• Basic Research •

Etomidate protects retinal ganglion cells from hydrogen peroxide-induced injury *via* Nrf2/HO-1 pathway

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Received: 2024-03-17 Accepted: 2024-05-10

Abstract

● **AIM:** To determine whether etomidate (ET) has a protective effect on retinal ganglion cells (RGCs) injured with hydrogen peroxide (H_2O_2) and to explore the potential mechanism underlying the antioxidative stress effect of ET.

● **METHODS:** Cultured RGCs were identified by double immunofluorescent labeling of microtubule-associated protein 2 and Thy1.1. An injury model of H_2O_2-induced RGCs oxidative stress was established *in vitro*. Cells were pretreated with different concentrations of ET (1, 5, and 10 μmol/L) for 4h, followed by further exposure to H_2O_2 at 1000 μmol/L. Cell counting kit 8 and Annexin V/propidium iodide assays were applied to detect the viabilities and apoptosis rates of the RGCs at 12, 24, and 48h after H_2O_2 stimulation. The levels of nitric oxide, malondialdehyde, and glutathione in culture media were measured at these time points. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blot were performed to observe the effects of ET on the messenger RNA and protein expression of inducible nitric oxide synthase (iNOS), nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase 1 (HO-1), glutathione peroxidase 1 and the level of conjugated acrolein in RGCs at 12, 24, and 48h after H_2O_2 stimulation and in the retina at 12h after optic nerve transection (ONT).

● **RESULTS:** The applications of 5 and 10 μmol/L of ET significantly increased the viability of RGCs. Results from qRT-PCR indicated a decrease in the expression of iNOS and an increase in the expressions of Nrf2 and HO-1 in ET-pretreated RGCs at 12, 24 and 48h after H_2O_2 stimulation, as well as in ET-treated retinas at 12h after ONT. Western blot analysis revealed a decrease in the expression of iNOS and levels of conjugated acrolein, along with an increase in the expressions of Nrf2 and HO-1 in ET-pretreated RGCs *in vitro* and ET-treated retinas *in vivo*.

● **CONCLUSION:** ET is a neuroprotective agent in primary cultured RGCs injured by H_2O_2. The effect of ET is dose-dependent with the greatest effect being at 10 μmol/L. ET plays an antioxidant role by inhibiting iNOS, up-regulating Nrf2/HO-1, decreasing the production of acrolein, and increasing the scavenge of acrolein.

● **KEYWORDS:** etomidate; retinal ganglion cell; neuroprotection; hydrogen peroxide-induced injury; nuclear factor erythroid 2-related factor 2; heme oxygenase 1

DOI:10.18240/ijo.2024.09.05

Citation: Zhao X, Fan DG, Zhang XC, You SW, Kuang F, Wu MM. Etomidate protects retinal ganglion cells from hydrogen peroxide-induced injury *via* Nrf2/HO-1 pathway. *Int J Ophthalmol* 2024;17(9):1606-1613

INTRODUCTION

Trauma and ophthalmic diseases can lead to the injury of the optic nerve (ON) and apoptosis of retinal ganglion cells (RGCs), causing permanent vision loss as the central neurons cannot proliferate and divide[1-2]. Therefore, the prevention of RGC apoptosis is a precondition for any treatment of ON injury, and many drug therapies could effectively protect injured RGCs. Etomidate (ET), a non-barbiturate anesthetic, selectively acts on γ-aminobutyric acid type A receptors containing β2 or β3 subunits. ET leads to chloride influx and hyperpolarization and thus induces anesthesia[3]. ET has also been found to have neuroprotective effects in recent years. ET may protect hippocampal neurons from kainic acid-induced damage[4] and promote

functional recovery after spinal cord injury in rats[5]. The neuroprotective effect of ET is related to antioxidant effects. In previous studies, ET reduced oxidative stress of the central nervous system (CNS) in hyperglycemic rats[6] and had a strong neuroprotective effect for ischemia/reperfusion spinal cord injury in rabbits[7]. This effect may be related to its enhancement of endogenous antioxidant activity and its influence on tissue ion balance. ET attenuated myocardial injury by inhibiting ischemia/reperfusion-induced ferroptosis *via* the nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase 1 (HO-1) pathway[8] and attenuated hyperoxia-induced acute lung injury in mice by modulating the Nrf2/HO-1 signaling pathway[9]. Our previous studies demonstrated that ET had protective effects on RGCs within 7d of optic nerve transection (ONT) and that antioxidative stress may be involved in the protective mechanism of ET[10]. However, the detailed mechanism remains unclear and requires further investigation. In the present study, we investigated the effects of ET on cultured RGCs injured with hydrogen peroxide (H_2O_2) and the role of Nrf2/HO-1.

MATERIALS AND METHODS

Ethical Approval This study was conducted in accordance with the United States National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication No.85-23, revised 1996). The Animal Ethics Committee of the Fourth Military Medical University, China approved all animal protocols (approval No.20210123).

