Regulation of Estrogen Receptors and MMP-2 Expression by Estrogens in Human Retinal Pigment Epithelium

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PURPOSE. Age-related macular degeneration (ARMD) is characterized by progressive thickening and accumulation of various lipid-rich extracellular matrix (ECM) deposits under the retinal pigment epithelium (RPE). ECM dysregulation probably contributes to the pathologic course of ARMD. By activating estrogen receptors (ERs), estrogens regulate the expression of genes relevant in the turnover of ECM, among them matrix metalloproteinase (MMP)-2. Estrogen deficiency may predispose to dysregulated synthesis and degradation of ECM, leading to accumulation of collagen and other proteins between the RPE and its basement membrane. The purposes in the current study were to confirm the expression of ERs in human RPE, to elucidate whether these ERs are functional, and to test whether 17β-estradiol (E2) regulates expression of ERs and MMP-2.

METHODS. Expression of ERs was examined in freshly isolated human RPE monolayer and in cultured human RPE cells, by using total RNA for RT-PCR and protein extracts for Western blot analysis. Supernatants were collected from freshly isolated human RPE and from cultured human RPE to assess MMP-2 activity by zymography and protein expression by Western blot. The transcriptional activity of ERs was studied in transfection experiments with an estrogen-responsive reporter construct. All these tests were performed in the presence or absence of E2 (10⁻¹¹ and 10⁻⁷ M).

RESULTS. Human RPE isolated from female and male individuals expressed both ER subtypes α and β at the mRNA and protein levels. Treatment of cultured RPE cells with 10⁻¹⁰ M E2 increased expression of mRNA and protein of both receptor subtypes. E2 (10⁻¹⁰ M) also increased MMP-2 activity (~2.2-fold) and protein expression (~2.5-fold). In contrast, there was no change in ER levels and MMP-2 activity at higher E2 concentrations (10⁻⁸ M), compared with baseline. Preincubation of cells with 10⁻⁷ M pyrrolidine dithiocarbamate (PDTC), an inhibitor of nuclear factor (NF)-κB, abolished the increase in MMP-2 activity and protein expression induced by E2 at 10⁻¹⁰ M.

CONCLUSIONS. Both ER subtypes are expressed in RPE and regulated in a dose-dependent fashion by E2. Estrogens similarly regulate MMP-2. This estrogen-induced effect is, at least in part, mediated through NF-κB. These data support the hypothesis that estrogens may exert biological function in RPE through ERs and that estrogen deficiency or excess may cause dysregulation of molecules that influence the turnover of ECM in Bruch’s membrane associated with ARMD. (Invest Ophthalmol Vis Sci. 2003;44:50–59) DOI:10.1167/iovs.01-1276

The incidence and severity of many diseases of the eye have been associated with estrogen status in women.1–4 Several observational studies have suggested that estrogens provide some protection for women from the development or progression of glaucoma,5 cataracts,6 and uveitis.7 However, detailed information on the role of estrogens in retinal diseases, especially age-related macular degeneration (ARMD) is limited. Early menopause may be associated with the development of ARMD in women,8,9 implying that estrogen deficiency may be important in the pathogenesis of ARMD.10 Nonetheless, these few observational studies have provided the basis for a large clinical trial to evaluate the effectiveness of estrogen replacement therapy (ERT) in preventing the onset or progression of ARMD.

The effects of estrogens are mediated by two estrogen receptor (ER) subtypes, ERα and β, which belong to the superfamily of nuclear receptors.11–14 Kobayashi et al.15 reported the expression of ERs in rat and bovine retinas, without differentiating between ERα and β. In the human eye, Oguta et al.16 suggested that ERα is present in the young female retina. In another recent report, both ER subtypes were found in the female and male retinal pigment epithelium (RPE)-choroid complex.17 These findings suggest that estrogens probably serve a physiological function in the outer retina, especially the RPE. However, the regulation of ER subtype expression and their function in the RPE has not been examined.

Among their many actions, estrogens regulate the expression of genes important for extracellular matrix (ECM) turnover, including collagen and matrix metalloproteinases (MMP).18,19 For example, estrogens have been shown to inhibit transforming growth factor-β-mediated type IV collagen production, to suppress expression of type I collagen through activation of activator protein (AP)-1, to increase both MMP-9 mRNA and activity in mesangial cells,20–22 and to increase MMP-2 activity and protein expression in human granulosa lutein cells.19 However, the regulatory effects of estrogens on MMP expression in the retina are unknown.

The retinal pigment epithelium (RPE) is a crucial target tissue in the progression of ARMD. Estrogen-mediated regulation of genes, which are expressed in RPE and are important for the turnover of ECM, may provide a pathogenic mechanism to explain the link between estrogen status and ARMD. In this regard, MMP-2 may be important, because it preferentially degrades ECM components such as type IV and I collagens and laminin.23–25 Dysregulation in the relative production of

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MMP-2 and collagen leads to net deposition of Bruch's membrane (BrM) and contributes to sub-RPE deposit formation.

