Protective effects of estrogen in a rat model of age-related cataracts

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ABSTRACT   Women have a higher incidence of cataracts, and epidemiologic data suggest that the increased risk may be caused by a lack of estrogen in postmenopausal years. We have examined the effects of estrogen on methylnitrosourea (MNU)-induced cataractogenesis in Sprague–Dawley rats. Animals were ovariectomized, injected with MNU, and treated with estradiol or estrone by a continuous-release, subcutaneous Silastic implant, or they received an empty Silastic implant (no hormone). In the no-hormone group, rats developed opaque lenses approximately 6 months after MNU treatment. By 8 months, 74% (14/19) of the no-hormone rats had evident opacity in one or both eyes by simple gross inspection; 58% (22/38) of the eyes in this group were opaque. Estradiol or estrone treatment reduced the incidence of cataractous eyes to 12% or 25%, respectively. Lenses were examined under a dissecting microscope for light transmission. The lenses of the group treated with no hormone had light transmission of 26% ± 9.2%, whereas lenses from the estradiol-treated animals had light transmission of 72% ± 5.8%. Histological examination revealed that the anterior cortices of the opaque lenses were disrupted and showed the hallmark signs of age-related cataracts; in addition, some eyes that appeared clear by macroscopic examination showed the early histologic signs of cataractogenesis. It was demonstrated with reverse transcription–PCR that lens cells express both α and β types of estrogen receptor, suggesting that the protective effects of the hormones may be a direct, receptor-mediated phenomenon. Thus, the MNU-treated, ovariectomized rat serves as a model for age-related cataractogenesis, and observation of a clear protective effect of estrogens in this system supports the implications of epidemiologic data.

More than 75% of people ≥75 years old have some degree of lens opacification (1), and it is estimated that >50% of blindness is caused by cataracts (2). Age-related cataracts can be classified according to their anatomic location within the lens: cortical, nuclear, posterior, or mixed (3). Women exhibit an increased incidence of cataracts compared with age-matched men (4–7), mainly because of a higher rate of cortical cataracts (8, 9). Thus, age-related cataracts present a significant health problem, one that exhibits a sexual dichotomy.

Epidemiologic evidence suggests that estrogens may protect against cataracts. Although women are at a higher risk of developing cataracts than are men, this increased risk comes after menopause, when estrogen levels have waned (9, 10). In one study of 544 women, early onset of menopause was associated with a 2.9-fold risk of developing cataracts (11). Moreover, the results of three small epidemiologic studies suggest that postmenopausal estrogen replacement therapy reduces the incidence of cataracts (12–14). The role of estro-gen in modifying the onset of age-related cataractogenesis requires suitable experimental models for further study.

Results of animal studies have produced conflicting observations, but overall, they suggest that a lack of estrogen is associated with cataractogenesis. Rats treated with oral contraceptives containing estrogen and progestogen have an increased incidence of cataracts compared with nontreated controls, suggesting that estrogens may actually promote cataract formation (15). However, the effectiveness of estrogen in these treatments is not certain, because the uteri of the treated animals were atrophic (15); progestogen may have produced a predominantly antagonist effect. In another study, long-term treatment with the antiestrogen tamoxifen increased the incidence of cataracts in rats (16). However, tamoxifen exerts both antagonist and agonist estrogen activity in different end organs (17), making conclusions about the mechanism of its cataract-promoting action difficult to draw. A brief report of cataractogenesis in transgenic mice expressing a dominant negative form of the estrogen receptor (ER) supports the notion that inhibition of estrogen action promotes cataractogenesis (18), but the negative dominance of the mutant ER required that it be activated by endogenous estrogen supplied by the ovaries or by treatment of ovariectomized animals with the potent synthetic estrogen diethylstilbestrol. Thus, although these studies point to an association of decreased estrogen action with increased cataractogenesis, they contain caveats that make definitive conclusions difficult.

