowledge, this is the first report of an effect of estrogen on ocular inflammation. EIU is a widely used animal model of acute ocular inflammation that is characterized by an increase in protein level and cellular infiltration in the aqueous humor, both of which peak at 20–24 h after LPS injection (13, 21). Both male and female rats have been used for experiments, but direct comparison between the two sexes in susceptibility to EIU has not been reported. In this study, the gender difference observed clinically in some forms of anterior uveitis, such as Behcet’s disease and ankylosing spondylitis, was also shown to apply to the EIU model; more severe anterior inflammation was observed in adult male rats than in females. Also, ovariectomized female rats showed more severe inflammation than nonovariectomized rats, and cellular infiltration as well as protein level was again reduced by E₂ in ovariectomized EIU rats. These results indicate that E₂ has a distinct inhibitory effect on cellular infiltration and protein increase in the aqueous humor in EIU. The more marked effect of E₂ on aqueous protein levels in females than in males (Figs. 1B and 2B) may have resulted from a possibility that expression levels of estrogen receptors would be higher in females.
Because male rats showed more marked cellular infiltration in EIU, and because we have previously observed the up-regulation of several cytokines, NO, and selectins in EIU in male rats (13–15, 21, 25), we performed additional experiments using male rats. Although the circulating levels of estrogen are low in males, the physiological and pathological functions of estrogen receptors in males have been suggested previously (i.e., estrogen administration was shown to improve vascular function in men (26) and estrogen reduces plaque formation in knockout male mice that express high levels of apoE (27)). In addition, estrogen receptors were reported to be present in the vascular smooth muscle cells from both males and females (28). To confirm whether estrogen receptors are also present in the anterior eye in our model, we performed immunohistochemical staining for estrogen receptor. Estrogen receptor-like immunoreactivity was found in the nucleus of the vascular endothelium and in the nucleus of some stromal cells in the iris-ciliary body.

The breakdown of the blood-aqueous barrier in uveitis involves cellular infiltration and an increase in protein permeability. Previous reports showed that these two features do not always occur in parallel and may involve independent mechanisms (14, 15); selectins significantly reduced cellular infiltration in EIU, but their effect on protein concentration was not significant. Similarly in the present study, the effect of estrogen was more apparent on cellular infiltration than on the increase in protein content in the aqueous humor in males. This implies that estrogen may act on some specific factors related to cellular infiltration, such as selectins. Selectins are a group of molecules that are involved in the rolling phase, the first step of cellular infiltration (6–8, 29). We reported previously that E-selectin is expressed on the vascular endothelium of the iris-ciliary body from 7 to 24 h after LPS treatment (15). We also showed that the expression of E-selectin seems to contribute to the retention of cellular infiltration during uveitis, and the inhibition of this molecule at any time during uveitis reduced subsequent cellular infiltration. In the present study, semiquantitative PCR revealed that E-selectin expression was reduced by 40% by E2 in the iris-ciliary body. Furthermore, tamoxifen reversed the effect of E2 on E-selectin expression. The parallel changes in E-selectin gene expression and cellular infiltration indicated that the effect of estrogen is likely to be mediated by its receptor in part by modulating the expression of E-selectin. Considering the marked effect of estrogen on cellular infiltration, we further screened for several other proinflammatory cytokines that are known to be up-regulated in EIU to determine whether estrogen could also modulate their gene expressions in vivo. Although E2 did not inhibit the LPS-induced expression of IL-1, TNF, and iNOS, it did have a significant inhibitory effect on IL-6 gene expression. The IL-6 gene was reported to be up-regulated in the anterior eye in EIU, and the IL-6 levels in the aqueous humor were shown to be well correlated with the severity of inflammation (19, 30), suggesting that the reduced production of IL-6 by E2 may contribute to the minimization of inflammatory reactions. The effect of estrogen on these cytokines, however, is somewhat disputable. For instance, Zuckerman et al. reported that pretreatment of estriol enhanced the increases in serum levels of TNF and IL-6 after LPS treatment (31), whereas an inhibitory effect of E2 on IL-6 production was reported in relation to the pathogenesis of osteoporosis (17) and in HeLa cells cotransfected with IL-6/chloramphenicol acetyltransferase constructs and estrogen receptor expression plasmid (32).