Animals Adult male Sprague-Dawley (SD) rats (age, 8–10wk; weight, 200–250 g), and neonatal SD rats were obtained from the Laboratory Animal Center of the Fourth Military Medical University, China [license No. SCXK (Shann) 2014-002]. The adult rats were housed in a temperature- and humidity-controlled room with a 12-hour/12-hour light/dark cycle. Food and water were freely available.

Primary Culture and Identification of RGCs The eyeballs were harvested from decapitated neonatal SD rats aged 0–3d and rinsed three times in ice-cold D-Hank's solution (containing 100 U/mL penicillin and streptomycin 100 μg/mL). The retinas were quickly separated, washed twice with ice-cold D-Hank's solution, minced and digested with 0.125% trypsin at 37℃ for 10min. The digestion was terminated with Dulbecco's Modified Eagle medium containing 10% fetal bovine serum. After centrifugation, cells were collected and suspended in Neurobasal-A medium containing 2% B27 and 2 mmol/L glutamine. They were then seeded onto either a 24-well plate at 5×10^5 cells/well for performance of a cell counting kit 8 (CCK-8) assay and detection of biochemical indicators, or onto a 6-well plate at 2×10^6 cells/well for Annexin V/propidium iodide (PI), quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and Western blot assays. Microtubule-associated protein 2 (MAP2) and Thy1.1 double immunofluorescent labeling were conducted to identify RGCs in culture. Cells grown on coverslips were fixed by 4% paraformaldehyde at 4℃ for 4h, washed three times with 0.01 mol/L phosphate-buffered saline (PBS, pH 7.4), blocked with 3% bovine serum albumin and 0.3% Triton X-100 at room temperature for 30min, and incubated with anti-MAP2 (1:1000, AB5622, Merck Millipore, Billerica, USA) and anti-Thy1.1 (1:100, MAB1406, Merck Millipore) antibodies at 4℃ overnight. The cells were then washed with PBS three times and incubated with secondary antibodies (Alexa Fluor 488, Alexa Fluor 594, 1:800, Jackson ImmunoResearch, West Grove, USA) at room temperature for 2h. After being washed again with PBS three times, the cells were covered using 50% glycerin.

Treatment of RGCs The RGCs were cultured for 7d and divided into the following groups: 1 control group in which RGCs were not treated, 1 control group in which RGCs were exposed to H_2O_2, and 3 experimental groups in which RGCs were pretreated with 1, 5, and 10 μmol/L ET, respectively, before exposure to H_2O_2. The cells were injured by the addition of 1000 μmol/L H_2O_2 following a 4-hour pretreatment of ET at concentrations of 1, 5, and 10 μmol/L.

CCK-8 Assay Cell viability was measured using the CCK-8 assay kit (Sant-bio, Shanghai, China) according to the manufacturer's protocols. Following H_2O_2 stimulation, the culture medium was removed, and 50 μL of CCK-8 reagent along with 500 μL of Neurobasal-A medium were added to each well. The cells were then cultured for 4h, after that the absorbance was measured at 450 nm. Cell viability was determined as the percentage of untreated control cells (100%).

Annexin V/PI Assay Apoptosis of RGCs was detected using an Annexin V/PI assay kit (7seapharmtech, Shanghai, China) according to the manufacturer's protocol. At 12, 24, and 48h after H_2O_2 stimulation, the treated cells were harvested and washed with cold PBS. The cells were resuspended in 100 μL binding buffer and stained with 10 μL Annexin V/FITC on ice for 15min in the dark. Next, 400 μL binding buffer and 5 μL PI were added for 5min in the dark. The percentage of apoptosis was analyzed using flow cytometry (Beckman, Miami, USA) within 1h.

Detection of Biochemical Indicators Related to Oxidative Stress Injury At 12, 24 and 48h after H_2O_2 stimulation, cell culture supernatants of each group were collected and centrifuged at 4°C and 1000×g for 5min. The levels of nitric oxide (NO), malondialdehyde (MDA) and glutathione in the cell-free supernatant were then measured in accordance with the instructions of the respective assay kits (NO, Jiancheng Bioengineering Institute, Nanjing, China; MDA and glutathione, Wanleibio, Shenyang, China).

Establishment of ON Injury Model and Design of Experimental Groups Fifty-four male rats were randomly divided into control, ONT, and ONT with ET groups (n=18 for each group). ONT of the rats in the ONT and ONT with ET groups was conducted as previously described[10]. The rats were anesthetized with 0.1% pentobarbital sodium. Those in the ONT and ONT with ET groups were injected intraperitoneally with either normal saline (2 mL/kg) or ET (4 mg/kg; ET injectable emulsion, license No. H20020511, Jiangsu Nhwa Pharmaceutical Co. Ltd., Xuzhou, China) immediately after ONT. The left retinas were isolated from these 54 aninals at 12h after ONT and stored at -80℃.

