Published in final edited form as:

Exp Eye Res. 2013 July ; 112: 1–9. doi:10.1016/j.exer.2013.04.002.

High Affinity Nuclear and Nongenomic Estradiol Binding Sites in the Human and Mouse Lens

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Abstract

Estrogen is reported to be protective against cataracts in women and animal models. Immunodection methods have identified the classic estrogen receptors (ER), ER α and ER β , in human lens epithelial cells and their RNAs have been detected in the rat and human lens. To verify that estrogen binding occurs in the lens, sensitive [125 I]-17 β -estradiol binding analyses were performed on subcellular lens fractions from women (ages 39–78 years). The presence of high affinity estradiol binding sites in the nuclear, cytoplasmic, and membrane fractions indicate the lens is able to respond to estrogens, even up to age 78, although fewer binding sites were detected in the postmenopausal women. Additionally, due to the importance of mouse models in estrogen action and lens research, lenses from intact female mice were also analyzed. Both the C57BL/6 and FVB/N mouse strains also possessed high affinity binding sites in all three lens fractions. Furthermore, transcripts for ER α , ER β , and G protein-coupled estrogen receptor (GPER; previously called GPR30) that bind estradiol with high affinity were expressed in the human and mouse lenses. These data provide the first evidence of GPER expression in the lens. Its role, functions, and subcellular location are currently unknown, but a G-shift assay in the membrane fractions of human and mouse lenses did not show evidence that estradiol induced classic G protein-coupled receptor activation. All three receptor transcripts were also detected in the lens capsule region isolated from female C57BL/6 mice, which is mainly comprised of epithelial cells. In contrast, only ER α and GPER were expressed in the cortex/nuclear region, which is primarily composed of differentiating and organelle-free fiber cells. No significant differences in specific estradiol binding and receptor RNA expression was observed in the lenses between male and female C57BL/6 mice. These findings indicate that the lens is an estrogen target tissue in both sexes. The identification of GPER, in addition to ER α and ER β , in the lens also adds to the complexity of possible estrogen responses in the lens. Accordingly, the protective effects of estrogen in women and animals may be mediated by all three estrogen receptors in the lens. In addition, the similarities in binding and receptor RNA expression in the lenses of both species suggest that mice can be used to model estrogen action in the human lens.

Keywords

age; binding analysis; estradiol; estrogen receptor; female; GPER; lens; male

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1. Introduction

The risk for several diseases increases in women after menopause when the ovaries stop producing estrogens, including age-related cataracts. The resulting estrogen deficiency may explain why cataracts are more common in aging women compared to men (Congdon et al., 2001; Kanthan et al., 2008; Klein et al., 2002; Klein et al., 2008). Multiple studies suggest that the length of exposure to endogenous estrogens influences cataract risk in women. That is, a shorter lifetime exposure due to later age of menarche (onset of menstruation) or earlier onset of menopause increases risk, while longer exposure from later onset of menopause or earlier menarche decreases cataract risk (Cumming and Mitchell, 1997; Freeman et al., 2004; Klein et al., 1994; Noran et al., 2007; Younan et al., 2002). Additionally, exogenous estrogens influence cataract risk. For example, cataracts are a side effect of tamoxifen therapy, which is given to breast cancer patients to inhibit the growth of estrogen-dependent tumors (Paganini-Hill and Clark, 2000). In contrast, augmenting declining estrogen levels with hormone replacement therapy in peri- and post-menopausal women has a protective effect on cataract risk (Freeman et al., 2001; Klein et al., 1994; Noran et al., 2007; Worzala et al., 2001; Younan et al., 2002).

Evidence for estrogen protection of lens transparency is also observed in rodent models and in vitro studies. Estrogen has been shown to protect against TGF- β -induced (Hales et al., 1997; Chen et al., 2004), radiation-induced (Dymlacht et al., 2006), and MNU-induced cataracts (Bigsby et al., 1999). In addition, inhibition of estrogen action with a dominant negative estrogen receptor in the ER-3 mouse model induces cataracts in intact female mice (Davis et al., 2002). Plus, in cultured, normal human epithelial cells, ER expressed in the mitochondria protects against oxidative stress (Flynn et al., 2008).

Detection of estrogen receptor (ER) transcripts and proteins in the lens has suggested that the eye can respond to endogenous and exogenous estrogens. Cultured human lens epithelial cells express the mRNAs for ER α and ER β (Cammarata et al., 2004). Transcripts for ER α and ER β in rats (Bigsby et al., 1999) and for ER α in rats and rabbits (Wickham et al., 2000) have also been detected in the lens. In addition to their transcripts, the receptor proteins have also been identified by immunohistochemistry for ER α in human lens epithelium (Ogueta, 1999) and for ER α and ER β in cultured human lens epithelial cells (Cammarata et al., 2004) as well as for ER β by western blot analysis in nuclear and mitochondrial preparations from an immortalized human lens epithelial cell line (Cammarata et al., 2004).

