Rapid action of oestradiol against hydrogen peroxide-induced oxidative stress in cataractous lens epithelium: an *in vitro* study

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Abstract

Purpose To evaluate the short-term protective effects of oestradiol against damages because of oxidative stress in human lens epithelial cells (LECs).

Methods The central zone of human lens epithelium was obtained from the cataract surgery and cultured in MEM culture medium. These cultured LECs were treated with 17β -oestradiol for varying time intervals from 1 to 5 min followed by treatment with H_2O_2 (5×10^{-6} M) in the culture medium. Catalase activity was measured to access the oxidative stress levels.

Results LECs exposed to H_2O_2 (5×10^{-6} M) showed a fourfold increase in catalase activity (407.03 ± 89.11 U/ μ g protein) after 6 h when compared to cultured unexposed LECs (97.124 ± 9.4 U/ μ g protein). When the cultured LECs were treated with oestradiol (5×10^{-8} M) before H_2O_2 treatment, the increase in catalase activity was inhibited, whereas simultaneous and post-treatments showed no effect. The catalase activity of LECs pretreated with oestradiol for 1, 2, 3, and 5 min was 259.92 ± 18.37 , 200.24 ± 14.39 , 140.50 ± 19.83 , and 110.01 ± 14.66 , respectively ($P < 0.0001$).

Conclusion Antioxidative enzymes are synthesized in response to the oxidative stress signal. Upon treatment with oestrogen catalase is not synthesized. The pretreatment time of oestrogen required for its antioxidative effect can be seen within 5 min indicating non-genomic mode of action of oestrogen. *Eye* (2009) 23, 1456–1463; doi:10.1038/eye.2008.284; published online 19 September 2008

Keywords: cataract; 17β oestradiol; oxidative stress; catalase; lens epithelial cells (LECs)

Introduction

The first indication of the eye as a target organ for sex steroid hormones emerged from epidemiological studies that indicated an increased incidence of ocular disorders in women.¹ Various epidemiological studies suggest that women are at a higher risk of developing cataracts than men of the same age group. This risk increases only after menopause, thus leading to the hypothesis that oestrogen is the protective molecule during the reproductive age. As far as the cumulative incidence of cataract is concerned, women are more likely than men to have nuclear cataract.² Prevalence data from the Barbados Eye Study, which had a predominantly black population, demonstrated that cortical and nuclear opacities are more frequent in women than in men.³ Other studies on the beneficial effects of oestrogen, such as the Beaver Dam Eye Study and the Salisbury Eye Evaluation Project found protective associations between hormone use and lens nuclear opacity. Weintraub *et al*⁴ evaluated hormone replacement therapy and lens opacities in a population of 480 postmenopausal women and reported a 49% decreased risk of nuclear opacities in oestrogen users. Studies using tissue culture and animal models also suggest the beneficial effects of oestrogen in lens. Oestrogen protects the lens against TGF- β -induced cataract in the lens culture system.⁵ Oestrogen has also been reported to exert protective effects in the rat model of age-related cataracts induced by methyl nitrosourea (MNU).⁶

Oxidative stress is widely recognized as one of the major causes of cataract. An elevated level of hydrogen peroxide H_2O_2 in the aqueous

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humour and whole lenses has been associated with cataractogenesis. Oxidative stress causes profound injury to a number of intracellular macromolecules in eukaryotic cells, including lipid peroxidation, protein alterations, breakage of covalent bonds of carbohydrates, and cleavage of DNA. The conventional intracellular antioxidant enzymes are overburdened under the oxidative insult. The protection provided by oestrogen against oxidative stress in neuronal tissues has been well documented.⁷ The antioxidative property of oestrogen has been attributed to the phenolic ring, which binds to H₂O₂.⁸ The precise mechanism of these cytoprotective effects of oestrogen against H₂O₂ in lens epithelial cells (LECs) is not known, but a plethora of cellular responses to the steroid has been reported, including the protection of the mitochondrial function, stimulation of antiapoptotic proteins, and stimulation of protective signalling pathways.^{9–11} Wang *et al*¹² have demonstrated that oestrogens are potent cytoprotectants that preserve the mitochondrial function during H₂O₂-induced oxidant insult. Nevertheless, the exact mechanism of oestrogen action against oxidative stress is not known. Moreover, it is not clear how oestrogen modulates the antioxidative defense system in response to the oxidative insult, and the precise time for its antioxidative action in the visual system is not known.

