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Neuroactive steroids protect retinal pigment epithelium against oxidative stress

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Abstract

This study was undertaken to assess whether neuroactive steroids, 17 β -estradiol and dehydroepiandrosterone-sulfate, enhance survival and protect DNA of human retinal pigment epithelial cells challenged by oxidative stress, and to investigate the role of σ_1 receptors in the effects of neuroactive steroids. Retinal pigment epithelial cells were treated with various concentrations of neuroactive steroids and then exposed to hydrogen peroxide. Pretreatment with steroids resulted in significant increased viability in a dose-related manner. DNA damage induced by oxidative insult was significantly lower with steroid pretreatment. The effects of 17 β -estradiol and dehydroepiandrosterone-sulfate were antagonized by pretreatment with a σ_1 receptor antagonist. The results suggest that neuroactive steroids protect retinal cells from oxidative stress, and that this effect is mediated by σ_1 receptors.

Keywords

Neuroactive steroids; Oxidative stress; Retinal pigment epithelial cells; σ_1 receptors

INTRODUCTION

Retinal pigment epithelium is a single layer of cells situated between the photoreceptors of neural retina and the choriocapillaris and choroid. Retinal pigment epithelium provides many important functions essential to the visual process. Defects in the retinal pigment epithelium contribute to initiation and progression of age-related macular degeneration in humans. Age-related macular degeneration is the leading cause of vision loss and blindness in individuals over the age of 65 in the developed world, and no pharmacological treatment is available. Age-related macular degeneration is characterized by the loss of central vision that arises from altered retinal function in the macula, the central portion of the retina. Larger areas of retinal pigment epithelium degeneration in the center of the macula result in vision loss. It has been demonstrated that phagocytosis of photoreceptor outer segments by retinal pigment epithelium causes an increase in intracellular and extracellular hydrogen peroxide generation [1,2]. Cytotoxic levels of hydrogen peroxide can cause significant DNA damage and cell death in human retinal pigment epithelial cells [3–5]. The term ‘neuroactive steroids’ has been adopted for steroids, including 17 β -estradiol and dehydroepiandrosterone-sulfate (DHEA-S), that might alter neuronal excitability via the cell surface through interaction with specific neurotransmitter receptors. It has been shown that 17 β -estradiol protects neurons (mouse hippocampal cell line HT22) from oxidative stress-induced cell death *in vitro* by hydrogen

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peroxide [6]. Recent studies [7] have indicated a neuroprotective effect of estrogen on retinal neurons *in vitro* against oxidative stress. Furthermore, it has been demonstrated that DHEA-S protects rat primary hippocampal neurons against the toxicity induced by hydrogen peroxide [8]. The mechanism or mechanisms by which neuroactive steroids exert these protective effects remain unclear. Direct interaction between neuroactive steroids and σ_1 receptors has been hypothesized from the evidence that several steroids inhibit the binding of σ_1 -receptor radioligands *in vitro* and *in vivo* [9]. Recently, it has been shown [10] that administration of 17 β -estradiol attenuates retinal ischemia-reperfusion injury in rats. More recently, it has been demonstrated [11] that the protective effect of 17 β -estradiol and DHEA-S on ischemia/reperfusion injury in the rat retina is mediated, at least in part, by activation of σ_1 receptors. The term 'sigma' (σ) is used to refer to a unique class of nonopioid, nonphencyclidine-binding sites heterogeneously distributed in the nervous system and in peripheral organs that may serve as receptors for any, as yet unidentified, endogenous ligand [12,13]. According to biochemical and radioligand-binding data, σ recognition sites have been classified into at least two types, termed σ_1 and σ_2 [12]. A σ_1 -binding protein was cloned [14], and its sequence shows significant similarities to sterol C₈-C₇ isomerases from fungi. Recently, the presence of σ_1 receptors in rat Müller cells, rat ganglion cells and human retinal pigment epithelial cells has been demonstrated [15]. The functional role of σ recognition sites has not yet been clearly determined. The present study was designed to determine whether 17 β -estradiol and DHEA-S enhance cell survival and protect DNA of human retinal pigment epithelial cells challenged by oxidative stress, and to investigate the role of σ_1 receptors in the effects of neuroactive steroids.