qRT-PCR Total RNA was extracted from the cultured RGCs and 18 retinas (6 retinas for each group and 2 retinas were used as 1 sample) using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. Complementary DNA was synthesized with PrimeScript RT Master Mix (TaKaRa, Beijing, China) according to the manufacturer's instructions. The qRT-PCR was performed using specific primers in the SYBR® Premix Ex Taq™ system (TaKaRa, Beijing, China) under the following conditions: 95°C for 2min, 40 cycles at 95°C for 10s and 60°C for 30s, and 72°C for 30s. The expression level of target mRNA was measured and normalized to the control gene β-actin. Primer sequences were: inducible nitric oxide synthase (iNOS), 5'-AGC TCG GGC TGA AGT GGT AT-3' (forward), 5'-CTC GGT GCC CAT GTA CCA AC-3' (reverse); Nrf2, 5'-GCC ACG TTG AGA GCT CAG TC-3' (forward), 5'-ATC AGT CAT GGC CGT CTC CA-3' (reverse); HO-1, 5'-TCG TGC TCG CAT GAA CAC TC-3' (forward), 5'-GGC CTC TGG CGA AGA AAC TC-3' (reverse); glutathione peroxidase1 (GPX1), 5'-AGA ATG TCG CGT CCC TCT GA-3' (forward), 5'-TGA TTG CAC GGG AAA CCG AG-3' (reverse); β-actin, 5'-TAC AAC CTC CTT GCA GCT CC-3 (forward), 5'-GGA TCT TCA TGA GGT AGT CAG TC-3' (reverse).

Western Blot For RGCs, 100 μL of RIPA lysis solution was added to each well of a 6-well plate. The cells at the bottom of the plate were scraped and collected in an Eppendorf tube along with the lysis solution. As for 36 retinas (12 retinas for each group and 4 retinas were used as 1 sample), 200 μL of RIPA lysis solution was added to an Eppendorf tube containing fragments from four retinas. Both the cells and retinas underwent disruption by sonication, with cells at 20% amplitude and retinas at 30%, 5 times with 5-second intervals at a low temperature. Subsequently, the samples were centrifuged at 4°C and 12 000×g for 15min. The supernatant was used for protein quantification using the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher, Waltham, USA). Proteins were separated using CFAS any KD PAGE protein electrophoresis kit (Gensharebio, Xi'an, China) and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). The PVDF membranes were blocked using 5% skim milk powder for 1h and incubated with primary antibodies at 4°C overnight. They were cut prior to hybridization with antibodies during blotting. The primary antibodies and dilutions were as follows: anti-iNOS (1:250, ab178945, Abcam, Cambridge, UK), anti-acrolein (1:500, ab240918, Abcam), anti-HO-1 (1:3000, ab13243, Abcam), anti-Nrf2 (1:250, NBP3-13682, Novus Biologicals, Littleton, USA), anti-β-actin (1:5000, A5316, Sigma-Aldrich, St. Louis, USA). After washing with Tris-buffered saline with Tween-20 (TBST), horseradish peroxidase-conjugated secondary antibodies were added and the solution was incubated for 2h at room temperature. An enhanced chemiluminescence reagent was added to visualize immunoreactive bands. Protein expression was normalized to β-actin and then quantitated using Image J software.

Statistical Analysis The data were analyzed by one-way analysis of variance followed by Tukey's post hoc test using SPSS version 13.0 (SPSS Inc., Chicago, USA). All data were expressed as mean±standard deviation, and the criterion for statistical significance was P<0.05.

RESULTS

Effect of H_2O_2 Stimulation on RGCs Viability The CCK-8 assay showed that the RGC viability decreased with an increasing duration of stimulation at a constant H_2O_2 concentration. The RGC viability also decreased with an increase in the H_2O_2 concentration. An H_2O_2 concentration of 1000 μmol/L was selected as optimal for subsequent experiments (Figure 1).

Alleviation of H_2O_2-Induced Injury with ET in Primary Cultured RGCs The CCK-8 Assay showed that H_2O_2 decreased the RGC viability and that ET (5 and 10 μmol/L) significantly increased the RGC viability. ET at 1 μmol/L did not affect the RGC viability at 12, 24, and 48h after H_2O_2 stimulation (P>0.05; Figure 2). However, ET at 5 and 10 μmol/L significantly increased the RGC viability. The RGC viability in the 10-μmol/L ET group was significantly higher than that in the 5-μmol/L ET groups (P<0.05; Figure 2).