In this study, we examined the expression of the ER subtypes α and β and their regulation in normal RPE. We also studied the effects of estrogens on MMP-2 expression and activity. We found that both ER subtypes are expressed and regulated by 17β-estradiol (E2) in RPE. In addition, E2 similarly regulated MMP-2 activity and protein expression. This regulation was mediated, at least in part, through the transcription nuclear factor (NF)-κB.

Materials and Methods

Materials

Culture media, supplements, collagen-striped fetal bovine serum (FBS), gentamicin, and oligonucleotides were obtained from Gibco-BRL-Life Technologies (Grand Island, NY). E2, pyrroldinedithiocarbamate (PDTC), bovine albumin (BSA), and EDTA were purchased from Sigma (St. Louis, MO). A cDNA synthesis kit for reverse transcription (RT)-PCR, avian myeloblastosis virus[AMV] reverse transcriptase) and Tag polymerase were purchased from Roche Molecular Biochemicals (Indianapolis, IN). For Western blot analysis, protein concentrations were measured by using the bicinchoninic acid assay (BCA; Bio-Rad Laboratories, Hercules, CA). Prestained molecular weight markers were purchased from Bio-Rad Laboratories. ER and MMP-2 antibodies and their respective blocking peptides were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Chemicon International, Inc. (Temecula, CA). The ERα antibody H-184 is a rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 2 to 185, mapping at the amino terminus of ERα of human origin. This antibody recognizes both mouse and human ERα protein. N-19, the antibody against human ERβ, is a goat polyclonal antibody raised against a peptide mapping at the amino terminus of the ERβ of human origin. maB 15405 MMP-2 is a mouse anti-human monoclonal antibody that recognizes a protein of 72 kDa which is identified as the pro (latent) form of matrix MMP-2 (also known as 72-kDa collagenase IV, or gelatinase A). Noncellulose membranes (Hybond ECL) and films (hyperfilm ECL) for chemiluminescence detection were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). For transfection studies, transfection reagent (TransFast) and lysis buffer were purchased from Promega (Madison, WI). Zymography gels were purchased from Novex (San Diego, CA).

Isolation of Human RPE

Ten pairs of human eyes (five female and five male donors, age range 52 to 84 years) not suitable for transplantation were obtained from the Lions Eye Bank (Miami, FL) within 32 hours after death. The eyes were rinsed twice with 10% gentamicin. The anterior segment was removed and the vitreous-retina was separated from the RPE and rinsed two times with 10% gentamicin. The anterior segment was then minced into smaller fragments under a dissecting microscope. The fragments were transferred into individual Eppendorf tubes (Eppendorf, Fremont, CA), containing 500 μL cold lysis buffer or (1 X) Earle’s balanced salt solution (EBSS), and homogenized on ice with a pestle. All tissues were stored at −80°C until protein extraction and analysis. Our experiments were conducted in accordance with the provisions of the Declaration of Helsinki for research involving human subjects.

Cell Culture

Human RPE cell primary cultures (one pair of eyes from a 50-year-old female donor) were established from eye bank eyes as previously described. These primary cultured cells were generously provided by the Missouri Lion’s Eye Tissue Bank (Columbia, MO). The cells were plated onto collagen IV/laminin and subcultured, propagated, and maintained in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (1/1 vol/vol) supplemented with 10% fetal bovine serum (FBS), 1 mM 1-glutamine, 100 μg/mL penicillin-streptomycin, and 0.075% NaHCO3 in a 5% CO2 humidified air incubator at 37°C. The RPE origin of the cultures was confirmed by positive staining for keratin and PHM-5 (Silenius Laboratories, Hawthorn, Victoria, Australia; data not shown). All experiments were performed using confluent RPE cells from passages 4 to 7.

Experimental Culture Conditions

We initially maintained the primary human RPE in DMEM/F12 (1:1 vol/vol) supplemented with 10% FBS. To characterize the presence of ER as well as expression and activity of MMP-2, 4 days before performing the experiments, the confluent cells were transferred into phenol red-free medium supplemented with 10% charcoal-stripped FBS, a condition generally accepted for studying steroid hormone effects. Phenol red-free medium was selected, because phenol red supplements may contain lipophilic impurities, which have weak estrogen agonist activity. Charcoal treatment removes steroid hormones and numerous other substances, including growth factors.

Confluent cells were plated in T-25 (25 cm2) flasks coated with collagen IV/laminin and cultured in phenol red-free medium supplemented with 10% charcoal-stripped FBS for 72 hours. The medium was changed to 1% charcoal-stripped FBS for 24 hours. Then the medium was changed to 0.1% charcoal-stripped FBS for 32 hours. Eighteen hours before collection of cells layers, the medium was changed to 0.1% charcoal-stripped BSA and total RNA and protein were collected.