Chao et al. reported that E2 at concentrations of >10^{-2} ng/ml reduced the amount of TNF released by peritoneal macrophages, whereas E2 at concentrations below this level increased the amount of TNF release (16). IL-1 is also reported to be both positively and negatively affected by estrogens, and the effect of E2 on IL-1 release was not dose-dependent (18, 33). These findings suggest that the effect of estrogen may well be complicated, especially in vivo; its effect is often not dose-dependent or even contradictory according to its concentrations. This may be due in part to its complicated roles as a receptor agonist and as an antioxidant and in part to the fact that estrogen could act on several types of cells capable of cytokine production, including endothelium and macrophages. These observations may also explain the discrepancy that estrogens have both positive and negative effects on autoimmune inflammatory reactions; it is known that premenopausal women are more susceptible to autoimmune diseases, and estrogen is reported to exacerbate Lupus disease (34); in contrast, estrogen is reported to reduce the incidence and severity of arthritis induced with type II collagen (35), and oral contraceptives reduced the incidence rate of rheumatoid arthritis (36).

We subsequently studied whether the effects of E2 observed in our study were receptor-mediated or antioxidative ones. In many pathological conditions, estrogen seems to exert its effect by functioning as an antioxidant. Estrogen has been shown to attenuate the oxidative impairment of synaptic Na+/K+ -ATPase activity, glucose transport, and glutamate transport induced by amyloid β-peptide and iron (37). The formation of atherosclerotic plaques is considered to be due in part to the in vivo oxidative modification of low-density lipoprotein cholesterol, and the cardioprotective effects of estrogen may be related to the antioxidant abilities of estrogen (38); estrogen protects against the endothelial and myocardial dysfunction resulting from brief ischemia/reperfusion, which is considered to be antioxidant effect (39). Interestingly in our model, estrogen is likely to have exerted its effect on cellular infiltration and E-selectin expression mainly through its receptor, because the estrogen receptor antagonist tamoxifen reversed these effects of estrogen (Figs. 3 and 5). In contrast, the effect of estrogen on the expression of IL-6 mRNA was not reversed by tamoxifen, which suggested the effect of estrogen as an antioxidant (Fig. 6).

These findings indicate that estrogen could act both through the receptor and as an antioxidant in our EIU model. Although much is known about the mechanisms of gene activation by steroid receptors, including the estrogen receptor, which typically involve
binding of the ligand-activated receptor to the respective response element of target genes, the mechanisms of their gene repression are not well understood. In HaCaT cells cotransfected with IL-6 and estrogen receptor expression plasmid, E2 was shown to inhibit IL-6 gene expression through a receptor-mediated indirect effect on the transcriptional activity of the IL-6 promoter site, which lacks a classical estrogen response element (32). Glucocorticoid inhibited the cytokine-induced expression of adhesion molecules including E-selectin through its receptor by interfering with the transcriptional activation potential of DNA-bound NF-kB complexes; NF-kB is an inducible transcription factor that participates in the induction of numerous cytokine genes and other inflammation-associated genes, including E-selectin (40–42). These observations may imply that estrogen could also modulate the expression of other important genes in addition to the ones tested in our study.

In the present study, we show gender differences in the susceptibility to EU as well as the protective effects of estrogen against EU. Considering the higher incidence of anterior uveitis in men, our observation that estrogen reduced the gene expressions of E-selectin and IL-6 in EU may suggest a possible role for estrogen as an in vivo modulator of several important genes involved in the pathogenesis of human anterior uveitis.

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References