We report here on a clear protective effect of estrogen in a rat model of accelerated, age-related cataractogenesis. The tumor initiator methylnitrosourea (MNU) causes cataracts to appear 6 to 8 months after a single intravenous injection into outbred rats (19). MNU induces cortical cataracts with many of the hallmarks associated with age-related cataracts in women. We show that a much reduced incidence of cataractogenesis occurs when estrogen is supplied to ovariectomized rats that have been treated with MNU.

METHODS

Animal Treatments. All procedures performed on experimental animals were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine. Sixty female, 49-day-old Sprague–Dawley rats (Harlan, Indianapolis, IN) were received. Within one week, 50 animals were ovariectomized (one group of 10 rats was not ovariectomized) and all were treated while under general anesthesia induced with ketamine. Each animal received a single intravenous injection of 50 mg/kg MNU (Sigma), and a treatment Silastic capsule was placed subcutaneously on the back. MNU was dissolved in PBS and injected intravenously

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through the tail within 15 min of preparing the solution. Silastic capsules containing approximately 20 mg of crystalline estradiol (E2) (20) were applied to 20 ovariectomized animals; likewise capsules containing 20 mg of estrone were implanted into 10 ovariectomized animals. Twenty ovariectomized rats received an empty Silastic capsule; these are referred to as the "no-hormone" treatment group. The 10 ovary-intact animals were anesthetized and injected with MNU. Animals were observed on a weekly basis by simple visual inspection for any gross changes in eye appearance. Animals were carried through 40 weeks after MNU treatment before being killed. Serum estradiol levels were determined with a solid-phase radioimmunoassay as described earlier (21) in 5 animals taken at random from each of the hormone and the no-hormone treatment groups.

Lens Histology. Entire eyes were removed from animals, slit open across the cornea, and immersed in fixative (neutral formalin/ethanol/acetic acid/water, 2:3:1:3) for 2 weeks, as described by Roy *et al.* (19). These eyes were processed and embedded in paraffin. Six-micrometer sections were prepared and stained with hematoxylin and eosin.

Light Transmission Through Lenses. The eyes of seven estradiol-treated, ovariectomized animals and seven no-hormone, ovariectomized animals were extruded and slit open around the cornea, and the lenses were carefully removed. The lens from each eye was placed in a shallow culture dish containing PBS. The dish was placed on the stage of a dissecting microscope with its zoom objective lens set at 1.5×; a charge-coupled device color video camera (Model DXC-960MD, Sony) was attached to one ocular. The lens was viewed with transmitted light and the image was captured by using an imaging program (IPLAB SPECTRUM, Signal Analytics, Vienna, VA) run on a computer (Macintosh Power PC, Apple). A 2-mm-thick piece (1 cm square) of opaque, white Teflon was included in the microscopic field for measurement of zero transmitted light. The intensity of the light (in arbitrary units) transmitted at the center of the lens was measured with the IPLAB SPECTRUM program. Likewise, the intensity of light transmitted through the culture dish to a position just outside the lens was measured and used to define 100% transmission. The units of light intensity measured from the Teflon piece were considered background and used to correct the light transmission measurements made at the lens and outside the lens. The light passing through the lens was calculated as the percentage transmission.

ER Reverse Transcription (RT)-PCR. Lenses were collected from six adult rats. The lenses were immediately frozen in liquid nitrogen, pulverized, and homogenized to extract total RNA by using a kit (RNeasy, Qiagen, Chatsworth, CA). A sample of lens RNA (0.5 μg) was subjected to RT, primed by random hexamer oligonucleotides, and subjected to PCR (Gene Amp RNA PCR, Perkin–Elmer). Aliquots of the RT reaction were used in PCRs for ERα with oligonucleotide primers kgb5 and kgb6, and for ERβ with oligonucleotide primers erbkg1 and erbkg2, as described by Kuiper *et al.* (22); these primer pairs yield amplicons of 344 bp and 262 bp for ERα and ERβ, respectively. As positive controls, 0.5 μg of total RNA from uterus and prostate were subjected to the same RT–PCR procedures. As a negative control, lens RNA that was not subjected to the RT reaction was used in PCR with both primer sets. PCR was carried out over 35 cycles of 95°C for 1 min, 55°C for 45 sec, and 72° for 2 min, followed by 7 min at 72°C for product extension. The products of the RT–PCRs were subjected to electrophoresis through 1.0% agarose and visualized by ethidium bromide fluorescence. The ERα and ERβ amplicons from lenses were ligated into a bacterial plasmid with the TA Cloning System (Invitrogen), and the cloned cDNAs were sequenced and analyzed at the Indiana University School of Medicine Biotechnology Facility to verify their origin.