In this study, we tested the interim protective effects of oestrogens against oxidative stress using *in vitro* human cultured LECs. The ability of oestrogen to modulate the adverse effects of H₂O₂ by stopping the cells sensing the stress signal is elucidated in this study.

Materials and methods

The research followed the tenets of the declaration of Helsinki and the institutional ethical review board.

Chemicals and biochemicals

All the routinely used chemicals, including culture media, were obtained from Sigma (St Louis, MO, USA). Fetal calf serum was obtained from Hyclone Laboratories (UT, USA). H₂O₂ and tetraethoxypropane was obtained from Merck India Ltd. (Mumbai, India). All chemicals were of reagent grade quality.

Primary culture of human LECs

Circular pieces of cataractous human anterior lens capsule (rhexis), about 6 mm in diameter, were obtained postoperatively in sterile normal saline. The cataract type was recorded using the slit lamp according to the zone of opacification. The grading of nuclei based on the degree of hardness was performed according to the

Emery–Little classification. All the anterior capsules belonged to a mixed cataract type (nuclear + cortical), with 2–3 grades from male or female patients of the same age group (50–65 years).

A single rhexis was placed in Eagle's minimal essential medium (MEM) containing 10% fetal calf serum in a single well of 24-well plate and incubated in a 5% CO₂ incubator at 37°C. The rhexis was allowed to attach itself to the wall of the plate followed by acclimatization of the LECs for 48 h in the culture medium. This primary culture of LECs was used for all further experiments. Each anterior capsule was used for development of one primary culture for 48 h after which cells were given respective treatments with H₂O₂ or oestrogen as mentioned. The biochemical property of the LECs in culture was analysed by measuring the catalase activity at various time periods as described later. A total of 188 anterior capsules were used for the development of primary cultures for this study.

H₂O₂ treatment

The LECs in the primary culture were exposed to oxidative stress conditions by the addition of H₂O₂ to a final concentration of 5 μ M in the culture medium. In the preliminary experiments, concentrations of 50 and 100 μ M H₂O₂ were also used to decide the toxic and subtoxic doses of H₂O₂. Catalase was measured to study the effect of H₂O₂ on the LECs at various points of time. The H₂O₂ concentration of 5 μ M was found to induce apoptosis and minimum necrosis, and hence was used for all further experiments.

Oestradiol treatment

A total of 17 β -oestradiol was added at a concentration of 5 $\times$ 10^{–8} M to the culture medium either before, during or after H₂O₂ treatment at various points of time as mentioned. After each treatment, the catalase and peroxidase activity, viable count, lipid peroxidation, total antioxidant status TAS, and DNA damage in the LECs were determined as described later. Morphological changes were observed under an inverted microscope at $\times$ 40 magnification.

The trypan blue exclusion method for cell viability

The viable count of cells was carried out through the vital staining method using trypan blue stain. Trypan blue is a dye that cannot enter cells through an intact membrane, and therefore stains only those cells that are undergoing membrane disruption. The cells were then washed twice with PBS and stained with 0.025% trypan blue in PBS for 3 min. Blue cells were then counted and these counts

were used to calculate the relative proportion of the dead cells.

Catalase assay

Catalase activity was assayed using Luck's method.¹³ The enzyme extract was prepared by homogenizing the cells in a lysis buffer (0.25 M sucrose, 20 mM tris-HCl, 100 mM KCl, 40 mM NaCl, and 10 mM MgCl₂), centrifuging them at 20 000 g for 30 min at 4°C, and re-extracting the pellet in a microsomal dilution buffer (0.1 M KH₂PO₄, 20% glycerol, 10 mM EDTA, and 0.1 mM β-mercaptoethanol). The pooled extract was added to 10 mM H₂O₂, and the rate of decomposition of H₂O₂ was measured spectrophotometrically at 240 nm. Decomposition of 1 μM H₂O₂ in 1 min corresponds to 1 U of catalase.