MATERIALS AND METHODS

Drugs:

The compound *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino) ethylamine (BD1047) was purchased from Tocris (Avonmouth, UK) and all other compounds from Sigma (St Louis, Missouri, USA).

Cell cultures:

The human retinal pigment epithelial cell line, ARPE-19, and primary cultures of adult human retinal pigment epithelium were used in this study. ARPE-19 cells were obtained from American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, New York, USA)/F12 containing 10% heat-inactivated fetal bovine serum (FBS; Gibco) and 2.5 mM glutamine. Human primary retinal pigment epithelial cells were obtained from eye bank donor eyes. The eye was cut across the posterior pole, and the vitreous and neural retina were removed. The remaining eyecup was washed with phosphate-buffered saline (PBS; Gibco) and 0.025% trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco) was added to the eyecup and incubated at 37°C in a humidified chamber. The cells were then gently scraped and seeded in DMEM containing 15% FBS in a 100 × 20 mm culture dish. These primary cultures of retinal pigment epithelial cells were grown in DMEM containing 15% FBS in a 5% CO₂ environment at 37°C. After checking for attachment, the medium was replaced with the same medium every other day. The human retinal pigment epithelial cells used in this study were from passage numbers 2 and 3, and each experiment on the cells was performed in quadruplicate.

DNA single-cell strand breakage assay:

DNA strand breaks were assessed after exposure to 50 μ M H₂O₂ by single-cell gel electrophoresis as described by Singh *et al.* [16] with minor modifications. Cells treated with H₂O₂ were harvested and mixed with 0.8% low-melting-temperature agarose (Sigma) at 37°C. They were then placed onto a frosted microscope slide that was already covered with a thin

layer of 0.8% normal melting agarose (Sigma) to promote adhesion of the second layer. The slides were covered with a coverslip and kept at 4°C for 5 min. After removing the coverslip, the slides were covered with a second layer of 0.8% low-melting agarose containing the sample cells. To protect the cells, this layer was covered with another layer of 0.8% normal-melting agarose and then covered with a coverslip and kept at 4°C for 5 min. The coverslip was removed, and the cells were incubated for 1 h in the dark with freshly prepared lysing solution [1% *N*-lauroylsarcosine sodium, 2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10.0) and 1% Triton X-100; Sigma]. The sample slides were placed into electrophoresis buffer (1 mM EDTA with 300 mM NaOH; Sigma) for 20 min at 4°C in the dark, and then electrophoresed with 17 V for 20 min at 4°C in the dark. These slides were put into 0.4 M Tris at pH 7.5 for 5 min to neutralize the NaOH. After staining with 20 µg/ml ethidium bromide (Sigma), the sample gel was covered with a coverslip and photographed on 35-mm film at 200 × magnification with a fluorescence microscope (VANOX-S; Olympus, Lake Success, New York, USA) and enlarged to 5 × 7 in. prints to measure the comet tails. DNA migration length is directly proportional to damage rates.

Cytotoxicity assay:

Cell viability was determined by spectroscopic measurement of the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT). ARPE-19 cells were plated in 96-well plates and after confirming cell attachment, the DMEM containing 10% fetal calf serum was replaced by serum-free DMEM containing the testing agents (prepared in serum-free medium) and cultured for 24 h. The next day, the cells were preincubated for 2 h with various concentrations (1–100 µM) of 17β-estradiol or DHEA-S, and then exposed to H₂O₂ (400 µM) for 4 h. In another series, the cells were pretreated (10 min) with BD1047 (100 µM), then treated with the neuroactive steroids at the highest dose (100 µM) and then exposed to H₂O₂. In another series, the cells were pretreated (10 min) with neuroactive steroids at the highest dose (100 µM), then treated with BD1047 (100 µM) and then exposed to H₂O₂. In a different set of experiments, the cells were preincubated (2 h) with different concentrations (1–100 µM) of BD1047. The culture media were discarded after the incubation period, and the cells were washed three times with PBS and each well received 150 µl of an MTT solution in serum-free medium. The plates were incubated for 2 h at 37°C; after discarding the MTT solution, 100 µl of dimethylsulfoxide was added to each well, and the plates were shaken for 5 min. The absorbances of the wells were determined at 555 nm with a microtiter plate reader.

Statistical analysis:

The results are expressed as mean ± SD. The comparison of data obtained was performed by the Student's *t*-test. Statistical significance was accepted at a level of $p < 0.05$.