Cells were plated, as described previously in T-25 flasks to determine the effects of E2 on the regulation of ER subtypes mRNA and protein expression, the regulation of platelet-derived growth factor (PDGF)-β, VEGF, and cyclooxygenase (COX)-2 mRNA, as well as the regulation of MMP-2 expression and activity by E2, the cells were plated, as described previously, in T-25 flasks. After 4 days, in phenol red-free medium containing 10% (72 hours) and 1% (24 hours) charcoal-stripped FBS respectively, the cells were treated with 0.1% charcoal-stripped FBS in presence of E2 (10−11-10−7 M) for 32 hours. Eighteen hours before collection of cells layers, the medium was changed to 0.1% charcoal-stripped BSA. Confluent cells were harvested for RNA and/or protein collection, whereas the supernatants were used to measure MMP-2 activity (number of cells and density were kept identical). Three or four independent experiments (triplicate flasks for each condition) were performed on cultured RPE cells with reproducible results.

Treatment of Cultured RPE Cells with ICI 182780

To determine whether the effects of E2 on MMP-2 activity were ER-mediated, we treated the RPE cells with the pure estrogen antagonist ICI 182780 (ICI). Confluent RPE cells were grown for 4 days in phenol red-free DMEM/F12 supplemented with 10% charcoal-stripped FBS. The medium was changed to 1% charcoal-stripped FBS for 24 hours. Then, the medium was replaced with 0.1% charcoal-stripped FBS with vehicle, 10−10 M E2, or 10−6 M ICI alone, or in combination with 10−10 M E2 for 32 hours. When ICI was used in combination with E2, the cells were incubated for 1 hour with the antiestrogen before the addition of E2. Eighteen hours before collection of the supernatants, the medium was changed to 0.1% charcoal-stripped BSA. The supernatants were used to measure MMP-2 activity (number and density of cells were kept identical). All experiments were performed in triplicate (duplicate flask for each condition) on cultured cells with reproducible results.

Treatment of Cultured RPE Cells with PDTC

Confluent RPE cells were grown for 4 days in phenol red-free DMEM/F12 supplemented with 10% charcoal-stripped FBS. The cells were preincubated with PDTC, an inhibitor of NF-κB activation. Before treatment with PDTC, the medium was replaced with 1% charcoal-stripped FBS for 24 hours. The medium was replaced with 0.1% charcoal-stripped FBS with vehicle, 10−10 and 10−8 M E2, or 10−7 M PDTC, alone or in combination with E2 for 32 hours. When PDTC was...
To determine the PCR assay range, we plotted the number of PCR cycles against the integrated density obtained from the densitometry analysis. GAPDH was used as an internal standard and housekeeping gene. PCR data obtained for ERα and β were normalized to GAPDH signals. Samples from four different experiments were run in triplicate on cultured cells with reproducible results.

**Real-Time PCR**

A computer was used (Primer Express software; Applied Biosystems, Foster City, CA) was used to design primer pairs and probe sequences for human VEGF, PDGFβ, and COX-2. Primers pairs were selected so that they were located in different exons to prevent the amplification of contaminating genomic DNA. The sequence of probe for VEGF was 5'-CCAAGTGTCGCAGGCTGCAGCAG-3' and labeled 6-carboxyfluorescein (FAM) fluorescent spectrum as a reporter. The amplification primers pairs were 5'-CTGGTGTCTTTGCGAGTTG-3' and 5'-TCCAGCAGCAGCAGCAGCAG-3', respectively. For the PDGFβ and COX-2 amplification, the sequences of the primers used for PDGFβ and COX-2 amplification were 5'-CGATCCAGGCTCTTGTGTGAT-3' and 5'-TCCAATGCGCCCGCATC-3' and 5'-GAACTTACACGACGCAAATGTG-3' and 5'-TCTGCATGGCGTGGGACACA-3', respectively. RT-PCR reactions were performed with a kit and a sequence-detection system (TaqMan One-step RT PCR Master Mix reagents kit and Prism 7700; Applied Biosystems) in a total volume of

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### Isolation of mRNA and RT-PCR

