Estrogen Receptor Expression in Bovine and Rat Retinas

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PURPOSE. To investigate the expression and distribution of estrogen receptor protein and mRNA in bovine and rat retinas.

METHODS. Western blot analysis with an antiestrogen receptor monoclonal antibody (mAb) was used to detect estrogen receptor protein in the bovine retina. Immunohistochemistry with an antiestrogen receptor mAb and in situ hybridization with an oligodeoxynucleotide sequence coding for estrogen receptor were applied to study the cellular distribution of estrogen receptor protein and its mRNA in male bovine retina and rat retina of both sexes.

RESULTS. Estrogen receptor protein was detected in bovine retina by Western blot analysis. Immunohistochemical staining with the antiestrogen receptor mAb was widespread throughout the neural retina. Specific staining showed extensive distribution localizing in the nerve fiber layer, the ganglion cell layer, the inner nuclear layer, and the outer plexiform layer. Retinal pigment epithelium and choroid were also stained with the antiestrogen receptor mAb. By in situ hybridization, the expression of estrogen receptor mRNA was predominantly observed in ganglion cell layer, the inner nuclear layer, and outer portion of the outer nuclear layer. No significant difference was found between male and female rats in the immunostaining of retinas with the antiestrogen receptor mAb.

CONCLUSIONS. This study provides the first evidence for the presence of estrogen receptor in bovine and rat retinas. Expression of estrogen receptor throughout the retina suggests that estrogen may have important functions in the retina. (Invest Ophthalmol Vis Sci. 1998;39:2105-2110)

In a previous study, we reported that retinal pigment epithelium (RPE) of embryonic chick eye expressed 17β-hydroxysteroid dehydrogenase type IV,1 which mediates the nicotinamide adenine dinucleotide (NAD)1-dependent oxidative transformation of estradiol to estrone. This dehydrogenase was identified as a highly hydrophobic fraction of 17β-hydroxysteroid dehydrogenase activity isolated from porcine uteri.2-7 17β-Hydroxysteroid dehydrogenase type IV is the key enzyme that inactivates estradiol, providing protection against excessive amounts. The presence of 17β-hydroxysteroid dehydrogenase type IV in RPE cells suggests that RPE may participate in estradiol inactivation, the expression of estrogen receptor mRNA was predominantly observed in ganglion cell layer, the inner nuclear layer, and outer portion of the outer nuclear layer. No significant difference was found between male and female rats in the immunostaining of retinas with the antiestrogen receptor mAb.

It has been shown that human and rat retinas contain measurable amounts of several sex steroids and steroid metabolizing activity.8-10 Rat retinal cells have been found to synthesize steroids and have been proposed as an in vitro model for neurosterogenesis in the central nervous system.11 In the trout, estrogen receptor mRNA has been detected in the pineal gland and retina.12 These findings account for a specific role of neurosteroids in the retina.

The association between steroids and cerebral function has been reconsidered in the discovery of a synthesis of neurosteroids and steroidogenic enzymes in the central nervous system.13 Estrogen plays a critical role in sexual differentiation in the brain.14,15 Several biologic effects of neurosteroids have been observed, such as electrical stimulation of neurons,16 modulation of the function of γ-aminobutyric acid (GABA)17 receptor,17 and growth and differentiation of glial cells in vitro.18 These findings suggest that neurosteroids have an important function in the central nervous system and the retina. The purpose of this study was to determine for the first time the expression and distribution of estrogen receptor protein and mRNA in bovine and rat retinas.

MATERIALS AND METHODS

Western Blot Analysis

Bovine retina was dissected from fresh adult male bovine eyes (purchased from Fukuseibutu Kumiai, Kyoto, Japan). We used male eyes because female eyes were not available from the abattoir. Retinal cells were solubilized in sample buffer containing 2% sodium dodecyl sulfate (SDS), 100 mM dithiothreitol, 60 mM Tris-HCl (pH 6.8), 20% glycerol, and 0.1% bromophenol blue; boiled for 2 minutes; and centrifuged at 10,000g for 15 minutes at 4°C. Recombinant human estrogen receptor (Pan Vera, Madison, WI) was used as a positive control. The proteins were subjected to electrophoresis on 10% SDS-polyacrylamide gels and were transferred electrophoretically to
membranes (Immobilon; Millipore, Bedford, MA). The membranes were blocked in 5% bovine serum albumin in phosphate-buffered saline (PBS) overnight at 4°C. The blots were incubated overnight at 4°C with an antiestrogen receptor monoclonal antibody (mAb; AER 314; Neomarkers, Fremont, CA) or, in the case of control samples, with an antitrinitrophenol mAb, which was an irrelevant mAb in the same class as the antiestrogen receptor mAb. The blots were washed three times in PBS before incubation for 1 hour with a 1:500 dilution of horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dakopatts, Glostrup, Denmark). After three washes in PBS, the staining was visualized with 0.05% 3,3'-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in PBS.