Lipid peroxidation

The extent of lipid peroxidation was examined by the thiobarbituric acid-reactive substances, such as malondialdehyde TBARS assay, as described by Fujiwara *et al.*¹⁴ Cells were homogenized in distilled water and centrifuged at 12 000 g, at 4°C for 30 min. Aliquots of this homogenate or standards (1 mM tetraethoxypropane in 0.01 N HCl) were combined with 1 ml 29 nM 2thiobarbituric acid (in 8.75 M acetic acid) followed by placing in a water bath at 95°C for 1 h and cooling under a water tap. A unit of 5 μl of 5 M HCl and 3.5 ml butanol were added to the aliquots, after which the tubes were agitated for 5 min and centrifuged in a table top centrifuge to facilitate separation of layers. The upper butanol phase was then removed and the absorbance was quantified at 525 nm. The TBARS or malondialdehyde levels were calculated from the standard graph and expressed as nmol/mg of protein.

Total antioxidant activity assay

The ABTS-H₂O₂-HRP assay system as described by Chen *et al.*¹⁵ was used to determine the antioxidant status. The ABTS-H₂O₂-HRP assay system measures the total antioxidant activity. In principle, when ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) is incubated with peroxidase and H₂O₂, the relatively long-lived cation ABTS⁺ is formed which is green in colour. In the presence of antioxidant reductant and hydrogen donors, the radical cation is quenched and green colour formation is delayed. This lag time is proportional to the total antioxidant activity in the sample. The wet weight of the rhexis was taken using a mettler weighing machine followed by homogenization in 6% orthophosphoric acid with 1 μM EDTA and centrifugation at 4°C, 12 000 g. The

working solution contained 2.0 mM ABTS and 0.86 nM horseradish peroxidase HRP in 50 mM phosphate buffer pH7.0. In the cuvette, 240 μl of working solution, 10 μl of sample homogenate, and finally 25 μl of 1 mM H₂O₂ in 50 mM phosphate buffer was added to initiate the reaction, and readings were taken immediately at 414 nm. The time taken for the formation of the green ABTS radical formation was calculated. A standard curve was plotted with different concentrations of L-ascorbic acid and the measurement expressed in mg L-ascorbic acid equivalent to per microgram of tissue.

Comet assay for DNA damage

After the respective treatments, the cells were harvested and washed with PBS and proceeded for comet assay. The comet assay was performed as described by Singh *et al.*¹⁶ Slides were prepared by coating two layers of 1% high melting point agarose and two layers of low melting point agarose of which one contained cells. The cells were lysed by placing the slides in an alkaline buffer followed by electrophoresis in the same buffer for 20 min. After neutralization, in 0.4 M Tris base pH7.5, they were stained with 0.01% Yo-Pro for 5 min and observed under a florescent microscope. The percentage of cells with comets was calculated. On the basis of their tail lengths, the comets were classified as: score 0, healthy undamaged cells with absence of tail (Normal); score 1, small comet 26–50 μM; score 2, medium comet 51–75 μM; and score 3, large comet >76 μM.

Protein estimation

Protein estimation was performed by the eosin Y method as described by Waheed and Gupta.¹⁷

Statistical analysis

Statistical analysis was performed using the Kruskal–Wallis (ANOVA) test. The difference between the groups was found by Bonferroni contrast.

Results

Effect of H₂O₂ on LECs

After the capsule was placed in the culture medium, it was allowed to get acclimatized for 48 h, after which it was attached to the plate; cells with distinct nuclei were seen. The acclimatization period of 48 h was standardized by measuring the catalase activity at regular time points after placing the rhexis in the media. After placing the cataractous epithelium in the culture medium, a continuous decrease was observed till 48 h followed by no further decrease in activity until 72 h (data not shown). Morphologically, after 48 h of primary

culture, the capsule was attached to the plate and cells were seen with distinct nucleus with stable catalase activity, and hence used for further experiments.