Protection of RGCs Against H_2O_2-Induced Apoptosis with ET After 12h of H_2O_2 stimulation, the apoptosis rate of RGCs was (12.47±0.45)% in the H_2O_2 group. The apoptosis rates of RGCs in the groups with 1, 5, and 10 μmol/L ET were (12.57±0.97)%, (9.81±0.62)%, and (7.16±0.31)%, respectively. Compared with the control group (2.63±0.37)%, the apoptosis rate was significantly higher after H_2O_2 stimulation. Compared with H_2O_2 stimulation, the rate was significantly lower in the 5- and 10-μmol/L ET groups (P<0.05; Figure 3B). Following a 24-hour stimulation with H_2O_2, the apoptosis rate was (14.31±1.06)% in the H_2O_2 group. In contrast, the apoptosis rates of RGCs were (13.74±0.96)%, (10.18±0.60)% and

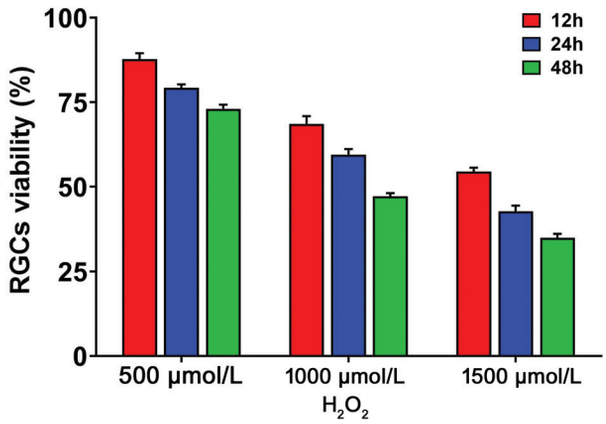

Figure 1 Viability of primary cultured RGCs exposed to various concentrations of H_2O_2 stimulation at 12, 24, and 48h after H_2O_2-induced injury Cell viability was determined as the percentage of untreated control cells (100%). *n*=6. RGCs: Retinal ganglion cells.

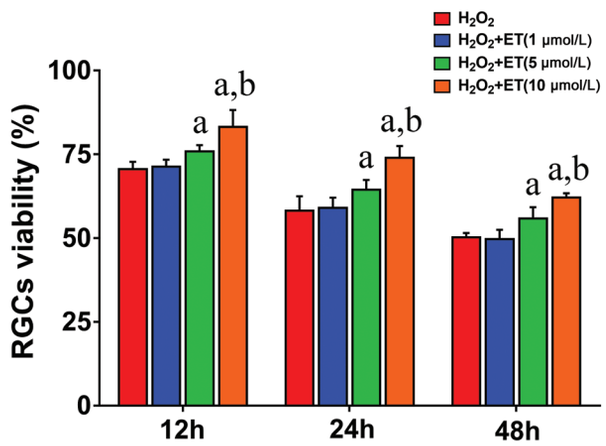

Figure 2 Increase in viability of primary cultured RGCs with ET at 12, 24, and 48h after H_2O_2-induced injury In the quantification of the RGC viability, the cell viability was determined as the percentage of untreated control cells (100%). [a]*P*<0.05 *vs* H_2O_2 group; [b]*P*<0.05 *vs* H_2O_2+ET (5 μmol/L) group; *n*=6. RGCs: Retinal ganglion cells; ET: Etomidate.

(6.25±0.34)% when exposed to 1, 5 and 10 μmol/L of ET, respectively. Compared with the H_2O_2 stimulation group, the rate was significantly lower in the 5- and 10-μmol/L ET groups (*P*<0.05; Figure 3D). Following 48h of H_2O_2 stimulation, the apoptosis rate of RGCs was (20.44±0.93)% in the H_2O_2 group. In contrast, the apoptosis rates of RGCs were (19.79±0.94)%, (17.31±0.79)% and (13.95±0.77)% when treated with 1, 5, and 10 μmol/L ET, respectively. The rate was significantly lower in the 5- and 10-μmol/L ET groups than in the H_2O_2 stimulation group (*P*<0.05; Figure 3F). The rate with 1 μmol/L ET was similar to that in the H_2O_2 group at 12, 24, and 48h after H_2O_2 stimulation (*P*>0.05; Figure 3). The reduction in the apoptosis rate was greater with 10 than with 5 μmol/L ET (*P*<0.05; Figure 3).

Amelioration of H_2O_2-induced Oxidative Stress with ET Compared with the control group, the NO and MDA levels increased significantly, and the glutathione levels decreased significantly at 12, 24, and 48h after H_2O_2 stimulation (*P*<0.05; Figure 4). The NO and MDA levels decreased and the glutathione levels increased in the H_2O_2 with 5 and 10 μmol/L ET groups compared with H_2O_2-only stimulation, the effects being more significant in the 10- than 5-μmol/L ET group (*P*<0.05; Figure 4).

Upregulation of Nrf2 and HO-1 and Downregulation of iNOS and Conjugated Acrolein with ET The qRT-PCR assay showed that mRNA expression of Nrf2 and HO-1 was significantly increased and that the iNOS level was decreased in the ET group (*P*<0.05; Figure 5). However, the GPX1 mRNA expression remained unchanged (*P*>0.05; Figure 5). Western blot assay showed that ET promoted the protein expression of Nrf2 and HO-1 and decreased the expression of iNOS and the level of conjugated acrolein at 12, 24, and 48h after H_2O_2 stimulation (*P*<0.05; Figure 5). The same results were observed *in vivo* after ONT. The mRNA and protein expression of iNOS and the level of conjugated acrolein in the retina decreased and that of Nrf2 and HO-1 increased with intraperitoneal injection of ET (Figure 6).