**Immunohistochemistry**

Rat eyes were removed from anesthetized female and male Lewis rats (8 weeks of age). Animals were managed according to ARVO Statement for Use of Animals in Ophthalmic and Vision Research. Bovine retina and rat eyeballs were immediately embedded in ornithine carbamoyltransferase compound (Tissue-Tec; Miles Scientific, Naperville, IL), and snap frozen in liquid nitrogen. Frozen sections 8 μm in thickness were cut using a cryostat microtome (Reichert Histostat, Buffalo, NY) and air dried onto coated glass slides (Neoprene; Nisshin, Tokyo, Japan). The sections were fixed with 4% paraformaldehyde for 10 minutes and incubated overnight at 4°C with an antiestrogen receptor mAb (MA1-310; Affinity Bioreagents, Golden, CO). The antitrinitrophenol mAb was incubated as a control. The sections were then washed three times in PBS and incubated for 1 hour at room temperature with a 1:500 dilution of horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dakopatts, Glostrup, Denmark). Reaction products were visualized by immersion of slides for 5 minutes in 0.05% 3,3'-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in PBS. After a final washing, the nuclei were counterstained with hematoxylin. The sections were dehydrated in a graded ethanol series, cleared in xylene, mounted, and viewed with a light microscope (Nikon, Tokyo, Japan).

**In Situ Hybridization**

The estrogen receptor antisense oligo-DNA, selected as described by Koji and Brenner, was complementary to the mRNA sequence coding for amino acids 13 to 27 of the human estrogen receptor protein as follows: 5'-GTT CAG GGG CTC CAG CTC GTC CCC TCG CAT GAT CAG TAG GGC-3'. The estrogen receptor sense oligo-DNA selected corresponded to the mRNA sequence as follows: 5'-GCC CTA CTG CAT CAG ATC CAA GGG AAC GAG CTG GAG CCC CTG AAC-3'. The oligo-DNA was synthesized on an automatic DNA synthesizer (Expedite 8900; Applied Biosystems, Foster City, CA) and labeled at their 3'-end with digoxigenin-11-deoxyuridine triphosphate by terminal transferase.

For in situ hybridization, 8-μm frozen sections were mounted on slides coated with aminopropyltriethoxy silane and air dried. The sections were fixed with 4% paraformaldehyde for 15 minutes. After washing with PBS, the sections were treated successively with 0.2 M HCl for 20 minutes, 0.2% Triton X-100 for 10 minutes, and 1 μg/ml proteinase K for 15 minutes. After fixation with 4% paraformaldehyde for 10 minutes, the sections were kept in 40% deionized formamide in 4× SSC until used for hybridization. Hybridization was performed overnight at 37°C in a solution containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.6 M NaCl, 1× Denhardt's solution, 500 μg/ml transfer RNA, 250 μg/ml salmon testicular DNA, 0.01% SDS, 50% deionized formamide, and a digoxigenin-labeled oligonucleotide probe. They were then washed five times with 2× SSC at 37°C for 30 minutes. After incubation for 1 hour with blocking solution (Boehringer Mannheim, Mannheim, Germany), the sections were incubated overnight at 4°C with alkaline phosphatase-conjugated antidigoxigenin antibody (Boehringer Mannheim). After the sections were washed with

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933202/)
RESULTS

Western Blot Analysis
The presence of estrogen receptor in the bovine retina was confirmed by Western blot analysis with the antiestrogen receptor mAb (Fig. 1A). Bovine retina contained a band of estrogen receptor protein at a relative molecular mass of 67 kDa. The blot incubated with the antitrinitrophenol mAb served as a control sample, in which no 67-kDa band was observed (Fig. 1B). Recombinant human estrogen receptor, a positive control, was also reacted with the antibody (Fig. 1C).

Immunohistochemistry
The staining of the antiestrogen receptor mAb was distributed widely throughout the neural retina (Fig. 2B). Intense staining by the antiestrogen receptor mAb was observed in the nerve fiber layer, the ganglion cell layer, the inner nuclear layer, and the outer plexiform layer. Most cell bodies in the ganglion cell layer were stained for the antiestrogen receptor mAb, but some of them were not (Figs. 2B, 2C). Some stained cell bodies were localized in the inner plexiform layer. In the inner nuclear layer, the antiestrogen receptor mAb reacted strongly with some of the cell bodies and cell processes. Stained cell processes were fine and extended vertically to the inner nuclear and inner plexiform layers. The processes in the outer plexiform layer also were stained. The outer nuclear layer showed weak staining. RPE and choroid were stained, especially vessels in the choroid. In the control section, no specific staining was seen (Fig. 2A). Rat retina showed staining similar to that in bovine retina. The staining of the antiestrogen receptor mAb was widespread throughout the neural retina. No significant difference was found in the staining between both sexes (Figs. 3A, 3B).