Total RNA was extracted from confluent cell cultures by the guanidium thiocyanate-phenol-chloroform method (Tri-reagent; Sigma). RT was performed on 2 µg total RNA in a total volume of 20 µL. After the total volume was adjusted to 100 µL with diethylpyrocarbonate water, 2 µL of the cDNA solution was used as a template for PCR. PCR amplifications were performed in a total volume of 50 µL with 1.5 U Taq polymerase. The specificity of each reaction was monitored in control reactions, where amplifications were performed on samples after omission of RT. Amplifications of human ER subtypes α and β in human RPE in culture were performed using specific primer pairs previously described by Enmark et al., which resulted in amplicons of 344 bp and 392 bp, respectively. Restriction enzyme analysis was used to confirm the correct sequence of the amplicons (data not shown). For amplification of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), samples were denatured for three minutes at 94°C, then PCR was performed for 27 cycles (45 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C) followed by 7 minutes at 72°C. The GAPDH oligonucleotides were designed from the human gene. The sequences of the GAPDH oligonucleotides were 5'-TCTTGACTGGCCTGACTGAC-3' and 5'-CAGCGGAGGCTGACGAG-3', respectively. The expected size of the product for GAPDH is 598 bp. PCR products were separated on 2% agarose gels containing 0.05% ethidium bromide gels and were photographed with a digital imaging system (Alpha Innotech, San Leandro, CA). Analysis was performed by computer-aided densitometry (NIH Image, produced by W. Rasband, National Institutes of Health, and available by ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, Springfield, VA).

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**Figure 1.** Western blot analysis of ERα and β expression in dissected freshly human RPE of two representative couples of women and men. Dissected RPE homogenates from each donor were analyzed by Western blot analysis with ERα and β antisera (H-184 and N-19, respectively). Ten µg ERα and 40 µg ERβ from isolated RPE monolayer homogenates were loaded in each lane.
FIGURE 3. Transcriptional activity of RPE ERs. Cultured RPE cells were grown in phenol red-free DMEM/F-12 supplemented with 10% charcoal-stripped FBS for 4 days. Transfection was then performed. After transfection, cells were treated with vehicle or 10^{-10} M E_2 for 24 hours in phenol red-free medium containing 10% charcoal stripped FBS. Data are expressed in relative luciferase units. Results are representative of three independent experiments performed in duplicate on cultured cells.

Western Blot Analysis
Dissected pieces from freshly isolated RPE were homogenized with a pestle in lysis buffer. In parallel, confluent cell layers were washed with phosphate-buffered saline PBS (1×) and collected in presence of lysis buffer. Freshly isolated RPE and cell homogenates were centrifuged 30 minutes at 15,000 g at 4°C. Supernatant was collected and protein concentration was determined by BCA protein assay. All samples were then diluted in Laemmli buffer and boiled. Ten micrograms of samples for ERs and MMP-2 or 40 μg for ERβ were loaded on a 10% polyacrylamide gel. Prestained markers were used to estimate molecular weight. Electrophoresis was performed by electroelution. Immunohybrid analysis was performed with each anti ERα, ERβ, and MMP-2 antibody (H-184 and N-19 from Santa Cruz Biotechnologies and mAb 13405 from Chemicon) and immunoreactive bands were determined by exposing the nitrocellulose blots to a chemiluminescent detection system using ECL reagents kit (Amersham Biosciences). The freshly isolated RPE homogenates supernatants were collected, and MMP-2 activity was assessed using 10% zymography gels, as described previously. Briefly, 10-μg samples of RPE tissue were used. One microgram of recombinant miacins was added to each of the 5% Laemmli buffer under nonreducing conditions. After electrophoresis, gels were washed for 1 hour in 2.5% Triton X-100 and incubated 24 hours in 50 mM Tris buffer. The gels were stained with Coomassie blue and air dried. Densitometry, using NIH image (ver. 1.6), was used to analyze the relative activity of MMP-2. Each zymographic assay was performed in duplicate by the manufacturer’s protocol. The standard curves for VEGF, PDGFβ, COX-2, and 18S were generated with serially diluted solutions of mRNA (0.001–10 ng) from human RPE in culture. PCR assays were performed with the reporter construct, 4ERE-TATA-Luc (0.5 μg/well). A generous gift from David J. Shapiro, University of Illinois, Urbana, IL, using transfection reagent (TransFast; Promega), according to the manufacturer’s recommendations. The reporter construct 4ERE-TATA-Luc contains four consensus estrogen-responsive elements (EREs) proximal to the TATA box, which drives the expression of the luciferase reporter gene in an estrogen-dependent manner. The TATA-Luc vector, which does not contain an ERE, served as a control. To adjust for transfection efficiency, RPE cells were cotransfected with pSV-βgal (0.2 μg/well), a vector that constitutively expresses the β-galactosidase gene. One hour later, phenol red-free medium supplemented with 12.5% of charcoal-stripped FBS was added to the transfected cells. Cells were incubated for an additional 24 hours in presence of 10^{-10} M E_2 or vehicle (ethanol). The final ethanol concentration was 0.001% in both conditions. For luciferase and galactosidase assays, cells were lysed in 100 μL of reporter lysis buffer at room temperature. Light emission was detected with a luminometer (AutoLumatPlus; PerkinElmer Life Sciences, Boston, MA) after addition of luciferin to 40 μL of cell lysate. Data are expressed as arbitrary light units normalized to the β-galactosidase activity of each sample.