Immunodetection methods for ER in the lens do not confirm the receptors are able to bind estrogen and are less sensitive than [125 I]-17 β -estradiol binding assays. Therefore, in this study, saturation binding analyses were examined in nuclear, cytosol, and membrane fractions from human lenses. Also, no studies have examined estrogen receptor expression in mice. With the diverse types of transgenic and other mouse models available for lens research, it is important to compare estrogen receptor expression in humans and mice. Two mouse strains, C57BL/6 and FVB/N, were examined for the presence of estrogen binding sites in the lens. The C57BL/6 strain is commonly used to generate gene targeted mouse models (knockout and knockin mice) as well as transgenic models. FVB/N mice are frequently used to generate transgenic mice with randomly inserted genes. C57BL/6 mice have pigmented eyes with normal retinas, whereas FVB/N mice are albino and homozygous for the mutation for retinal degeneration (*Pde6b^{rd1}*) (Taketo et al., 1991). Additionally, a membrane-bound G protein-coupled receptor, named G protein-coupled estrogen receptor 1 (GPER; previously called GPR30) (Filardo and Thomas, 2012), has recently been identified to bind estradiol with high affinity (Thomas et al., 2005), but its expression has not been examined in the lens. Accordingly, RNA levels for GPER, ER α , and ER β were compared in

the human and mouse lenses in order to investigate the different capabilities of the lens to respond to estrogens.

2. Material and methods

2.1. Animal Care

All animal work was approved by the Institutional Animal Care and Use Committee at Duquesne University in accordance with NIH guidelines. Animals were housed with 12h:12h light:dark cycle with food and water provided ad libitum. The C57BL/6 (Charles River Laboratories, Wilmington, MA) and FVB/N mice (Jackson Laboratory, Bar Harbor, ME) were bred in-house on a purified, isoflavone-free diet in order to eliminate the exposure to estrogenic soy isoflavones that are present in standard rodent chow (Thigpen et al., 1999; Degen et al., 2002). This diet is a modification of AIN-93G using corn oil with 20% protein, 16% fat, 64% carbohydrates, and 3,713 kcal/kg (Harlan-Teklad, Madison, WI). After euthanasia with inhaled carbon dioxide, mouse eyes were enucleated and then microdissected to collect the entire lens for the binding and RNA analyses or the lens capsule and cortex/nucleus regions for evaluating receptor RNA expression.

2.2. Human Lens Collection

All human lens tissues were obtained from the National Disease Research Interchange (NDRI) in compliance with Duquesne University Institution Review Board (IRB). Lens donors were female without a history of eye disease or oophorectomy. As some cancer therapies can influence the lens and/or expression of estrogen receptors, donors also could not have a history of radiation therapy or chemotherapy (i.e., tamoxifen or fulvestrant). Donor information is listed in Table 2. After the individual frozen lenses were received, the entire lens was pulverized in liquid nitrogen and stored at - 80° C. Frozen aliquots, representative of the entire lens, were prepared for the binding and RNA analyses.

2.3. Subcellular Fractions from Lens Tissues for Binding Analysis

Approximately 100 mg of the individual human lens tissue or pooled mouse lenses were homogenized on ice in TEMMG buffer (10 mM Tris, 1.5 mM EDTA, 1 mM monothioglycerol, 25 mM sodium molybdate, and 10% (v/v) glycerol, pH 7.4) with Protease Inhibitor (PI) Cocktail (Roche, Nutley, NJ) added at 1:5 (wt:vol). Each homogenate was centrifuged at 4° C for 10 minutes at 1200 × g to obtain the nuclear pellet. The supernatant was transferred to another tube and then centrifuged at 4° C for 1 hour at 106,000 × g to obtain the cytosol (supernatant) and membrane fraction (pellet). The nuclear fractions were resuspended in 7 mL TEMMG + PI + 0.5 M KCl and incubated for 1 hour on ice to extract the nuclear proteins, while being vortexed every 15 min. The cytosol and membrane fractions were resuspended in 7 mL of TEMMG + PI. All fractions were stored on ice until use in the binding assay. Protein concentrations for the nuclear, cytosolic and membrane fractions were determined using a 96-well format of the BCA protein assay kit (Pierce Biotechnology, ThermoFisher Scientific, Waltham, MA) based on a standard curve using bovine serum albumin.