In Situ Hybridization
To identify cells expressing estrogen receptor mRNA, we performed in situ hybridization in bovine and rat retinas. No specific staining was seen in sections hybridized with the sense probe (Fig. 4A). Estrogen receptor mRNA was detected in some cell bodies in the ganglion cell layer (Fig. 4B). Intense staining was observed in the inner nuclear layer and outer portion of the outer nuclear layer. Estrogen receptor mRNA localized to the RPE and endothelium of blood vessels in the choroid.

DISCUSSION
In this study, we showed widespread expression of estrogen receptor protein and its mRNA in bovine and rat retinas. This is the first report describing the presence of estrogen receptor in the mammalian retina.

In the present study, immunohistochemistry with the antiestrogen receptor mAb showed that estrogen receptor was distributed widely throughout the neural retina. The staining of processes of the retinal cells was observed. Several immunohistochemical studies have shown cytoplasmic estrogen receptors in the rat brain, most likely representing complete receptors rather than fragments. These investigators have suggested that there may be fundamental differences in the function of some estrogen receptors in the brain, compared with function in peripheral reproductive tissues such as uterus and oviduct.

Estrogen receptor mRNA, detected by in situ hybridization, was distributed widely in the neural retina. Recent in
FIGURE 3. Immunohistochemical localization of estrogen receptor in frozen sections of rat retinas of male (A) and female (B). No significant difference was found in the staining between both sexes. GCL, ganglion cell layer. Original magnification, ×200.

situ hybridization studies have documented the widespread distribution of estrogen receptor mRNA in the adult rat brain. Clearly labeled cells were found in regions with widespread connections throughout the brain, suggesting that estrogen receptor may modulate a wide variety of neural functions. In culture, estrogen receptor mRNA was expressed in different types of glial cell. In our study, the cell types of the stained cells were not determined. Further

FIGURE 4. In situ hybridization with digoxigenin-labeled oligodeoxynucleotides corresponding to the estrogen receptor sequence in bovine retina. (A) No hybridization was seen with the control probe. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. (B) The expression of the estrogen receptor mRNA was predominantly observed in the GCL, INL, and the outer portion of the ONL. Original magnification, ×100.
investigations should attempt to determine the cell types of the estrogen receptor-positive cells in retina.

The immunohistochemical studies of the distribution of estrogen receptor protein yielded results largely in agreement with those in the in situ hybridization studies. The protein staining of the outer portion of the outer nuclear layer appeared to be weak, whereas there appeared to be strong mRNA signal. It cannot be excluded that the relative abundance of mRNA and protein may differ in cell type because of synthesis, degradation, or secretion of protein in various cell types. The differences between mRNA and protein distributions may be caused by different levels of sensitivity. The oligonucleotide probe used in this study was designed to be specific for estrogen receptor. However, it cannot be excluded that the probe reacts with unknown mRNA.

The presence of estrogen receptor suggests that estrogen has functions in the retina. However, the biologic role of estrogen in the retina remains obscure. Recent evidence has shown that neurosteroids have genomic and nongenomic effects on neural activity in the central nervous system.28 Steroid hormone action involves binding to cognate intracellular receptors, which in turn change conformation, bind to the respective response elements, and modulate gene expression.29 In addition, neuroactive steroids regulate neuronal function through their effects on transmitter-gated ion channels.30-32 Estrogen enhances growth and differentiation of neurites within the developing forebrain.33 Estrogen and neurotrophins, which influence one another’s actions by regulating ligand availability at the level of signal transduction or gene transcription, stimulate synthesis of proteins required for neural differentiation, survival, and maintenance of function.33-34 Therefore, in the normal retina, estrogen is likely to have genomic and nongenomic effects on neuronal activity to enhance growth, differentiation, and survival of neurons and to modulate neuronal functions. Further investigation is needed to study the functional role for estrogen and its receptor in the retina.

The importance of sex in ophthalmologic research is emphasized by focusing on what is known about sex differences in relation to the influence of female hormones.35 There are several studies that have evaluated an association between estrogen and retinal diseases. In women, risk of central retinal vein occlusion and idiopathic macular hole decreases with use of postmenopausal exogenous estrogen.36,37 The associations with postmenopausal exogenous estrogen use are consistent with a hypothesis linking risk factors for cardiovascular disease with neurovascular age-related macular degeneration.38 In our study, no significant sexual difference was found in the distribution of estrogen receptor protein. In the rat brain, there were no absolute sexual dimorphisms in the distributions of estrogen receptor mRNA-containing cells between male and female rats.26 However, more subtle quantitative differences between levels of estrogen receptor expression of the male and female retinas cannot be ruled out.

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References