Statistics. The incidence of gross lens opacities, as identified by visual inspection, was compared by χ^2 analysis (23). The means of light transmission through lenses were compared by *t* test (STATVIEW, Abacus Concepts, Berkeley, CA).

RESULTS

One of the no-hormone, ovariectomized animals died before the end of the experiment and three of the estradiol-treated animals were killed early because of the presence of large tumors: two animals had mammary tumors and one had a salivary gland tumor. At the end of the 8-month experimental period, treatment capsules still contained approximately one-half of the original mass of crystalline steroid. Serum estradiol levels were 23.0 ± 1.24 pg/ml or 12.5 ± 2.53 pg/ml (±SEM) for estradiol-treated or estrone-treated animals, respectively; the no-hormone, ovariectomized animals had serum estradiol levels of 0.72 ± 0.42 pg/ml.

Gross examination first revealed cataracts in the ovariectomized animals approximately 6 months after MNU injection (Fig. 1). Treatment of ovariectomized animals with either estradiol or estrone significantly reduced the incidence of cataracts (Table 1). By 8 months, 74% (14/19) of the no-hormone, ovariectomized animals had cataracts; of the 14 animals with cataracts, 8 had bilateral cataracts. The estradiol-treated group had a significantly lower incidence ($P < 0.01$)
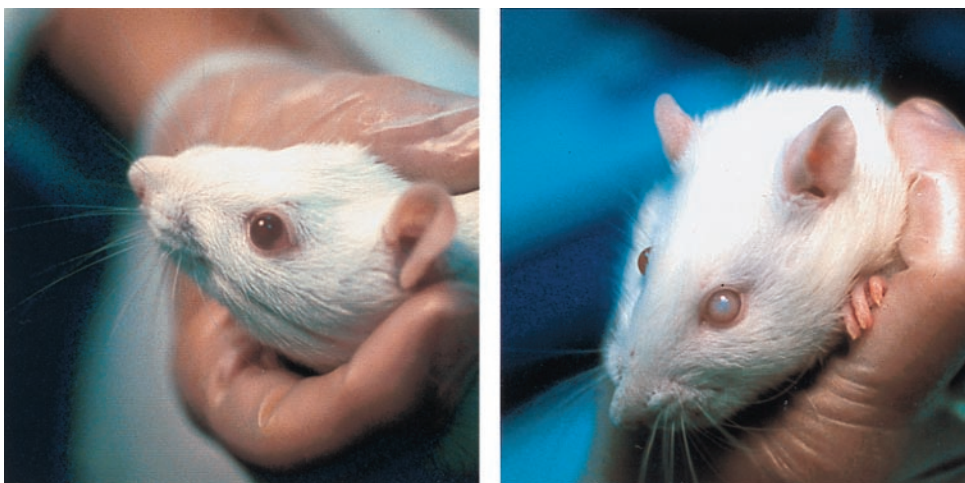

FIG. 1. Gross examination of rat eyes. (*Right*) An ovariectomized animal with one opaque eye. (*Left*) An animal that had been treated with estradiol is devoid of cataracts.