2.4. Saturation Binding Assays

Nuclear, cytosolic, and membrane extracts from mouse or human lenses were incubated for 1 hour at room temperature with varying concentrations of 16 -[¹²⁵I]-iodo-3,17 -estradiol (2,200 Ci/mmol; Perkin-Elmer, Boston, MA) in the absence (total binding) or presence (non-specific binding) of 100-fold excess diethylstilbestrol (DES; Sigma-Aldrich, St. Louis, MO). The reaction was stopped by the addition of 50 mM ice-cold Tris-HCl buffer (pH7.4) and rapid filtration over glass-fiber filters (Whatman, Piscataway, NJ) soaked in 0.5% polyethylenimine glycol solution. Each filter was washed twice with 5 mL of ice-cold Tris-

HCl buffer (pH 7.4) and counted in a gamma counter. Saturation binding curves, performed in duplicate, were analyzed by non-linear regression analysis using GraphPad Prism® software for calculating the affinity (K_D) and total binding (B_{max}) of 16 - [125 I]-iodo-3,17 - estradiol binding in the lens.

2.5. G-shift Assay

Competition with various concentrations of DES or 17 -estradiol (estradiol) (1 pM to 1 μ M) for 16 - [125 I]-iodo-3,17 -estradiol binding to mouse and human lenses was determined in the absence or presence of 5 μ M GTP S. Membrane extracts from C57BL/6 female mouse or human lenses were incubated for 1 hour at room temperature with approximately 200 pM 16 - [125 I]-iodo-3,17 -estradiol plus increasing concentrations of DES or estradiol, with or without GTP S. The reaction was stopped and counted as described for the binding assays (Section 2.4).

2.6. Real-time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA from lenses from individual animals or human donors was isolated using the Absolutely RNA Miniprep Kit (Stratagene, La Jolla, CA). Total RNA samples were reverse transcribed with the qScript cDNA Synthesis Kit (Quanta Bioscience, Gaithersburg, MD). The resulting cDNA samples were analyzed for relative RNA levels using the iCycler iQ Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA) with B-R SYBR Green Supermix for iQ (Quanta BioSciences, Gaithersburg, MD) using the primers listed in Table 1. Primers spanned at least one intron/exon boundary to prevent detection of genomic DNA. Fifty cycles at 95° C for 30 seconds and 60° C for 30 seconds were performed. To verify the amplified product, melting curves on all samples and agarose gel electrophoresis on a subset of samples were performed. Relative expression was calculated using the 2^{-Ct} method and normalized with the cyclophilin A (*Piaa*; for mouse RNA) or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; for human RNA) housekeeping genes.

2.7. Statistical Analysis

Statistical analyses were performed using GraphPad® Prism Version 5.0 (San Diego, CA). A p value <0.05 was considered significant.

3. Results

Nuclear, cytoplasmic, and membrane fractions were prepared from donor lenses obtained from four women (Table 2). The affinity of [125 I]-17 -estradiol binding to human lens was similar for the nuclear, K_D = 2.5 nM; cytoplasmic, K_D = 1.8 nM; and membrane fractions, K_D = 0.1 nM. The B_{max} values were higher for the nuclear, 688 fmol/mg protein, and membrane fractions, 605 fmol/mg protein, compared to the cytosol, 150 fmol/mg protein. Therefore, high affinity, saturable [125 I]-17 -estradiol binding sites were detectable in all 3 fractions of the human lens (Fig. 1). To compare whether age influenced the binding sites, the data for the four women donors were divided into either younger or older ages based on the average age of menopause (52 years). The two women that died prior to age 45 years (likely pre-or peri-menopausal) displayed higher levels of [125 I]-17 -estradiol binding sites than the older women (over age 55 years, estimated to be postmenopausal), when specific binding at high concentrations of labeled estradiol was compared (Fig.2). Even though the levels of specific binding were lower in the lens from the older women, saturable, high affinity binding sites were evident, such as in the nuclear fraction of the 78-year-old woman (inset Fig. 2).

Estradiol binding sites were also examined in the lenses from C57BL/6 female mice. As in the human lens, high affinity, saturable [125 I]-17 -estradiol binding sites were identified in

all 3 fractions of the lenses from the intact adult female mice (Fig. 3). The resulting K_D values in the nuclear (0.1 nM), cytosolic (0.06 nM), and membrane (0.7 nM) fractions of the mouse lens were in a similar range as observed in the human lens. The lens K_D values were comparable to the uterine nuclear fraction in these mice, 0.02 nM. The B_{max} value was highest in the membrane fraction at 155 fmol/mg protein, intermediate in the nuclear fraction, 19.5 fmol/mg protein, and lowest in the cytoplasmic fraction, 0.3 fmol/mg protein. In the FVB/N lenses, estradiol binding sites were also detected in all 3 fractions (Fig. 4). Membrane estradiol binding sites also displayed comparable affinity, $K_D=0.3$ nM, and B_{max} , 19.4 fmol/mg protein, to the lenses of C57BL/6 mice.