MMP-2 Activity
The freshly isolated RPE homogenate supernatants were collected, and protein concentration was determined. For cultured RPE cells, the supernatants were collected 18 hours after treatment. At the time the medium was collected, the cells at comparable density were counted for the purpose of adjusting the volume of the medium to the number of cells. MMP-2 activity was assessed using 10% zymography gels, as described previously. Briefly, 10-μg samples of RPE tissue were used. For RPE in culture, the medium was diluted to normalize for number of cells (approximately 30,000 cells/mL), before the addition of 5% Laemmli buffer under nonreducing conditions. After electrophoresis, gels were washed for 1 hour in 2.5% Triton X-100 and incubated 24 hours in 50 mM Tris buffer. The gels were stained with Coomassie blue and air dried. Densitometry, using NIH image (ver. 1.6), was used to analyze the relative activity of MMP-2. Each zymographic assay was performed in duplicate by the manufacturer’s protocol. The standard curves for VEGF, PDGFβ, COX-2, and 18S were generated with serially diluted solutions of mRNA (0.001–10 ng) from human RPE in culture. PCR assays were performed with the reporter construct, 4ERE-TATA-Luc (0.5 μg/well). A generous gift from David J. Shapiro, University of Illinois, Urbana, IL, using transfection reagent (TransFast; Promega), according to the manufacturer’s recommendations. The reporter construct 4ERE-TATA-Luc contains four consensus estrogen-responsive elements (EREs) proximal to the TATA box, which drives the expression of the luciferase reporter gene in an estrogen-dependent manner. The TATA-Luc vector, which does not contain an ERE, served as a control. To adjust for transfection efficiency, RPE cells were cotransfected with pSV-βgal (0.2 μg/well), a vector that constitutively expresses the β-galactosidase gene. One hour later, phenol red-free medium supplemented with 12.5% of charcoal-stripped FBS was added to the transfected cells. Cells were incubated for an additional 24 hours in presence of 10^{-10} M E_2 or vehicle (ethanol). The final ethanol concentration was 0.001% in both conditions. For luciferase and galactosidase assays, cells were lysed in 100 μL of reporter lysis buffer at room temperature. Light emission was detected with a luminometer (AutoLumatPlus; PerkinElmer Life Sciences, Boston, MA) after addition of luciferin to 40 μL of cell lysate. Data are expressed as arbitrary light units normalized to the β-galactosidase activity of each sample.

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Expression of ERα and β in Freshly Isolated Human RPE

We found that both ERα and β were expressed in freshly isolated human RPE from female and male eyes. By Western blot analysis, we detected signals at approximately 66 and 53 kDa by using antibodies to ERα blot analysis, we detected signals at approximately 66 and 53 kDa by using antibodies to ERα. By Western blot analysis, we detected signals at approximately 66 and 53 kDa. Preincubation of the ER antisera with their respective immunizing peptides completely abrogated these signals, confirming that the detected bands were ERα and β (data not shown). Thus, freshly isolated RPE from female and male eyes expressed both ERα and β.

Expression of ERα and β in Cultured Human RPE Cells

Total RNA and protein were extracted from RPE cells. We detected the transcripts of ERα and β by RT-PCR (shown are representative 344- and 392-bp amplicons of human ERα and β cDNA; Fig. 2A). The expression of ERα and β protein was confirmed by Western blot analysis (Fig. 2B). In summary, we demonstrated that both ER subtypes were expressed at the mRNA and protein level in cultured RPE cells.

Regulation of ERα and β mRNA and Protein Expression by Estrogens in Cultured Human RPE Cells

Estrogens have been shown to autoregulate the expression of both ER subtypes in nonocular tissues.11,13 Herein, we examined whether E2 modulated expression of ERα and β in cultured RPE cells. RPE cells were treated with E2 (10^-11-10^-7 M) for 52 hours to study the modulation of expression of ERα by 10^-11 to 10^-7 M estrogen. E2 at 10^-10 and 10^-9 M increased ERα mRNA levels. The maximal increase of ERα mRNA was found with E2 concentrations of 10^-11 M. E2 at 10^-7 M (1.6-fold, P < 0.05), equivalent to levels found in plasma of premenopausal women during the menstrual cycle and in postmenopausal women receiving ERT. However, at the physiological estrogen levels during the menstrual cycle and in postmenopausal women, or during pregnancy, at levels above 10^-9 M, higher than the mid-cycle ovulatory level but similar to levels present in women during pregnancy; at levels in women with long-term use of oral contraceptives; or

Statistical Analyses

All experiments were performed three or four times on cultured cells, with reproducible results. Data are expressed as a percentage of control or as arbitrary densitometry units. Results are the mean ± SEM of three or four independent experiments, performed either in duplicate or triplicate (as indicated). One-way ANOVA and the Dunnett multiple comparison post hoc test were performed. For transfection experiments, data are expressed as arbitrary light units, normalized to β-galactosidase activity for each sample (relative luciferase activity).