RESULTS

Protection against oxidation-induced DNA damage:

To evaluate the protective effect of neuroactive steroids on DNA damage induced by hydrogen peroxide, DNA strand breaks were evaluated by single-cell electrophoresis. Exposure of primary human retinal pigment epithelial cell cultures to H₂O₂ resulted in significant single-strand breaks. The DNA breakage of the human retinal pigment epithelial cells cultured in the presence of 17β-estradiol or DHEA-S (60 min) was significantly ($p < 0.05$; $p < 0.01$) lower than that of the control (H₂O₂ alone) at all concentrations, and in a dose-related manner (Figs 1a and 2a). Pretreatment (10 min) with BD1047 (100 µM) antagonized the effects of 17β-estradiol and DHEA-S. On the contrary, the protective effects of neuroactive steroids were significantly enhanced when we added 17β-estradiol or DHEA-S 10 min before the BD1047 treatment (Figs 1a and 2a). This effect was statistically significant ($p < 0.05$) when the cells were treated with DHEA-S. In a separate set of experiments, we evaluated the effects of

BD1047 alone at different concentrations (1–100 μM). The BD1047 caused a significant ($p < 0.01$) inhibition of DNA breakage of the human retinal pigment epithelial cells at the highest concentration (data not shown); however, this effect was of less magnitude compared with the cells pretreated with neuroactive steroids.

Enhancing survival of ARPE-19 cells:

Cell viability assay showed that 4-h exposure to hydrogen peroxide (400 μM) caused a significant decrease in cell survival ($p < 0.01$). Pretreatment with 17β -estradiol and DHEA-S resulted in significant increased viability ($p < 0.05$; $p < 0.01$) in a dose-related manner. Pretreatment (10 min) with BD1047 (100 μM) antagonized the effects of 17β -estradiol and DHEA-S (Figs 1b and 2b). Cell survival was particularly prominent when we added 17β -estradiol or DHEA-S 10 min before the BD1047 treatment (Figs 1b and 2b). In a separate set of experiments, we evaluated the effects of BD1047 alone at different concentrations (1–100 μM). The BD1047 caused a significant ($p < 0.01$) increase of cell survival at the highest concentration (data not shown); however, this effect was of less magnitude compared with the cells pretreated with neuroactive steroids.

DISCUSSION

Retinal pigment epithelial cells play a central role in maintaining visual function of the retina. In environments of oxidative stress, as in the aging retina, dysfunction of the retinal pigment epithelial cells is accelerated. Impairment of the retinal pigment epithelial cells may alter the extra-cellular environment of the photoreceptors and thereby contribute to the pathogenesis of age-related macular degeneration, the leading cause of permanent visual loss in older adults. In fact, retinal pigment epithelial cells generate H_2O_2 during phagocytosis and degradation of rod and cone outer segments, leading to DNA damage. The present study shows that retinal pigment epithelial cells are susceptible to H_2O_2 -mediated damage, and that this injury can be attenuated by neuroactive steroid treatment. We demonstrated that treatment with 17β -estradiol or DHEA-S can protect human retinal pigment epithelial cells from H_2O_2 -induced cell death and DNA damage, and that these effects are antagonized by pretreatment with the σ_1 receptor ligand. We also observed that pretreatment with neuroactive steroids followed by treatment with BD1047 enhanced the protective effects of 17β -estradiol and DHEA-S. This synergistic effect could be related to the fact that BD1047 also presented an affinity for the σ_2 receptor ($K_i = 47 \text{ nM}$). Because σ_1 receptors appear to be antiapoptotic and σ_2 receptors are proapoptotic [17,18], the activation of σ_1 receptors by an agonist and the inhibition of σ_2 receptors by an antagonist should lead to a synergistic effect in terms of cells protection. Indeed, cell protection does not occur when the σ_1 receptors are already blocked by an antagonist, as in the case of the pretreatment with BD1047. The term ‘neuroactive steroids’ has been introduced to designate certain steroids that may alter neuronal excitability via their action at the cell surface through interaction with certain neurotransmitter receptors. More specifically, these neuroactive steroids can influence neuronal functions by classical genomic effects, by binding to intracellular receptor-activating transcription factors and regulating gene expression, or through nongenomic, rapid effects by binding or indirectly modulating the activity of several neurotransmitter receptors and ion channels. A more recent concept is that, among these targets, some neuroactive steroids interact with an atypical neuromodulatory receptor, namely the σ_1 receptor. In this study, we demonstrated that neuroactive steroids increase cell viability of retinal cells in response to hydrogen peroxide, suggesting that the inhibition of mitochondrial function by hydrogen peroxide can be attenuated by 17β -estradiol and DHEA-S. It has been shown [7] that 17β -estradiol provides neuroprotection in retinal neurons against H_2O_2 -induced cell death, and that this effect is not caused by activation of estrogen receptors, because the 17α -estradiol, a biologically inactive stereoisomer, also provides retinal neuroprotection. These results are in accord with a study [6] on primary neurons and clonal hippocampal cells