Since binding experiments cannot specify which subtype of estrogen receptors are expressed in the human and mouse lens, RNA analyses for ER α , ER β , and GPR30/GPER were performed on lenses from both species using real-time RT-PCR. Except for ER α and ER β in the 78-year-old donor (with the longest post-enucleation time, PET), transcripts for ER α , ER β , and GPER were detected in human lens (Fig. 5A). Transcript levels for ER α and ER β declined slightly with age, unlike GPER, which appeared to increase with age. In C57BL/6 female mice (Fig. 5B), the opposite effect was observed for GPER (*Gpr30*), with the mice over 1 year old having the lowest expression ($p<0.05$, one-way ANOVA). In contrast, ER RNA levels were highest in the oldest mice ($p<0.04$, one-way ANOVA). ER RNA levels in the mouse lens did not show significant variation with age, but its levels were lower in the mice over 1 year and at 1 month of age relative to the 3-month-old females.

Due to the detection of GPER transcripts and membrane binding in the human and mouse lens, a G-shift assay was performed to determine if the membrane receptors behave like a G protein-coupled receptor (GPCR). [125 I]-17 β -estradiol binding with DES as a competitor was examined in the lens membrane fractions in the absence and presence of a non-hydrolyzable form of GTP, GTP γ S. In the absence of GTP γ S, in both human and mouse lens membrane fractions, estradiol binding was initially increased in the presence of 1 nM DES (Fig. 6A, 6C). At the highest concentration of DES (1 μ M), [125 I]-17 β -estradiol binding was reduced; however, binding only dropped below 100% in the human lens. Since DES has limited binding affinity with GPER (Thomas et al., 2005), the assay was also tested with estradiol as the cold competitor (Fig. 6B, 6D). Similar results were obtained with the initial increase in binding at 1 nM estradiol. However, the higher concentrations of estradiol were not effective at decreasing binding compared to DES in both species. In the presence of GTP γ S, a small shift was observed in the mouse and human membrane fractions at 25 nM DES, but not with estradiol as the competitor (Fig. 6).

To investigate which receptor transcripts were expressed in the capsule, which houses the epithelium, versus the cortex/nuclear regions of the lens, which primarily contains fiber cells with and without nuclei, real-time RT-PCR analyses were performed on these isolated regions from 3-month-old C57BL/6 female mice. RNA for ER α and GPER were detected in both regions, whereas ER β transcripts were only detectable in the capsule region of the lens (Fig. 7A). Relative RNA levels of all 3 receptors were slightly enriched in the capsule region compared to the entire lens. Although ER α and GPER transcripts were detected in the fiber cell-rich cortex/nuclear region of the lens, their levels were lower than in the entire lens and capsule region. The lower levels of expression would correlate with the presence of mRNA in only a few layers of differentiating fiber cells prior to the organelle-free zone compared to the entire lens, which would include epithelial and fiber cell expression. Therefore, expression of ER β appears to be restricted to epithelial cells in contrast to ER α and GPER, which are detected in both the epithelial and differentiating fiber cells.

To compare estradiol binding and receptor expression in males and females, 3-month-old C57BL/6 male mice were also analyzed. Similar levels of specific binding at 1.5 nM

[¹²⁵I]-17 β -estradiol were detected in the nuclear, cytosolic, and membrane fractions of the lenses from male and female mice (Fig. 7B). Additionally, no significant differences in the relative RNA levels for ER α , ER β , and GPER (*Gpr30*) were detected for the male and female lenses. Therefore, the lens is an estrogen target tissue in both genders.

4. Discussion

Detection of high affinity estradiol binding sites indicates that both the human and mouse lenses can respond to estrogen. Previous studies have detected ER α and ER β in the human lens (Cammarata et al., 2004; Ogueta et al., 1999); however, this study provides the first evidence of estrogen receptors in the mouse lens. Additionally, the binding experiments in the human and mouse lenses verify that the receptors detected in previous studies by immunohistochemistry and western blot are able to bind estrogens, an essential function of these ligand-activated receptors. The similarities in binding for the three subcellular fractions and RNA expression for the receptors that bind estrogens with high affinity, ER α , ER β , and GPER, suggest that mice can model estrogen action in the human lens. Therefore, future investigations in mice, including genetically manipulated models, may be beneficial for examining the role of estrogens in preserving lens transparency.

For the nuclear and cytoplasmic fractions, the dissociation constants (K_D) in the human and mouse lenses were within the range of those obtained from previous studies using cells transfected with ER α or ER β (0.1-0.6 nM) (Kuiper et al., 1997; Kuiper et al., 1996) and cell lines and samples from various estrogen target tissues (0.07-5 nM) (Attardi et al., 1976; Beattie et al., 1985; Chamness et al., 1975; Couse et al., 1995; Fiorelli et al., 1995; Garola and McGuire, 1977; Kim-Schulze et al., 1996; Migliaccio et al., 1992; Raam and Cohen, 1980; Razandi et al., 1999; Rubanyl et al., 1997). Specific estradiol binding in the nuclear fraction correlates with previous reports detecting ER α immunostained nuclei in human lens epithelial cells (Cammarata et al., 2004; Ogueta et al., 1999) and weak staining for ER α by western blot and immunohistochemistry in the nuclei of cultured human lens epithelial cells (Cammarata et al., 2004; Cammarata et al., 2005). In the lens cytosol, ER β likely accounts for the estradiol binding since immunostaining for ER α , and not ER β , was observed in the cytoplasm of a human lens epithelial cell line (Cammarata et al., 2004). However, due to the increased sensitivity of the [¹²⁵I]-17 β -estradiol binding assay compared to immunohistochemistry, ER α may also contribute to the specific binding measured in the lens cytoplasmic fraction. The lower B_{max} in the cytosol versus the nuclear fraction may be due to the use of intact mice with circulating estrogens, which should localize the majority of these classic ERs to the nucleus.