DISCUSSION

In the present study, cultured RGCs were obtained from the retinas of SD rats 3d after birth. Although the antigen Thy1.1 exists on the surfaces of several kinds of cells and is considered a marker of RGCs[11], this antigen is also expressed by fibroblasts *in vitro*[12]. The antigen MAP2 is a marker of mature neurons and is not expressed on fibroblasts. Therefore, RGCs were identified by double immunofluorescence labeling of Thy1.1 and MAP2. The results showed that almost all cells with Thy1.1 immunolabelling were MAP2-positive.

H_2O_2 stimulation is a common method for establishing a model of oxidative stress injury *in vitro*[13]. We observed the viability of RGCs at 12, 24, and 48h after administration of H_2O_2 at three concentrations (500, 1000, and 1500 μmol/L), with 1000 μmol/L being proved to be the optimal concentration. As described in a previous study, we used ET at three concentrations (1, 5, and 10 μmol/L)[14]. The results indicated that the neuroprotective effect of ET was dose-dependent and that 10 μmol/L ET had the strongest effect.

In order to explore the possible mechanism underlying antioxidative stress of ET, the mRNA and protein expressions of iNOS, Nrf2, HO-1, and GPX1 as well as the level of conjugated acrolein in cultured RGCs and retinas with ONT were observed. The results showed significantly-increased mRNA and protein levels of iNOS after H_2O_2 stimulation and ONT. Infection, endotoxin, ischemia, and trauma are well known to induce the expression of iNOS with sustained large-scale production of NO. Excessive NO can cause cell damage or even death[15]. Some studies have shown that a variety of substances play protective roles in RGCs by reducing the expression of iNOS after ON injury. For example, erinacine A