that demonstrated that steroids within the hydroxyl group in the C3 position on the A ring of the steroid molecule can act as powerful neuroprotectants in an estrogen-receptor-independent manner because of their antioxidative capacity. Despite these findings, the mechanism or mechanisms by which neuroactive steroids exert these protective effects against oxidative stress have not been completely elucidated. The evidence of a direct interaction between neuroactive steroids and σ_1 receptors was first suggested by the ability of several steroids to inhibit the binding of σ_1 -receptor radioligands *in vitro* and *in vivo* [9]. A crossed pharmacology between neuroactive steroids and σ_1 receptors was described in various physiological tests and behavioral responses [9]. Several *in-vitro* studies demonstrated that σ_1 -receptor ligands exert neuroprotective properties [19–21]. Senda *et al.* [20] showed that σ_1 -receptor agonists protect retinal cells against glutamate-induced neurotoxicity. The neuroactive steroids, 17 β -estradiol and DHEA-S, inhibited the binding to σ_1 receptors with K_i values in the low micromolar range [22]. Recently, we demonstrated [11] that 17 β -estradiol and DHEA-S exert a protective effect on ischemia-reperfusion injury in the rat retina, and that this effect is mediated by activation of the σ_1 receptors.

CONCLUSION

Although the exact mechanisms by which 17 β -estradiol and DHEA-S protect human retinal pigment epithelial cells against hydrogen peroxide toxicity are not clear, the present study supports the hypothesis that they act through nongenomic effects with an involvement, at least in part, of σ_1 receptors. A further understanding of the protective mechanisms of neuroactive steroids may provide new insights into the treatment of retinal degenerative diseases such as age-related macular degeneration.

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Fig.1.

Effects of 17β-estradiol (βE) on DNA damage and death of retinal pigment epithelial (RPE) cells induced by H_2O_2 . **(a)** Primary RPE cells were preincubated with βE (1–100 μM) and then exposed to 50 μM of H_2O_2 with or without *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino) ethylamine (BD1047) (the concentration used was 100 μM for both BD1047 and βE). DNA strand breaks were determined after 20 min of H_2O_2 exposure. **(b)** ARPE-19 cells were preincubated with βE (1–100 μM) with or without BD1047 (100 μM) and then exposed to 400 μM H_2O_2 . Cell viability was measured after 4 h of H_2O_2 exposure. Each bar shows the mean ± SD of 4–6 experiments. * $p < 0.05$, ** $p < 0.01$ versus control (CTR; H_2O_2 alone).

Fig.2.

Effects of dehydroepiandrosterone-sulfate (DHEA-S) on DNA damage and death of retinal pigment epithelial (RPE) cells induced by H_2O_2 . **(a)** Primary retinal pigment epithelial (RPE) cells were preincubated with DHEA-S (1–100 μ M) and then exposed to 50 μ M of H_2O_2 with or without *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino) ethylamine (BD1047) (the concentration used was 100 μ M for both BD1047 and DHEA-S). DNA strand breaks were determined after 20 min of H_2O_2 exposure. **(b)** ARPE-19 cells were preincubated with DHEA-S (1–100 μ M) with or without BD1047 (100 μ M) and then exposed to 400 μ M H_2O_2 . Cell viability was measured after 4 h of H_2O_2 exposure. Each bar shows the mean $\pm$ SD of 4–6 experiments. * p < 0.05, ** p < 0.01 versus control (CTR; H_2O_2 alone); *** p < 0.05 versus DHEA-S (100 μ M).