In the membrane fraction, estradiol binding in the human and mouse lens could be due to ER α , ER β , and GPER. All three receptors have been reported to be associated with the plasma membrane in multiple cell types, although there is some controversy with GPER as to whether it is expressed in the plasma or endoplasmic reticulum membrane (Maggiolini and Picard, 2010). The K_D for estradiol binding in the human and mouse lens membrane fractions are similar to those reported in membrane fractions for ER α and ER β (0.19-0.29 nM) (Razandi et al., 1999) and for GPER (2.7 nM) (Thomas et al., 2005). Since the mitochondria and endoplasmic reticulum would isolate with the plasma membranes in the membrane fraction, estradiol binding sites in this fraction would also correlate with previous studies detecting GPER in the endoplasmic reticulum (Revankar et al., 2005) or plasma membrane (Filardo et al., 2007; Funakoshi et al., 2006) in non-ocular tissues and immunostaining for ER α in the mitochondria of cultured normal human lens epithelial cells from male and female donors (Flynn et al., 2008).

Besides their expression in the lens capsule, transcripts for ER and GPER were also detected in the cortex/nuclear region of the mouse lens, which would include cortical cells undergoing differentiation before or just after they lose their nuclei. Although RNA does not indicate expression of the receptor proteins, RNA continues to be translated in differentiating fiber cells until organelle loss (Shestopalov and Bassnett, 1999). Therefore, ER and GPER messages in the cortex/nuclear region could be translated into their receptor proteins in nucleated fiber cells. With the imminent loss of the nuclei and other organelles as differentiation continues, localization of these receptors to the plasma membrane would allow estrogens to continue to influence organelle-free cells in the lower layers of the lens. In contrast, ER RNA was only detected in the mouse lens capsule, even though its receptor also localizes to the plasma membrane (Razandi et al., 1999).

Age-related decreases in ER message (non-quantitative RT-PCR) and protein (western blot and immunohistochemistry) have been detected in retinas from human donors (Ogueta et al., 1999), but have not been examined in the lens. Specific estradiol binding in human donor lenses also displays an age-related decrease in the nuclear, cytosol, and membrane fractions for the women presumed to be postmenopausal (Fig. 2). In a study examining cataractous donor lenses over age 50 years by immunohistochemistry, ER was not detected in the epithelial cells (Zhang et al., 2003). However, the lack of detection is likely due to immunohistochemistry being less sensitive than [¹²⁵I]-17 β -estradiol binding analyses. Additionally, cataract development may modify the expression of ER compared to the normal lenses examined for binding. Therefore, detection of specific binding in the older lenses indicates estrogen binding sites still exist that would allow the lens to respond to estrogen. Accordingly, estrogens produced systemically or locally via enzymes in ocular tissues (Cascio et al., 2007; Salyer et al., 2001) as well as exposure to pharmaceutical, dietary, and environmental estrogens could still induce estrogen-regulated responses in the lenses of postmenopausal women.

Expression of ER α , ER β , and GPER transcripts was also modified with age in the human lenses. However, RNA levels may also be affected by the stability of their messages due to the different PET. For example, the inability to detect ER α and ER β transcripts in the oldest donor is likely related to the long PET (22.5 hours). Additionally, low levels of ER RNA in human lenses may suggest that its message is vulnerable to degradation. However, ER expression was low or undetectable in the retina from postmenopausal women (ages 74 and 77 years), unlike in younger women (35 and 47 years of age) (Ogueta et al., 1999), suggesting its expression may decrease with age. In contrast, GPER transcripts appear to be fairly stable based on minimal variation in its levels in the 57- and 44-year-old donors (PET 2 hours versus 10 hours). However, with the shortest PET, ER α and ER β transcript levels appear to decrease in the 57-year-old donor (presumed postmenopausal) compared to the two younger donors. In contrast, relative GPER RNA levels increase in the 78-year-old donor lens (longest PET) compared to 57-year-old donor lens (shortest PET). These data suggest ER α and ER β levels, but not GPER levels, decrease after menopause. However, further studies will be required to confirm this effect.