The exposure of the LECs to H₂O₂ for various points of time caused an increase in the catalase activity (Figure 1). Within 6 h of administration, 5 μ M H₂O₂ initiated a linear increase in catalase activity in the control cells. A sharp decrease in catalase activity was recorded between the

Figure 1 Effect of H₂O₂ on catalase activity in cultured LECs. Time course of the effects of H₂O₂ on catalase activity in LECs. Increase in catalase activity was seen after 6 h of H₂O₂ administration. Data were computed after three experiments and expressed as mean $\pm$ SD. When SD bars are not shown, they are obscured by the symbol.

8 and 10 h of H₂O₂ administration, after which no further decrease was observed for 24 h.

Effect of 17 β -oestradiol

To test whether 17 β -oestradiol protects the cells against the H₂O₂ effect, the LECs were treated with 5×10^{-9} M 17 β -oestradiol before, simultaneously, and after the H₂O₂ exposure. As seen in Figure 2, no increase in catalase activity was observed when the LECs were given 3 min of oestradiol treatment before H₂O₂ exposure. However, simultaneous and post-treatment of oestradiol followed by H₂O₂ treatment showed high catalase activity. The 3 min preincubation period with oestradiol 5×10^{-9} was decided on after experimenting with a series of time periods (Figure 3). When the LECs were treated for 1 or 2 min, there was a slight increase in catalase activity, but the difference was not statistically significant (P -value = 1) when compared to the H₂O₂ treated group, whereas treatment for 3 min and more showed no increase in catalase activity in H₂O₂-treated cells and was statistically significant. A slight increase in catalase activity was seen when the LECs were treated only with 17 β -oestradiol; however, the increase was not statistically significant when compared to the cultured LECs. Thus, when the LECs were pretreated with oestradiol for 3 min, the oestradiol was found to nullify the effect of H₂O₂. Subsequently, the effectiveness of oestradiol on other stress parameters like viability, lipid peroxidation, total antioxidant status TAS, and DNA damage were

Figure 2 Effect of 17 β -oestradiol against H₂O₂ exposure for 6 h in cultured LECs. Catalase activity was measured in the LECs after three sets of treatment viz oestradiol pretreatment for 3 min of oestradiol pre, simultaneous treatment H₂O₂ + oestradiol, and post-treatment (oestradiol post) along with H₂O₂ exposure. The three sets of control were primary cultured LECs without any treatment, only oestradiol-treated cultured LECs, and only H₂O₂-treated LECs. Of the oestradiol treated sets, only pretreated LECs showed no increase in catalase activity, whereas both the other treatments showed high catalase activity after H₂O₂ exposure. The data represented are the average of the three experiments and expressed as Mean $\pm$ SD, * P < 0.005, significantly different from only H₂O₂ treatment.

Figure 3 Catalase activity in LECs pretreated with 17β-oestradiol for various time periods followed by H₂O₂ exposure for 6 h. Three-min pretreatment and more showed no increase in catalase activity. Data shown are the average of three experiments. #*P* = 1, **P* < 0.01, ***P* < 0.001, significantly different from only H₂O₂ treatment.

Table 1 Catalase activity, lipid peroxidation, TAS, and cell viability after pretreatment with 17β-oestradiol for 3 min followed by H₂O₂ treatment

Treatments	Control ^a	Control + H ₂ O ₂	Control + oestradiol	Control + oestradiol + H ₂ O ₂
Catalase (U/μg of protein)	97.124 ± 9.4	407.03 ± 89.11	198.00 ± 83.64	120.32 ± 43.52
Lipid peroxidation (nm/mg protein)	4.65 ± 1.37	11.73 ± 3.60	9.66 ± 3.94	11.66 ± 3.48
Total antioxidant status (μg/mg wet weight of sample)	0.17 ± 0.07	0.35 ± 0.05	0.13 ± 0.06	0.12 ± 0.07
Viability %	84.27 ± 6.5	46.85 ± 5.5	70.31 ± 1.5	67.32 ± 5.01

Values represent *n* = 5 mean ± SEM.