Table 1. Effects of estrogen on the incidence of lens opacities in ovariectomized, MNU-treated rats

Treatment	Incidence of cataracts			
	Animals	%	Eyes	%
No hormone	14/19	74	22/38	58
Estradiol	3/17	18**	4/34	12**
Estrone	4/10	40	5/20	25*

Within a treatment group, the proportion of animals (%) with either one or both eyes opaque, and the proportion of eyes (%) that were opaque are shown. The values for the estradiol-treated group and the estrone-treated group were compared separately against the no-hormone group by χ^2 analysis: *, $P < 0.05$; **, $P < 0.01$.

with 18% (3/17) of animals having obvious cataracts by visual inspection; only 1 animal had bilateral cataracts. Although the percentage of eyes with cataracts decreased with estrone treatment (25% for estrone-treated vs. 58% for control, $P < 0.05$, Table 1), the number of animals with cataracts in the estrone-treated group (4/10) was not statistically different from the controls. Most of the ovary-intact, MNU-treated animals had large mammary tumors and were killed before 5 months after treatment; one of the two intact animals that survived beyond 5 months had cataracts at the time of death.

The degree of lens opacity varied from eye to eye. The amount of light that was transmitted through isolated lenses was measured in eyes from seven control animals and seven estradiol-treated animals chosen at random at the time of death. The range in the level of clarity among lenses is depicted in Fig. 2 *A–E*. When a clear lens is placed over a grid and transilluminated, the underlying grid lines are obvious in the image (Fig. 2*A*); light transmission for such lenses was measured at 96% to 100%. Loss of clarity and light transmission varied widely, as shown in Figs. 2 *B–E*; some lenses, e.g., that seen in Fig. 2*E*, had 0% light transmission. The average light transmission differed between the control and estradiol-treated groups ($P < 0.01$). In the no-hormone group, the 14 lenses examined had percent transmissions over a range of 0% to 91% with an average of 26% ± 9.2% (±SEM). Lenses from the estradiol-treated animals exhibited an average of 72% ± 5.8% transmission (Fig. 2*F*); this latter group included one lens with a 12% light transmission whereas the remainder transmitted light at 51% to 92%.

Histological examination of clear and opaque lenses revealed that the opacities were due mainly to disruption of the cortex. The anterior cortex of the opaque lenses showed various degrees of disruption, with the most severe cases exhibiting balloon cells and liquefaction of the tissue (Fig. 3*B*). A lens taken from an estradiol-treated animal appeared clear on gross examination, but the nucleated fibers in the bow area of the equatorial region showed swelling and had a granular appearance, which are early signs of cataractogenesis (Fig. 3*C*). In the cataractous lens, the epithelium in the equatorial region was hyperplastic, producing a multilayered tissue (Fig. 3*D*).

Expression of ER within lens cells was assessed by RT–PCR analysis. As shown in Fig. 4, mRNAs for both ERα and ERβ were present; slightly more PCR product appears for ERα. No bands were present when the reverse transcriptase step was omitted (not shown), indicating that no genomic DNA contamination of the RNA occurred that would produce a false-positive result. Sequence analysis of the cloned amplicons verified that they represented ERα and ERβ. Similar analysis of the same amounts of RNA from rat prostate and uterus was used to validate the RT–PCR procedure. It is known that the prostate expresses high levels of ERβ and lower levels of ERα, whereas the uterus expresses high levels of ERα and little or no ERβ (22, 24). Our RT–PCR analysis reflected these relative expression levels in the prostate and uterus (Fig. 4).