In the mice, RNA levels for ER α and GPER (*Gpr30*) were significantly modified with age. The only difference between the human and mouse lenses for the binding and RNA analyses was the direction of ER α and GPER expression with age. ER α transcripts were more abundant and GPER levels were lower in the lenses of mice over 1 year old, compared to younger mice, unlike in postmenopausal women. However, with only two postmenopausal donors (one with a long PET), it is unclear if these age-related effects definitely differ between humans and mice.

Neither estradiol binding nor ER α , ER β , and GPER RNA expression differs significantly between male and female mouse lenses. These data agree with a study reporting similar ER RNA levels in human lens epithelial cell cultures from men and women (Flynn et al., 2008). With the retina being able to convert testosterone to estradiol (Cascio et al., 2007; Salyer et al., 2001) and the lens of both sexes expressing estrogen receptors, estrogen should provide similar protective effects in males and females. In fact, estrogen provides mitochondrial protection against acute oxidative stress in both male and female cultured human lens epithelial cells (Flynn et al., 2008).

G-shift assay results (Fig. 6) do not show evidence of a typical GPCR mechanism, which would predict a loss of [¹²⁵I]-17 β -estradiol binding with higher concentrations of the competitor (estradiol or DES) and a shift to the right (toward higher competitor concentrations) in the presence of GTP S. These data suggest that, if estradiol binding in the membrane fraction is due mainly to GPER, it does not act as a typical GPCR. Instead, these results could be due to ER α tethered to the lens membrane, since ER α interacts directly with the G α and G $\beta\gamma$ subunits (Kumar et al., 2007; Razandi et al., 2004). Despite binding to and mediating responses via G proteins, estradiol stimulation of membrane-bound ER α and G $\beta\gamma$ does not increase [³⁵S]GTP S binding (Kumar et al., 2007). Kumar and collaborators speculate that liberation of G $\beta\gamma$ from ER α and G α occurs via a conformation change induced by estradiol versus GTP binding, unlike classic GPCR activation, which requires GTP binding. Thus, with membrane ER α , GTP S may not shift the curve as would be expected with a GPCR. Additionally, ER α dimers induce nongenomic actions (Razandi et al., 2004), so the competitor may augment dimer formation to result in higher binding (as observed at 1 nM DES and estradiol). However, as DES concentrations increase, competition for labeled estradiol may result in the observed lower binding. This decreased binding is not evident with estradiol, which may be related to DES being a more effective competitor, due to its higher affinity for ER α (Kuiper et al., 1997). Another possibility for the unexpected findings is that GPER may cooperate with ER α for nongenomic signaling, as previously suggested (Levin, 2009; Prossnitz et al., 2008). Therefore, these G-shift results may reflect an interaction between ER α and GPER on the plasma membrane that augments specific binding at the lower competitor concentrations in the presence and absence of GTP S.

This study provides the first evidence of GPER expression in the lens. Its detection in women and mice adds to the complexity of the possible estrogen-mediated responses in the normal lens. Its function is unknown, but its expression in both species suggests GPER has a role in lens physiology. GPER is documented to bind estrogen and induce rapid, nongenomic signaling through multiple pathways (Maggiolini and Picard, 2010; Prossnitz et al., 2008). GPER also has physiological roles in multiple organ systems, including reproductive, cardiovascular, and nervous systems (Filardo and Thomas, 2012; Prossnitz and Barton, 2011). In the lens, it may be located in the plasma membrane or the endoplasmic reticulum, or possibly both, since one study suggests that estradiol binding may cause GPER to be rapidly shuttled from the plasma membrane to the endoplasmic reticulum (Wang et al., 2008). However, future studies will be needed to determine its location and function in the lens.

4.1. Conclusions

Detection of ER α , ER β , and GPER transcripts and estradiol binding sites in the three subcellular fractions in both women and mice highlights estrogen's potential to mediate diverse responses via genomic and nongenomic signaling in the lens. These findings also indicate that the epithelial and differentiating fiber cells are able to respond to estrogen. The presence of ER α and GPER messages in the cortical region of the lens also suggest these two receptors may have a role in fiber cell differentiation and, possibly, maintenance of the

fiber cells after organelle loss. Therefore, the protective effects of estrogen against age-related cataracts reported in epidemiological and animal studies may be mediated by all three receptors.

Acknowledgments

This work was supported by the National Institute of Health, National Eye Institute (EY014600).

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Highlights

- High affinity estradiol binding sites were detected in the lens from women and mice
- Estrogen binding sites are present in nuclear, cytosol, and membrane lens fractions
- Lenses of postmenopausal women remain estrogen responsive, but fewer sites exist
- ER α , ER β , and GPER transcripts were expressed in human and mouse lenses
- Binding and receptor RNA levels were similar in male and female mouse lenses

Figure 1.