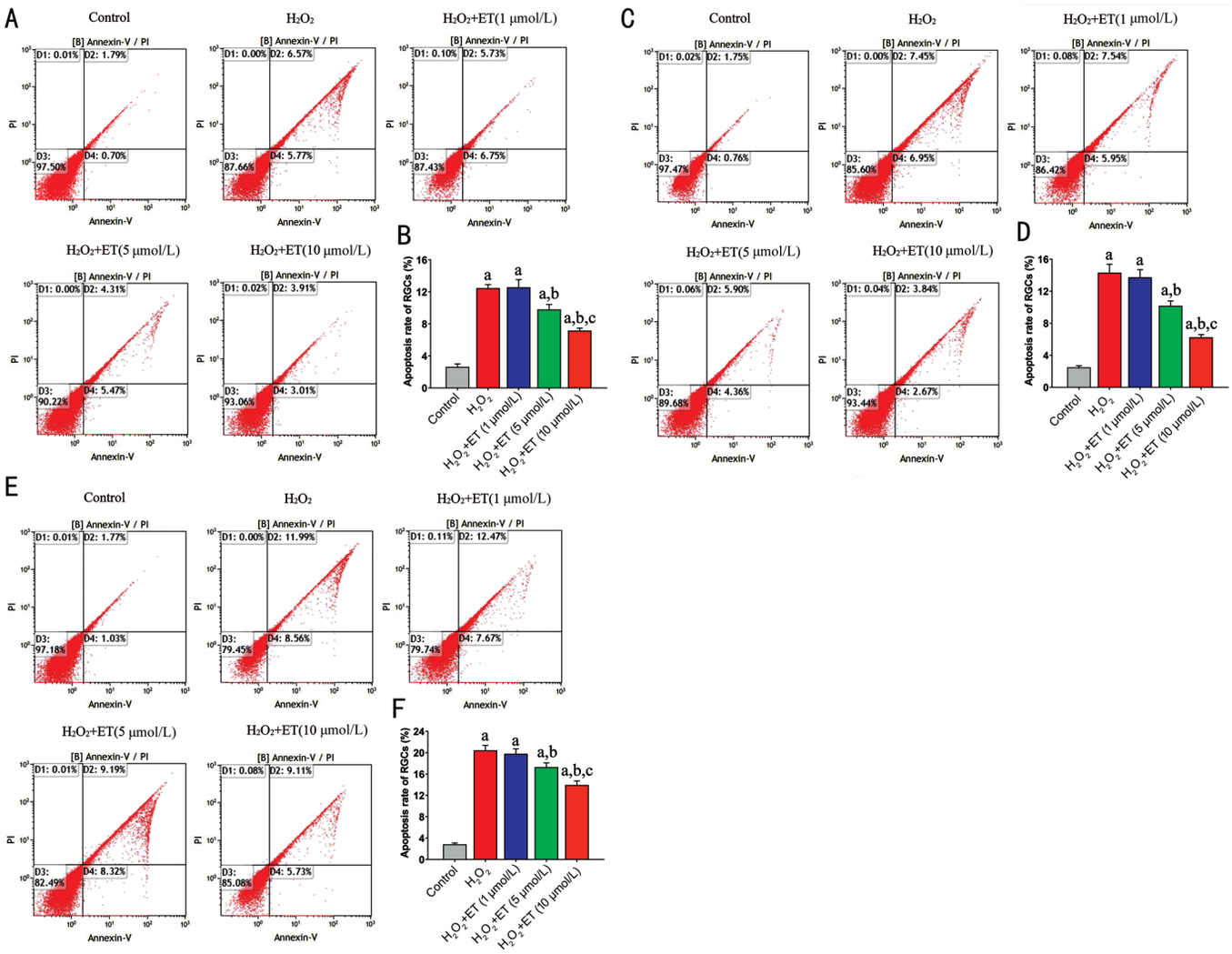

Figure 3 Decrease in the apoptosis rate of primary cultured RGCs with ET at 12 (A, B), 24 (C, D), and 48h (E, F) after H_2O_2-induced injury A, C, E: Results of the RGC apoptosis rates (D1: cellular debris or necrotic cells; D2: necrotic or late apoptotic cells; D3: viable cells; D4: early apoptotic cells). B, D, F: Statistical comparisons of the RGC apoptosis rates. [a]*P*<0.05 *vs* control group; [b]*P*<0.05 *vs* H_2O_2 group; [c]*P*<0.05 *vs* H_2O_2+ET (5 μmol/L) group; *n*=3. RGCs: Retinal ganglion cells; ET: Etomidate.

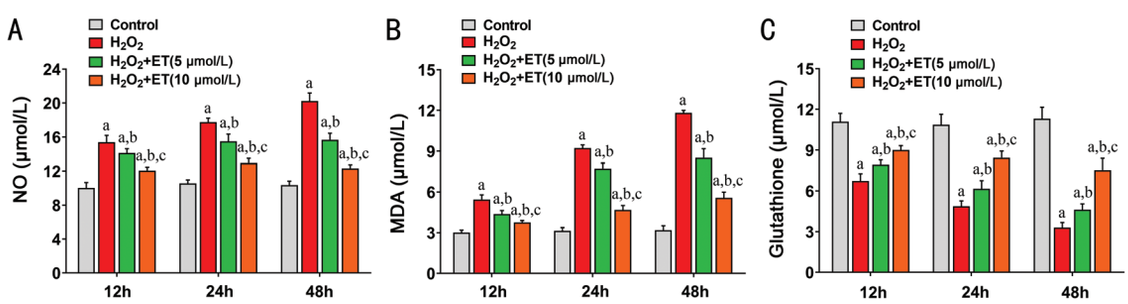

Figure 4 Amelioration of H_2O_2-induced oxidative stress with ET in primary cultured RGCs at 12, 24, and 48h after H_2O_2-induced injury Quantification of NO (A), MDA (B), and glutathione (C) in RGCs culture medium. [a]*P*<0.05 *vs* control group; [b]*P*<0.05 *vs* H_2O_2 group; [c]*P*<0.05 *vs* H_2O_2+ET (5 μmol/L) group; *n*=6. ET: Etomidate; RGCs: Retinal ganglion cells; NO: Nitric oxide; MDA: Malondialdehyde.

inhibited the expression of iNOS in the retina and promoted the survival of RGCs after ON crush[16]. Our study indicated that ET decreased the expression of iNOS in RGCs and retinas at both the mRNA and protein levels. ET may play a role in reducing oxidative stress by inhibiting the expression of iNOS and reducing NO content.

MDA is a marker of lipid peroxidation[17]. Our previous experiments showed that ET can reduce the MDA content of the retina after ON damage[10]. Consistent with this finding, ET reduced the MDA content of RGCs after H_2O_2 stimulation in the present study. The results indicated that ET has the effect of reducing lipid peroxidation. GPX is one of the most