DISCUSSION

In this report, an animal model of age-related cataractogenesis showed estrogen replacement to be protective. The induction of cataracts in rats by MNU was described in an earlier report but the sex of the animals used in those studies was not indicated (19); however, it is unlikely that females were used because MNU induces hormone-dependent mammary tumors in rats (25). The rate of growth of such tumors in ovary-intact animals would preclude carrying the animals through the 8 months of observation required (ref. 25 and this study). Our histological results indicate that the cataracts that develop in the MNU-treated rat are similar to those that develop in aging animals (26) and in postmenopausal women (27). In both cases, anterior cortical opacification occurs with the histological hallmarks of cellular swelling, balloon cells, vacuolization, and liquefaction. We also found hyperplasia of the equatorial epithelial cells, which has been described in posterior capsular cataracts in humans (28). Thus, the MNU-treated ovariectomized rat appears to be a suitable model of cataractogenesis
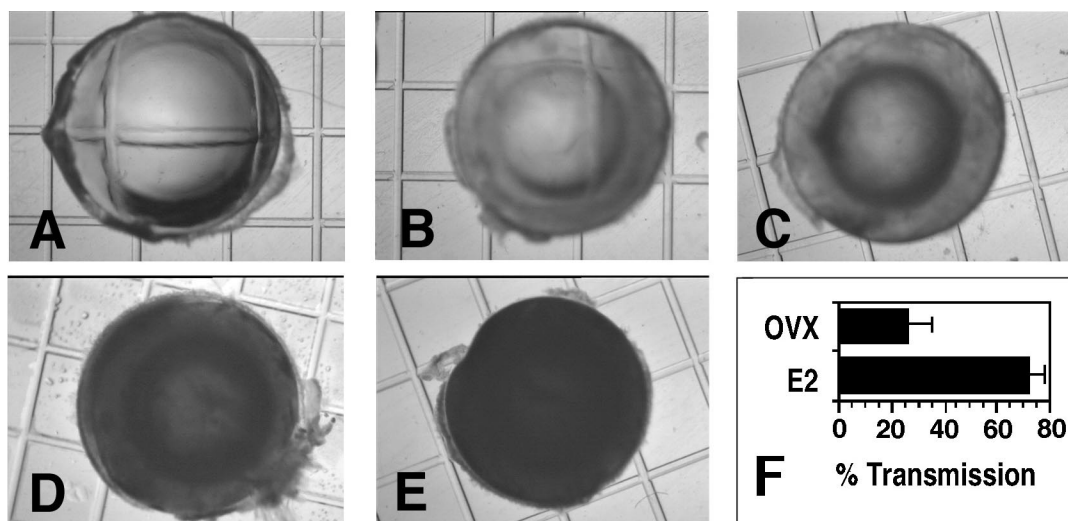