High affinity estradiol binding sites in the nuclear, cytosol, and membrane fractions of the female human lens. Nuclear, cytosol, and membrane fractions were isolated from the 4 human donor lenses to analyze estradiol specific binding. Aliquots of each fraction from each donor were incubated with increasing concentrations of [¹²⁵I]-17 β -estradiol in the absence (total binding) or presence of DES (non-specific binding). Three assays with unique [¹²⁵I]-17 β -estradiol concentrations were used to analyze binding for the 4 donor lenses. The curves display the specific binding determined by subtracting non-specific binding from total binding and normalized to the protein concentration for each fraction (fmol/mg protein). Data shown are individual data points plotted for the 4 different donor lenses. Detectable binding was observed in all three fractions from all donor lenses. K_D and B_{max} values were calculated using GraphPad® Prism software, non-linear regression analysis.

Figure 2.

Comparison of nuclear, cytosol, and membrane estradiol binding in the lens of younger and older women. Total specific binding of [125 I]-17 β -estradiol was determined in nuclear, cytosol, and membrane fractions from the lenses of the older women (ages 78 and 57 years; estimated to be postmenopausal), Old (Post), and younger women (ages 44 and 39 years, estimated to be premenopausal), Young (Pre). Although no bar is evident for the cytosol fraction for the older women, very low specific binding was detected (<0.01 fmol/mg protein). [125 I]-17 β -estradiol concentrations used to calculate specific binding were as follows: nuclear fractions for Old (Post), 0.58 and 0.55 nM, and Young (Pre), 2.31 nM; cytoplasmic fractions for Old (Post), 0.58 and 0.55 nM, and Young (Pre), 2.31 nM; and membrane fractions for Old (Post), 0.58 and 0.55 nM, and Young (Pre), 0.97 nM. With only an $n=2$ for each age group, no statistical significance was detected by two-way ANOVA for age, cellular fraction, or their interaction, though age approached significance ($p<0.065$). Saturation binding curve for the nuclear fraction of the lens from the oldest woman donor generated using non-linear regression analysis (GraphPad Prism) is shown in the inset above the bar graph. The K_D value was 0.6 nM.

Figure 3.

Estradiol binding sites in the nuclear, cytosol, and membrane fractions from the lenses of C57BL/6 female mice. Nuclear (A), cytosol (B), and membrane fractions (C-D) were prepared from pools of lenses ($n=3$) isolated from 3-month-old C57BL/6 mice. C) A biphasic curve was detected in the membrane fraction with 0.08 and 0.7 nM K_D values and 13 and 155 fmol/mg protein B_{max} values. In panel D, the low concentrations of [125 I]-17 - estradiol (0.017-0.41 nM) tested in panel C are shown separately to display the higher affinity, saturable binding sites (K_D of 0.08 nM) in the biphasic curve in the membrane fraction. Aliquots of each fraction were incubated with increasing concentrations of [125 I]-17 - estradiol in the absence (total binding) or presence of DES (non-specific binding). Specific binding points (fmol/mg protein) are shown as mean $\pm$ SEM. K_D and B_{max} values were generated using GraphPad® Prism software, non-linear regression analysis.

Figure 4.

Lens fractions from FVB/N female mice have high affinity estradiol binding sites. Subcellular fractions from pools of lenses (n=3) collected from 3-month-old FVB/N female mice were analyzed for specific binding. Specific binding in the nuclear (A) and cytosol (B) fractions were analyzed at 2 concentrations of [¹²⁵I]-17 β -estradiol (0.58 and 3.45 nM). C) Aliquots of the membrane fractions were incubated with increasing concentrations of [¹²⁵I]-17 β -estradiol in the absence (total binding) or presence of DES (non-specific binding). K_D and B_{max} values were generated using GraphPad® Prism software, non-linear regression analysis. Specific binding points (fmol/mg protein) are shown as mean $\pm$ SEM.

Figure 5.

Estrogen receptor transcripts in the human and mouse lens at different ages. Total RNA prepared from human and mouse lenses were analyzed by real-time RT-PCR. All 3 genes were examined from the same RT reaction for each sample. **A)** ER (*ESR1*), ER (*ESR2*), and GPER (*GPER*) expression levels relative to GADPH (*GADPH*) were examined in female human lenses at various ages in years (yr). Fold change in expression determined by the 2^{-C_t} method are shown within each bar relative to the 39-year-old donor. Threshold cycle (C_T) values for GADPH were >4 cycles later in the human donor (age 78 years) with the longest PET (22.5 hours), 26.8 vs. 22.2 (57 yr), 21.5 (44 yr), and 18.6 (39 yr). With only one human donor for each age, statistics could not be performed. **B)** ER (*Esr1*), ER (*Esr2*), and GPER (*Gpr30*) expression levels relative to cyclophilin A (*Ppia*) were examined in lenses isolated from C57BL/6 female mice at different ages. Both lenses from 3 mice at ages 4 weeks (1 mo) and 3 months (3 mo) and 3 pools of lenses from mice over 1 year of age (> 1 yr) were examined (n=2 for ER at age >1 yr). Mean $\pm$ SEM is shown. Fold change in expression for each gene are shown within each bar relative to lenses from the 3-month-old females. One-way ANOVA for the different ages for each gene were significant for ER ($p < 0.04$) and GPER ($p < 0.05$). **a** indicates significant compared to 3 mo and **b** indicates significant compared to 1 mo ($p < 0.05$, Tukey's test).