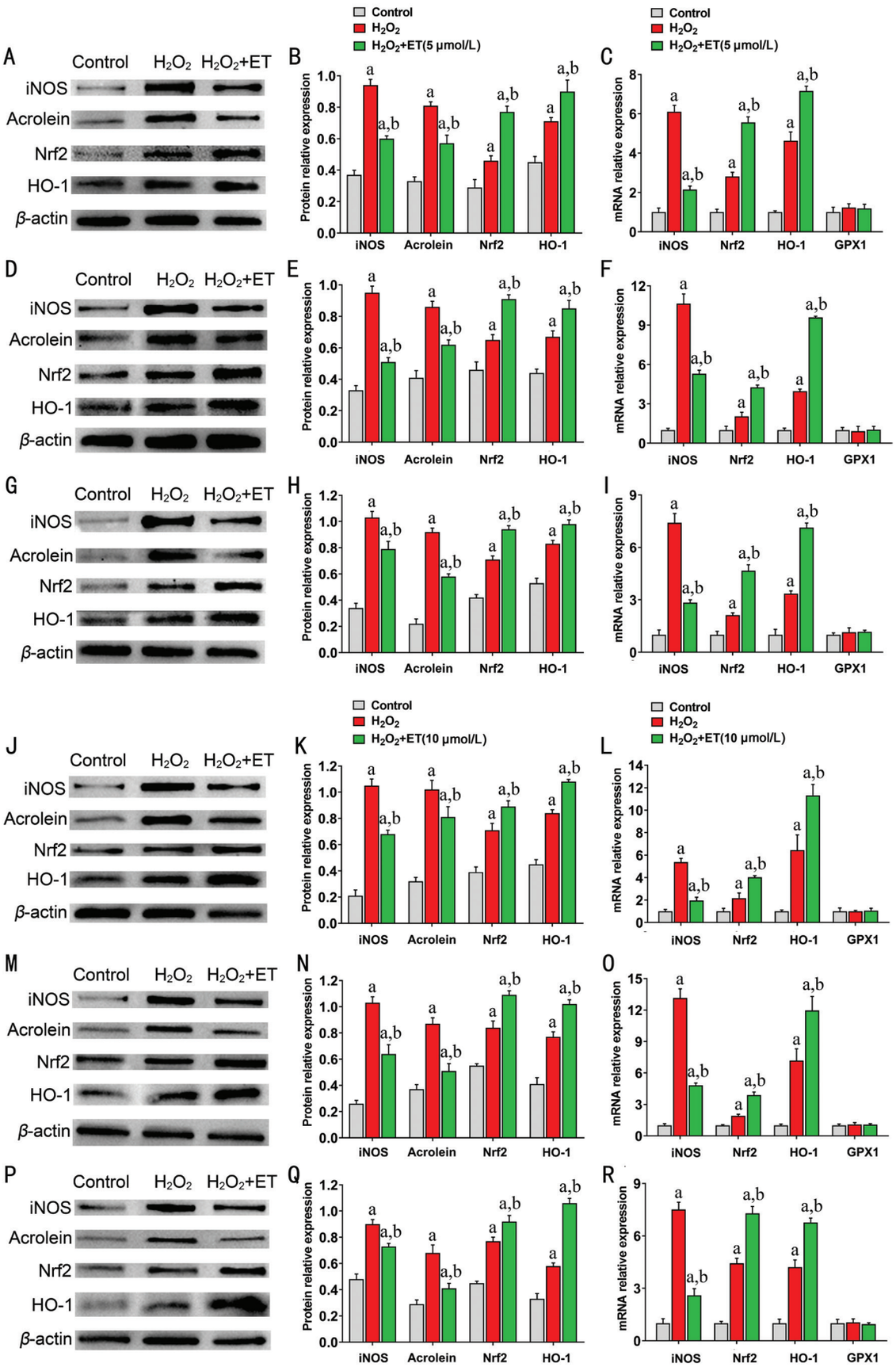

Figure 5 ET administered at 5 μmol/L (A–I) and 10 μmol/L (J–R) promoted upregulation of Nrf2 and HO-1 and downregulation of iNOS and conjugated acrolein in primary cultured RGCs at 12h (A–C, J–L), 24h (D–F, M–O), and 48h (G–I, P–R) after H_2O_2-induced injury A, D, G, J, M, P: Western blot images. B, E, H, K, N, Q: Statistical comparisons of relative protein expressions. C, F, I, L, O, R: Statistical comparisons of relative mRNA expressions. [a]*P*<0.05 *vs* control group; [b]*P*<0.05 *vs* H_2O_2 group; *n*=3. ET: Etomidate; Nrf2: Nuclear factor erythroid 2-related factor 2; HO-1: Heme oxygenase-1; iNOS: Inducible nitric oxide synthase; RGCs: Retinal ganglion cells; GPX1: Glutathione peroxidase1.

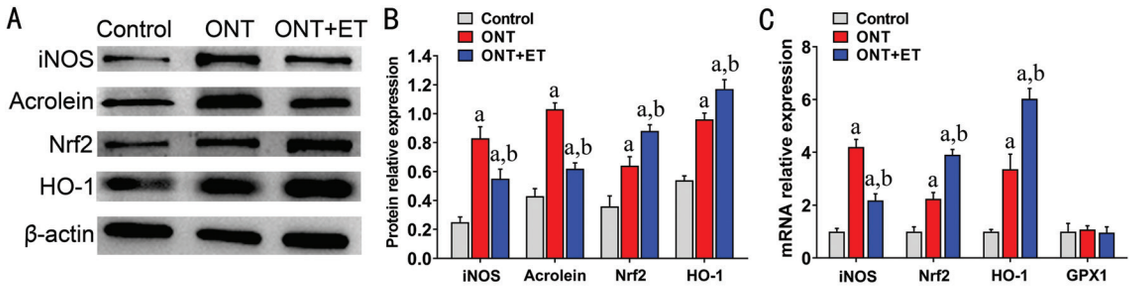

Figure 6 Upregulation of Nrf2 and HO-1 and downregulation of iNOS and conjugated acrolein with ET in the retina at 12h after ONT A: Western blot images. B: Statistical comparisons of relative protein expressions. C: Statistical comparison of relative mRNA expression. [a]*P*<0.05 *vs* control group; [b]*P*<0.05 *vs* ONT group; *n*=3. Nrf2: Nuclear factor erythroid 2-related factor 2; HO-1: Heme oxygenase-1; iNOS: Inducible nitric oxide synthase; ET: Etomidate; ONT: Optic nerve transection; GPX1: Glutathione peroxidase1.

important antioxidant enzymes, reducing lipid peroxidation by scavenging lipid hydroperoxides[18]. In the present study, however, the expression of GPX1 did not change significantly after H_2O_2 stimulation, and ET did not affect the expression of GPX1 mRNA.