FIG. 2. Transillumination of isolated lenses. Lenses were placed in a culture dish that had a grid etched in its bottom surface. The dish was placed on the stage of a dissecting microscope and viewed with transmitted light. Eyes that appeared clear (*A*) or that had various degrees of opacity (*B–E*) by gross examination showed a gradation of clarity and light transmission. (*F*) The average light transmission of lenses from the no-hormone (OVX) and the estradiol-treated (E2) animals; the means differed when compared by *t* test ($n = 14$; $P < 0.01$; error bars = SEM).
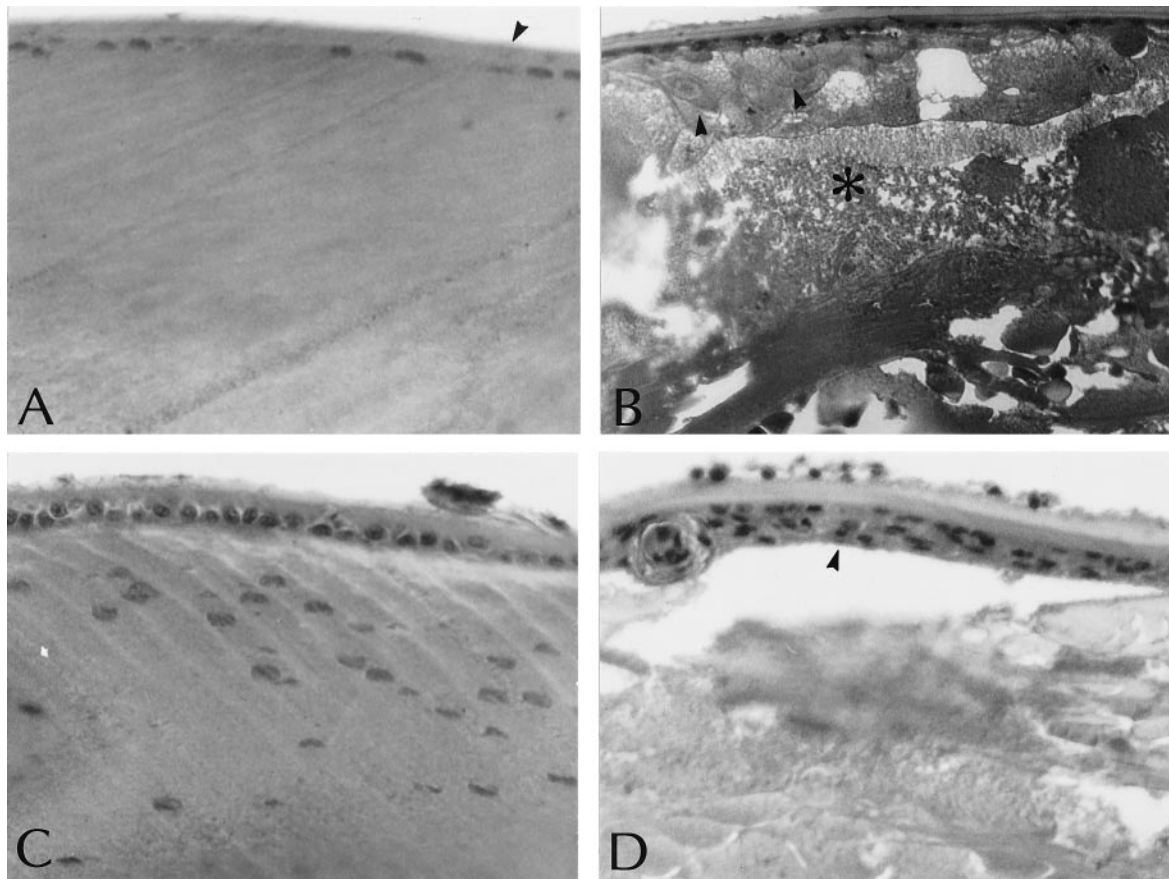
FIG. 3. Histology of lenses from clear or cataractous eyes. (*A* and *C*) Lenses from an estradiol-treated animal. (*B* and *D*) Lenses from a no-hormone animal. The eye of the estradiol-treated animal appears clear on gross examination, whereas the eye of the no-hormone-treated animal is opaque. (*A*) The anterior cortex of the estradiol-treated animal has a homogenous appearance and is covered by a lens capsule made of a thin epithelial cell layer and a normal, thick lens capsule (arrowhead). (*B*) The anterior cortex of the opaque eye is disrupted with the appearance of balloon cells (arrowhead) and areas of complete fiber degeneration and liquefaction (asterisk); the lens capsule is normal in appearance. (*C* and *D*) In the equatorial region, the clear lens (*C*) exhibits swelling of the nucleated fibers in the bow area and the fibers have a slight granular appearance. In the opaque lens (*D*), the fibers in the bow area are disrupted and the lens epithelium is hyperplastic (arrowhead). [Magnification = 250× (*A, C,* and *D*) and 125× (*B*).]

in postmenopausal women and should be useful in determining the mechanisms of the protective effects of estrogen.

As in humans, the incidence of cataracts in rats increases progressively with age, with the majority of lesions occurring after 14 months (26, 29). At 2 years of age, 11% of Sprague–Dawley rats exhibited some form of cataract, but the incidence of anterior cortical lesions was only 0.8% and 2.0% at 14 and 24 months, respectively (29, 30). MNU may enhance the normal aging processes that lead to lens opacification and

FIG. 4. Demonstration of ERα and ERβ expression in the lens by RT–PCR. Samples of total RNA (0.5 μg) extracted from rat lenses (Lens), prostate (Pros), or uterus (Uter) were subjected to RT–PCR for ERα (α) and ERβ (β). The reaction products were electrophoresed through a 1.0% agarose gel and stained with ethidium bromide. The relative intensities of α and β amplicons in the uterus and prostate reflect the known patterns of expression in these organs.

estrogen may have slowed this process. In the present study, the estrogen-treated groups were not completely devoid of cataracts. Microscopic examinations indicated that even the lenses that appeared clear macroscopically did not always fully transmit light, and some showed early histological signs of cataractogenesis. It will be important to carry these studies forward to determine whether the estrogen-treated animals show an increasing incidence of cataracts as they age beyond the 8 months after MNU treatment.