RESULTS

Expression of ERα and β in Freshly Isolated Human RPE

We found that both ERα and β were expressed in freshly isolated human RPE from female and male eyes. By Western blot analysis, we detected signals at approximately 66 and 53 kDa by using antibodies to ERα and β, respectively (Fig. 1). The estimated molecular weight of these bands corresponded to the sizes predicted for the wild-type human ERα and β.11,13 Preincubation of the ER antisera with their respective immunizing peptides completely abrogated these signals, confirming that the detected bands were ERα and β (data not shown). Thus, freshly isolated RPE from female and male eyes expressed both ERα and β.

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Transcriptional Activity of the ER in Cultured Human RPE Cells

To elucidate whether the ER expressed in RPE cells are transcriptionally active, we transfected RPE cells with a luciferase reporter construct, 4ERE-TATA-Luc, which contains four EREs in its promoter region. In the transfected RPE, there was an approximate 2.3-fold increase in luciferase activity after treatment with E2 (10^-10 M; Fig. 3). This demonstrated that the endogenous ER subtypes function as ligand-activated transcription factors in cultured RPE cells.

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at the levels in those who receiving ERT at supraphysiological doses, there was no difference in expression of ERα mRNA compared with vehicle treated cells (Figs. 4).

Thus, we focused our attention on these two doses of E₂. One in the physiological serum concentration range (10⁻¹⁰ M E₂) found in premenopausal women during the menstrual cycle and in postmenopausal women receiving ERT at physiological doses and in others in the physiological concentration range (10⁻¹⁰ M E₂) found during pregnancy and/or during ERT at supraphysiological doses. After treatment with E₂ (10⁻¹⁰ M), there was an approximately 2.3-fold (229.1% ± 12.4%; P < 0.01) and 1.7-fold increase (168% ± 6.3%; P < 0.05) in ERα and β mRNA levels, respectively (Fig. 5).

Similarly, we found an increase in expression of ERα and β protein after treatment with 10⁻¹⁰ M E₂. ERα and β protein levels increased approximately 2.2-fold (P < 0.01; Fig. 6A) and 1.78-fold (P < 0.05; Fig. 6B), respectively. At the higher E₂ concentration (10⁻⁸ M), there was no difference in ERα and β mRNA and protein expression compared with vehicle-treated cells. Thus, the levels of ERα and β protein and mRNA were regulated in a coordinate manner in cultured RPE cells after 32 hours of treatment with E₂.

**MMP-2 in Freshly Isolated Human RPE**

The presence of MMP-2 has been reported in conditioned media from RPE cells in culture and in human ocular tissues from female and male eyes. We confirmed the presence of this metalloproteinase in freshly isolated human RPE. Gelatin zymograms of homogenates of dissected RPE showed a prominent gelatinase activity corresponding to MMP-2 (Fig. 7A). This identification was confirmed by immunoblot analysis using MMP-2 antisera (Fig. 7B). In addition, two bands of activity corresponding to monomeric (~92 kDa) and dimeric (~220 kDa) forms of MMP-9 were observed in the same zymograms.

**Regulation of MMP-2 Expression and Activity by Estrogens in Cultured Human RPE Cells**

We found that E₂ regulated MMP-2 protein levels in a dose-dependent fashion similar to the regulation of ER. In the presence of 10⁻¹⁰ M E₂, MMP-2 protein levels increased approximately 2.5-fold (244.2% ± 28.4% of control; P < 0.05). In contrast, there was an approximately 50% decrease in MMP-2 protein (46.9% ± 12.7% of control; P < 0.01) after treatment with 10⁻⁸ M E₂ (Fig. 8A).

MMP-2 activity changed in a similar fashion after treatment with E₂ 10⁻¹⁰ M, increasing approximately 2.2-fold (227.3% ± 42.2%; P < 0.05). However, at 10⁻⁸ M there was no difference compared with the vehicle control (Fig. 8B, V). These studies demonstrate that E₂ modified MMP-2 expression in a dose-dependent (bimodal) fashion. A low physiologic E₂ concentration (10⁻¹⁰ M) increased expression and activity of MMP-2 protein, whereas higher E₂ concentrations (10⁻⁸ M) levels decreased MMP-2 protein levels.

To determine whether the effects of estrogen on MMP-2 activity were ER-mediated we treated the RPE cells with the pure estrogen antagonist ICI. ICI (10⁻⁸ M) alone did not change baseline MMP-2 activity in the cultured RPE cells.