^aPrimary cultured LECs for 48 h.

evaluated. Oestradiol prevented LEC loss due to H₂O₂ toxicity. H₂O₂ treatment of LECs resulted in a 55.59% decline in cell survival. A 3 min preincubation period with oestradiol before H₂O₂ treatment increased the cell survival to 79.88% (Table 1). The TAS of LECs treated with oestradiol before the H₂O₂ treatment was two times lower than those treated only with H₂O₂. However, oestradiol did not seem to alter lipid peroxidation in LECs as compared to the control LECs. To determine the effect of oestradiol on DNA damage, a comet assay was performed. Figure 4 demonstrates the highest percentage of DNA-damaged cells in H₂O₂-treated LECs, whereas the lowest number of the same was seen in oestradiol-treated cultured LECs.

Discussion

The indication of the eye as a target organ for gender-specific hormones has been well documented over the years.^{1,18} The protective role of oestrogen in cataractogenesis has been well studied in *in vivo* and *in vitro* conditions.^{5,6,12,15} This study elucidates the quick

protective role of 17β-oestradiol against H₂O₂-induced oxidative stress in cultured LECs.

Oxidative insult to the LECs is generated by the elevated H₂O₂ concentrations in the aqueous humour during cataractogenesis, and to cope with this oxidative stress, the enzyme activities get elevated as evidenced by the high catalase activity in cultured LECs exposed to H₂O₂ as compared to only cultured LECs in this study.

The effect of H₂O₂ on cultured human LECs (HLEB3 cell line) was studied by Xing and Lou.¹⁹ The authors observed no H₂O₂-induced DNA damage in cells exposed either to 0.5 mM for 3 h or 0.05 mM for 72 h, and upon enzymatic analysis, GPx showed 80% loss in activity. On the contrary, Kleiman *et al*²⁰ found that H₂O₂ at concentration as low as 25 μM induced DNA damage within 5 min in bovine LECs. In this study, low concentration (5 μM) of H₂O₂ also caused apoptosis and induced catalase activity. The discrepancy in the results in all these studies are difficult to explain other than perhaps that the cells come from different origin (*viz*, primary bovine cells and human transfected cell lines as against primary culture of cataractous LECs used in this study) and in different conditions. Moreover, most of the

Figure 4 Effect of oestradiol pretreatment on DNA damage caused by H₂O₂ in the LECs. LECs were treated with oestradiol for 3 min followed by H₂O₂ treatment for 6 h and they were processed for the comet assay and scored for various classes of DNA damage. Three hundred cells were scored for each treatment and the percentage of damaged cells was calculated. Score 0 represents healthy undamaged cells and scores 1, 2 and 3 represent small, medium, and large comets, respectively. The highest number of DNA damaged cells was found in H₂O₂-treated LECs whereas, oestradiol-treated LECs showed the highest number of healthy cells.

authors used confluent cultures (0.8 million), whereas approximately only 5000 cells per well were available for our study. Concentration-dependent effects of H₂O₂ on cultured rabbit LECs were studied by Ohguro *et al.*²¹ Low (1 μ M) dose of H₂O₂ led to proliferation, whereas high (100 μ M) dose killed the LECs. They showed that the survival of cells is greatly dependent on the initial number of cells seeded in the culture plate. With an initial low (<5000 cells per well), the cells were not able to survive (100 μ M) H₂O₂. Our results corroborate with the above finding as the number of cells per well in this study—the number of cells per well is approximately 5000—and 100 μ M H₂O₂ is lethal to LECs in our culture conditions (data not shown). Catalase was shown to be induced within 1 h of 50 μ M H₂O₂ treatment by Goswami *et al.*²² In this study, the time of induction for catalase is higher, probably because of the less concentration of H₂O₂ used in our study.