Acrolein is an aldehyde produced by lipid peroxidation and is highly reactive with biological molecules. Acrolein shows the greatest reactivity with thiols. Therefore, the reaction between acrolein and glutathione may be an important target in acrolein-mediated injury[19]. Acrolein can form stable conjugates with proteins and the detection of conjugated acrolein can indirectly reflect acrolein content[20]. Western blot results showed that the level of conjugated acrolein in RGCs and retinas increased after H_2O_2 stimulation and ONT. However, such elevated acrolein was significantly reduced with ET. Acrolein can undergo a Michael addition reaction with the nitrogen atoms of the imidazole ring of histidine to generate $N^{\pi}(^{\tau})$-formylethylhistidine[21]. Because ET is an imidazole derivative with an imidazole ring, we speculated that ET can also react with acrolein and promote the scavenging of acrolein. As the acrolein level decreased, acrolein-bound glutathione was released, leading to an increase in the glutathione content of injured RGCs. Our study demonstrated that ET can increase the glutathione content in injured RGCs. Because ET reduces lipid peroxidation, we suggest that ET may decrease the content of acrolein in injured RGCs by reduced production and increased scavenging of acrolein.

Nrf2/HO-1 pathway is an important antioxidative pathway. The transcription factor Nrf2 is a central regulator of cellular responses to oxidative stress stimulation and is inhibited by Keap 1 protein. Reactive oxygen species or electrophilic substances may destroy the cysteine residues of Keap 1 or phosphorylate Nrf2 through the signaling pathway of mitogen-activated protein kinase during oxidative stress caused by injury. Therefore, Nrf2 changes from a bound state to a free state. Free Nrf2 can enter the nucleus to form a heterodimer with Maf protein and bind to the upstream promoter region of the antioxidant response element (ARE), which promotes the expression and transcription of antioxidant enzyme genes. The antioxidant enzymes regulated by the Nrf2/ARE pathway include superoxide dismutase, glutathione S-transferase and HO-1. HO-1 is the rate-limiting enzyme of heme degradation and can convert heme into biliverdin, which is reduced to bilirubin. Bilirubin reduces inflammation and lipid peroxidation, inhibits apoptosis, dilates blood vessels and improves microcirculation[22]. The previous studies have shown that Nrf2/HO-1 is involved in the neuroprotective effect on RGCs. Overexpression of the Nrf2 gene could promote the survival of RGCs in mice with concurrent multiple sclerosis and experimental autoimmune encephalomyelitis[23]. SIRT6 promoted Nrf2/ARE signal transduction by inhibiting bach1, thus protecting RGCs from H_2O_2-induced oxidative stress and apoptosis[24]. Trimetazidine could protect RGCs by upregulating Nrf2/HO-1 in mice with acute glaucoma[25]. Our results showed that the mRNA and protein expression of Nrf2 and HO-1 in RGCs and retinas increased significantly after H_2O_2 stimulation and ONT. Our analysis suggests that the increased expressions of these factors after injury may be a response to oxidative stress compensation. This observation aligns with similar findings in RGCs exposed to high-glucose injury and retinas subjected to ischemia/reperfusion injury[26-27]. Despite the upregulation of Nrf2 following injury, experimental data indicated that it was not adequate to prevent RGC loss. ET further increased the mRNA and protein expression of Nrf2 and HO-1 after injury, indicating that the antioxidative stress effect of ET may be related to the upregulation of Nrf2/HO-1. Therefore, activation of the Nrf2/HO-1 pathway plays an important role in the protective effects exerted with ET.

In conclusion, our study showed that ET can rescue H_2O_2-induced apoptosis of RGCs in a dose-dependent manner within a defined period. The neuroprotective effect of ET was related to antioxidative stress through the Nrf2/HO-1 pathway. Our study provides the evidence for the neuroprotective effects of ET by the Nrf2/HO-1 pathway in CNS.

ACKNOWLEDGEMENTS

Foundations: Supported by the Ministry of Science and Technology of China (No.2021ZD0203104); the Science and Technology Plan Project of Shaanxi Province of China (No.2022SF-497); Xi'an Medical University Doctoral Research Fund (No.2020DOC18).

Conflicts of Interest: Zhao X, None; **Fan DG,** None; **Zhang XC,** None; **You SW,** None; **Kuang F,** None; **Wu MM,** None.

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