**Figure 6.** Treatment with E₂ for 32 hours increases expression of ERα and β protein in RPE cells. Human RPE homogenates were collected and analyzed by Western blot analysis using ERα (left) and β (right) H-184 and N-19 antisera, respectively. Ten micrograms of RPE cells homogenates for ERα and 40 μg for ERβ were loaded. (A) Expression ERα and (B) ERβ protein levels. Data are expressed as a percentage of control (V; vehicle = 0.001% EtOH). Shown are mean results ± SEM of four independent experiments run in triplicate for both ER subtypes. Statistical significance: *P < 0.05 and **P < 0.01.

**Figure 7.** (A) Gelatin zymography of freshly isolated human RPE from female and male eyes (two representative couples). Numbers on the left represent protein molecular weight in kilodaltons. Lane M: molecular weight standard. The cleared band near the 72 kDa marker migrated in a manner similar to MMP-2. The two bands near 92 and 220 kDa were similar to, respectively, the monomeric (M) and dimeric (D) forms of MMP-9. (B) Immunoblot analysis of MMP-2. Western blot analysis of dissected human RPE homogenates. Ten micrograms of protein were loaded in each lane. MMP-2 is clearly present in RPE homogenates from female and male eyes (B).
(92.7% ± 11.9%). However, it blocked the E₂-induced increase in MMP-2 activity (74.3% ± 12.5% of control), which confirmed that this was an ER-mediated effect (Fig. 9).

**Abolishment of Increase in MMP-2 Activity and Protein Expression Induced by E₂**

ERs have been shown to modulate gene expression through interaction with nuclear transcription factors such as NF-κB. To further elucidate the E₂-mediated effects on MMP-2 expression and activity, we treated RPE cells with PDTC, a known inhibitor of NF-κB activation, alone or in combination with E₂. PDTC did not affect baseline MMP-2 activity and protein expression (97.27% ± 9.9% and 105% ± 11.6%, respectively). A notable finding was that PDTC blocked the E₂-induced increase in the expression and activity of MMP-2 protein in cultured RPE cells (Fig. 10). These data suggest that in cultured RPE cells, the effects of estrogens on expression and activity of MMP-2 protein were, at least in part, mediated by activation of NF-κB.

**Estrogen Regulation of Other RPE Genes**

To show that the upregulation of expression of ERα, ERβ, and MMP-2 by E₂ (10⁻¹⁰ M) was specific and not a generalized transcriptional activation of all cellular genes, we studied the changes in mRNA content induced by 10⁻¹⁰ M estrogen in cultured RPE cells for three other genes: PDGFβ, VEGF, and COX-2 (Table 1). After treatment with E₂ (10⁻¹⁰ M), PDGFβ mRNA increased by 33%, whereas VEGF mRNA decreased by 38%. Minimal modification in levels of COX-2 mRNA were observed. Thus, as expected, transcriptional upregulation by estrogen is not generalized but is probably restricted to specific genes.

**DISCUSSION**

The hypothesis underlying this study is that estrogen may contribute to the RPE regulation of the synthesis and degradation of ECM. Accordingly, estrogen deficiency in postmenopausal women may cause dysregulation of the turnover of ECM, contributing to abnormalities in the basement membrane, thickening of BrM and accumulation of deposits under the RPE in ARMD. Limited information is available regarding the presence of functional estrogen receptors in RPE and their ability to regulate RPE synthesis of molecules important in the maintenance of ECM.
Analysis of ER function in RPE lines developed from ER knockout mice and use of ER subtype specific inhibitors will be necessary to determine the role of each ER subtype in RPE physiology.

In this study, we demonstrated the presence of both ER subtypes α and β at the mRNA and protein level, similar to the findings observed in studies of epithelial cells from other tissues.\(^{41-44}\) Also, RPE ERs are functional and transcriptionally active (i.e., maintain their function as ligand-activated transcription factors), and they regulate the RPE expression of MMP-2, a gelatinase potentially important in maintaining RPE basement membrane. These results confirm and extend the data of others who reported the expression of the two ER subtypes in the human RPE-choroid complex from female and male eyes.\(^{17}\)

### Table 1. Regulation of PDGFβ, VEGF, and COX-2 mRNA by E₂ in Cultured Human RPE Cells

<table>
<thead>
<tr>
<th>E₂</th>
<th>PDGFβ mRNA</th>
<th>VEGF mRNA</th>
<th>COX-2 mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 M</td>
<td>70.1</td>
<td>64.1</td>
<td>80.3</td>
</tr>
<tr>
<td>10⁻¹⁰ M</td>
<td>93.4*</td>
<td>40.0*</td>
<td>74.6</td>
</tr>
<tr>
<td>% Change</td>
<td>+33</td>
<td>-58</td>
<td>-7.1</td>
</tr>
</tbody>
</table>

Cells were grown for 4 days in phenol red-free DMEM/F12 supplemented with 10% charcoal-stripped FBS. Medium was replaced with phenol red-free DMEM/F12 medium supplemented with 0.1% charcoal-stripped FBS containing 0 or 10⁻¹⁰ M E₂ for 32 hours. PDGFβ, VEGF, and COX-2 mRNA expression was normalized to 18S transcript content. Data are expressed as a percentage of vehicle-treated RPE cells control (0.001% EtOH). Results are mean nanograms ± SEM of four independent experiments run in duplicate on cultured cells.