The mechanisms by which MNU induces cataracts are unknown. MNU is rapidly oxidized in a neutral solution and must be applied within minutes of preparation to maintain its carcinogenic effectiveness; it is fully degraded and cleared from the blood within just 15 min (31). MNU is an alkylating agent that produces DNA adducts in tissues within minutes of its administration to rats (32); although some forms of this DNA damage are repaired (33, 34), the DNA adducts may prove to be very persistent in specific tissues (35). MNU is also capable of acting as a methyl-group donor for glutathione and cysteine (36) and thus may produce protein adducts, although such findings have not been described. It has been suggested that alkylating agents are cataractogenic because of interference with cell proliferation (37) or gene expression (38). A determination of the type and persistence of adducts formed in the lens will be important to understanding how MNU induces cataracts.

We can only speculate about the mechanisms involved in the protective effects of estrogen. We have demonstrated that rat
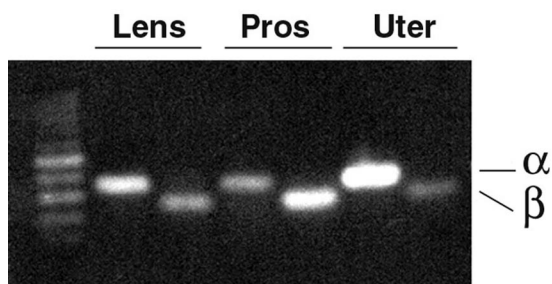
lens cells express both ERα and ERβ, but it is not known whether presence of the receptor is required for the protective effects of the hormone. Although reports have linked use of the antiestrogen tamoxifen with an increased incidence of cataracts (39), a lack of any such association was also reported (40). Experimental studies have shown that tamoxifen (16) and the related antiestrogen clomiphene (41) induce cataracts in rats. Zhang and coworkers (42) have shown that tamoxifen can induce lens opacification during short-term incubation in explant culture of lenses. These observations suggest that the ER does play a role in protection against cataractogenesis. However, the *in vitro* studies of Zhang *et al.* (43) suggest that the cataractogenic effect of tamoxifen is not mediated by the ER, but rather involves its ability to block chloride channels, thereby causing excess hydration of lens fibers (44).

On the other hand, Katzenellenbogen and coworkers have found that the ER enhances transcription of the gene for a Na^+/H^+ exchanger regulatory factor (45); in this way, estrogen may aid in the maintenance of proper ionic composition and cell hydration through a genomic effect. And finally, it is well established that antioxidants are protective in experimental cataractogenesis (for review, see ref. 46). Estrogens can behave as antioxidants either through a purely chemical, nongenomic mechanism (47) or through enhanced transcription of genes such as quinone reductase (48). However, the antioxidant hypothesis of the protective effect of estrogen is incongruent with the observation that antiestrogens, which are also chemical antioxidants (49), induce cataracts (16, 41). In addition, chemical antioxidant effects are likely to require high concentrations of steroid, but the serum levels achieved by the hormone treatments were in a physiologic range. Further study is required to determine whether genomic or nongenomic mechanisms mediate the protective effects of estrogen in cataractogenesis.

In summary, we have shown that estrogens are protective in an animal model of cataractogenesis. These studies provide strong experimental evidence supporting the suggestion, derived from recent epidemiologic studies (12–14), that postmenopausal hormone replacement therapy may inhibit cataractogenesis in women. This protective effect in the lens is one more benefit in a range of advantages, spanning maintenance of bone mineral density to protection against cardiovascular disease (50, 51), all of which might be attributed to estrogen therapy.

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