Figure 6.

DES and estradiol competition in GTP γ S binding assays in the human and mouse lens.

Membrane fractions from 2 human donors (ages 39 and 44 years, panels A-B) and 1 pool of 3-month-old C57BL/6 female mice (panels C-D) were incubated with 200 pM [¹²⁵I]-17 β -estradiol with increasing concentrations of DES (panels A and C) or estradiol (E₂; panels B and D) with and without 5 μ M GTP γ S. Specific binding relative to 1 pM of competitor E₂ or DES (100%) is shown. For the human lenses, each data point represents the mean $\pm$ SEM.

Figure 7.

C57BL/6 mouse estrogen receptor gene expression in the lens capsule and cortex/nucleus and transcripts and binding in male and female lenses. **A)** Total RNA prepared from the capsule and cortex/nucleus regions of lenses from 3-month-old C57BL/6 female mice were analyzed by real-time RT-PCR compared to the entire lens (from Fig. 5B) for ER (*Esr1*), ER (*Esr2*), and GPER (*Gpr30*) relative to cyclophilin A (*Ppia*). Fold change in expression for each gene are shown within each bar relative to the whole lenses from the 3-month-old females. 3 pools were examined for each region; genes were examined from the same RT reaction within each region. GPER expression was significant by one-way ANOVA, $p < 0.015$; * $p < 0.05$ vs. entire lens and capsule (Tukey's test). ER expression approached significance by one-way ANOVA ($p < 0.059$). **B)** Nuclear (Nuc), cytoplasmic (Cyt), and membrane (Mem) fractions from pools of lens from 3-month-old male and female C57BL/6 mice were incubated with approximately 1.5 nM [125 I]-17 β -estradiol (total binding) and [125 I]-17 β -estradiol plus 100-fold excess DES (non-specific binding). Specific binding (total - nonspecific binding) is shown for the mean $\pm$ SEM ($n=5$ pools/sex). No significant differences were detected between male and female lenses (Mann-Whitney, $p > 0.05$). **C)** Gene expression for each receptor in the lenses of 3-month-old male and female C57BL/6 mice were analyzed by real-time RT-PCR. Fold change for each gene in the male lenses is shown within each bar relative to the female lenses. No significant differences were detected between male and female lenses (Mann-Whitney, $p > 0.05$; $n=3$ pools/sex). Mean $\pm$ SEM is shown.

Table 1
Primers for Real-time RT-PCR

Species	Gene	Protein	F/R	Primer sequence
Mouse	<i>Esr1</i>	ER	F	TATGCCTCTGGCTACCATTAT
			R	CATCATGCCCACTTCGTAAC
	<i>Esr2</i>	ER	F	AAATGTGCTATGGCCAACTTC
			R	TTGGCGCTTGGACTAGTAAC
	<i>Gpr30</i>	GPR30 ^a ; GPER ^b	F	AGATCAGGACACCCAACAGA
			R	TTAAGGGGAGCAGAGTCCTT
	<i>Ppia</i>	Cyclophilin A	F	TATCTGCACTGCCAAGACTG
			R	ACAGTCGGAAATGGTGATCT
Human	<i>ESR1</i>	ER	F	GGCCCAGCTCCTCCTCATCC
			R	GCAGCAGGTCATAGAGGGGC
	<i>ESR2</i>	ER	F	GCTCAATTGACCACCCCGGC
			R	ACGCATTTCCCCTCATCCCTGT
	<i>GPER</i>	GPER ^b ; GPR30 ^a	F	GGCTGCAACCCAGGTACCCA
			R	GTGCATCCGTGGAGGCGAG
	<i>GADPH</i>	GADPH ^c	F	GAGTCAACGGATTTGGTCGT
			R	CATTGATGACAAGCTTCCCG

F/R Forward or reverse primer

^aG protein-coupled receptor 30

^bG protein-coupled estrogen receptor 1

^cGlyceraldehyde-3-phosphate dehydrogenase

Table 2
Human Lens Donors

Age (years)	Sex	Race	PET (hours)	Cause of Death
39	F	C	6.0	Subarachnoid hemorrhage
44	F	C	10.0	Intracerebral hemorrhage
57	F	C	2.0	Not determined (secondary to stroke)
78	F	C	22.5	Cardiac Arrest

PET, post-enucleation time; C, Caucasian