* Statistically significant at \(P < 0.05\).

Because pharmacological hormone replacement therapy in women can produce E₂ blood concentrations that vary over a relatively large range, often surpassing physiologic levels, tissue differences in the dose dependence of estrogen’s effect must be considered. In this study, we found a marked bimodal dose dependence: physiologic concentrations inducing significant ER expression and MMP-2 activity, but lower or higher concentrations resulting in inhibition, or failure to induce, production. In the complete absence of E₂ or in the presence of 10⁻¹¹ M E₂ (serum levels in postmenopausal women), RPE express very low levels of ER and MMP-2. Treatment with 10⁻¹⁰ M E₂ (physiologic serum concentration found in women during the follicular phase of an ovulatory menstrual cycle) increased the expression of both ER subtypes and MMP-2 expression and activity. In contrast, higher E₂ concentration (10⁻⁸ M), a level occasionally observed in women receiving hormone replacement therapy, failed to upregulate either ER or MMP-2 expression and activity. These E₂ effects were ER-mediated, in that they were abolished in the presence of a complete ER-antagonist (ICI).

The explanation for the bimodal dose dependence is likely multifactorial. Estrogens are known to autoregulate ER, and therefore it is not surprising that physiologic concentrations upregulated both ERs and MMP-2. ER subtypes are coexpressed at different levels and mediate different cellular functions. Possibly, dose-dependent differences in activation and expression of ERα and β may induce complex downstream interactions that could result in negative feedback of ER expression. Analysis of ER function in RPE lines developed from ERα and β knockout mice and use of ER subtype specific inhibitors will be...
performed in the future to test the relevance of this mechanism.

Another possibility may involve dose-dependent differential activation of NF-xB pathways by estrogen. Our preliminary data show that PTDC, a well-known inhibitor of the transcription factor NF-xB, suppressed estrogen-stimulated expression and activity of MMP-2, suggesting that NF-xB may be involved in estrogen-mediated regulation of MMP-2 in RPE. In support of these findings, several studies have shown that activity and production of MMP-2 are partly regulated through a NF-xB-dependent pathway.45,46 which may involve Sp1/NF-xB interactions.47 Because estrogen and NF-xB can be mutually antagonistic in some systems, high concentration of estrogen may markedly upregulate NF-xB resulting in the paradoxical inhibition of ER signaling. However, additional experimental validation for the contribution of NF-xB to estrogen-mediated MMP expression is necessary, and this mechanism will be more thoroughly evaluated in future studies.

The capacity of estrogens to regulate production of ECM, especially to modulate the expression and activity of MMP-2, has been observed in some other cell types.18–20 MMP-2 (gelatinase A) has type IV collagenolytic activity but also cleaves type I, V, VII, and XI collagen and laminin.25 Because many of these molecules are part of BrM, altered production or activity of MMP-2 may influence the accumulation of deposits, collagenous thickening, and the biochemical function of BrM. However, the molecular mechanisms by which estrogens regulate MMP-2 transcription and activity in RPE cells are incompletely understood. The human MMP-2 promoter does not have a consensus ERE but contains several other potential cis-acting regulatory elements, including cAMP response element-binding protein (CREB), AP-1, PEA3, C/EBP, P53, Est-1, AP-2, and Sp1 binding sites.48,49 We believe that these observations may have clinical relevance to ARMD. Consistent with our in vitro findings in the current study, we have performed in vivo studies in aged mice by using estrogen depletion and supplementation, producing similar results (Marin-Castaño ME, et al., manuscript submitted). In those studies, estrogen depletion by ovariectomy resulted in diminished production of MMP-2, low-dose estrogen replacement restored normal expression, but high-dose replacement failed to restore normal expression of MMP-2. Loss of MMP-2 and estrogen depletion correlated with increased accumulation of sub-RPE deposit and thickening of BrM in aged mice. High-dose estrogen replacement did not prevent the changes. Taken together, the data suggest that estrogen regulates ECM synthesis and turnover may, in part, explain gender differences in the severity of ARMD. However, although loss of estrogen is detrimental, replacement does not necessarily restore normal regulation, unless a specific physiological concentration is achieved. It is possible that hormone replacement therapy, currently under study in the Women’s Health Initiative clinical trial, may produce contradictory results, depending on the blood concentration achieved among individual women.

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Expression and regulation of estrogen receptors

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