The increase in catalase activity upon H₂O₂ treatment was prevented by treating the LECs with 17 β -oestradiol for 3 min before adding H₂O₂. Oestradiol pretreatment was found to prevent the detrimental effect of H₂O₂ as it was found to preserve cell viability, TAS, and DNA damage in LECs. Of the three treatments, only pretreatment with oestradiol was protective against H₂O₂. LECs, when pretreated with oestradiol for 1 and 2 min, showed little protection against H₂O₂ as evidenced by the slight increase in catalase activity. However, a pretreatment with 3 min showed complete inhibition of the increase in catalase activity even in the presence of H₂O₂. This short period of oestradiol treatment was sufficient for the oestradiol molecule to penetrate the target cell. Simultaneous application was not protective, probably because the effect of the peroxide molecule is very spontaneous and it takes very little time to diffuse

rapidly through the cell barriers.²³ Steroid hormones are known to exert their action by classical genomic as well as rapid non-genomic pathways. In genomic mode of action, effect of oestrogen on target tissues are mediated through hormone responsive elements and the entire process requires a few hours to few days,²⁴ whereas in non-genomic mode, the action is fast and short lived, and thus does not activate transcription or translation. Oestrogen has been reported to induce instant actions in various tissues.²⁵ In this study, it may be possible that the effect of oestradiol is non-genomic as it requires only 3 min to be protective, but only on the target cells as it does not protect the LECs, when added along with H₂O₂. Oestradiol has been previously reported to preserve the mitochondrial function, cell viability, and intracellular ATP levels in cultured human LECs during oxidative stress.^{12,26} Emerging published data suggest that these lipophilic hormones are also able to produce rapid effects within several seconds, which cannot be adequately explained through the classical mechanism. These rapid responses have been observed in several tissues, such as myometrial cells, neurons, endothelium, osteoblasts, granulosa cells, and some breast cancer cell lines.²⁷

The activities of superoxide dismutase, catalase, and glutathione peroxidase have been shown to be regulated by hormones, and oestradiol has been shown to increase catalase activity in macrophages.²⁸ In this study, when cultured LECs were treated with oestradiol alone in the absence of H₂O₂, a slight increase in catalase activity were observed. The results probably suggest that in presence of H₂O₂ oestradiol first functions as antioxidant and involved in binding to H₂O₂. In simultaneous application of both oestradiol and H₂O₂, probably an additive effect is seen.

The antioxidant nature of oestradiol has been well documented in neuronal cells.^{29,30} Han *et al*³¹ demonstrated that the protective potency of various oestrogens was dependent on the precise structure and 17 β -oestradiol, a phenolic ring oestrogen, acted in a way similar to the antioxidants taurine and vitamin C. Studies by Wang *et al*¹² using the oestrogen antagonist, ICI 182780, conclude the non-involvement of oestrogen receptors in the cytoprotective effects of oestrogen. Cytoprotection by oestradiol with only 3 min of pretreatment has been elucidated for the first time in our study.

Oestrogens have been shown to be powerful antioxidants, effectively preventing lipid peroxidation.^{32,33} However, in our data, oestradiol has no effect on the lipid peroxidation for some unknown reason. The normal physiological level of oestrogen is 0.5 ng/ml. The levels that we used were slightly above this range that is, 5×10^{-8} M (13.6 ng/ml) but within safe limits, and would not cause any harmful side effects. In most of the *in vitro* studies of lipid peroxidation oestradiol concentration of 54 μ M, hundreds of fold higher than normal plasma levels has been used.^{34,35} In a study by Santanam *et al*,³⁶ the authors support the conclusion that at physiologic concentrations it is unlikely that oestradiol could reduce LDL oxidation. The association of oestradiol with mitochondria has been recently confirmed by Camarata *et al*,⁹ with oestrogen receptors being shown on the mitochondrial membrane. The rapid and non-genomic action of oestrogen against acute oxidative stress has been recently shown by Moor *et al*,¹¹ wherein oestradiol activates an important antiapoptotic pathway within 15 min of administration. The protective effect of oestradiol mediated by preventing the LECs from undergoing apoptosis is evident from the comet analysis in this study. The stimulation of antiapoptotic proteins by oestrogen has been reported previously.³⁷ This study elucidates the rapid protective role of oestradiol against H₂O₂-induced stress. The protective function of oestradiol works by preventing the cells from undergoing cell death. Oestradiol regulates the AOD enzymes indirectly by neutralizing H₂O₂, and thereby providing the cells with a stress-free atmosphere. Thus, the cells do not sense the stress condition with just 3 min of pretreatment with oestradiol and do not induce antioxidative enzymes. Further studies are in progress to evaluate the antioxidative properties of oestradiol.

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