

17 β -estradiol suppresses hyperoxia-induced apoptosis of oligodendrocytes through paired-immunoglobulin-like receptor B

HUA WANG and JINLIN WU

Department of Pediatrics, West China Second University Hospital, Sichuan University, Chengdu, Sichuan 610041, P.R. China

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Abstract. Hyperoxia is a high risk factor for neurodevelopmental disorders and can cause nerve cell death. 17 β -Estradiol (E2) has been demonstrated as a neuroprotective agent. In the present study, the effect of hyperoxia on rat oligodendrocyte precursor cells (OPCs) *in vivo* and the neuroprotective effects of E2 on these cells were investigated. OPCs were treated with various concentrations of E2 and were harvested for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis at various time-points. RT-qPCR analysis demonstrated that paired immunoglobulin-like receptor B (PirB) PirB mRNA expression levels were markedly decreased following treatment with 10⁻⁶, 10⁻⁷ and 10⁻⁸ M E2. Cells treated with 10⁻⁷ M E2 for 24 h were selected for subsequent experiments. PirB was silenced with small interfering (si)RNA and the effects of E2 treatment and silencing of PirB on the viability and apoptosis of OPCs under hyperoxic stimulation was detected using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium-bromide (MTT) assay and flow cytometry analysis. The results revealed that hyperoxia induced apoptosis in OPCs and decreased their viability. Hyperoxia also induced the expression of caspases-3 and -8, and Fas cell surface death receptor (Fas). E2 treatment markedly downregulated the expression of PirB. E2 treatment or PirB silencing markedly decreased hyperoxia-induced apoptosis, increased cell viability and decreased the expression of caspases-3 and -8, and Fas in OPCs, indicating that E2 protects OPCs from hyperoxia-induced apoptosis, predominantly through the downregulation of PirB. The results of the present study provide a theoretical basis for the reasonable use of oxygen in Neonatal Intensive Care Units.

Introduction

Improvements in Neonatal Intensive Care Units, and advancements made in neonatal resuscitation and respiratory support and monitoring systems, have increased the survival rates of very-low-birth-weight (<1,500 g) and premature (<28 weeks) infants. However, this increase in survival rates has not resulted in improved neurodevelopmental outcomes (1,2). Previous studies have shown that ~40% of the surviving infants suffered from neurological deficits, including periventricular leukomalacia (PVL), a severe type of brain injury in premature infants characterized by extensive oligodendrocyte precursor cells (OPCs) migration and maturation (3-5). One of the reasons for the occurrence of neurological deficits may be oxygen administration. It is known that oxygen is widely administered to premature infants. Previous studies have shown that hyperoxia is important in the development of bronchial pulmonary hypoplasia and retinopathy of prematurity (3-5). Certain previous studies reported that hyperoxia-induced nerve cell death was observed during the developmental phase (6,7). To the best of our knowledge, the influence of hyperoxia on the human brain has not been reported.

PVL can reduce the levels of neurotrophic factor and nerve growth factor (NGF), resulting in chronic disability of cerebral white matter (8,9). Certain previous studies have shown that the reduced NGF may upregulate c-Jun NH2-terminal kinases (JNK)-p53-Bcl-2-associated X protein (Bax) cell death and the Fas cell surface death receptor (Fas) apoptosis pathway, which comprise the predominant parts of the extrinsic apoptotic signal transduction system (10). A reduction in the tyrosine kinase receptor cell survival pathway, as well as the increased JNK-p53-Bax and Fas apoptotic pathways may therefore promote brain cell death (11-13).

Paired immunoglobulin-like receptor B (PirB) is a receptor expressed on myeloid cells. The inhibition of PirB in animal models of spinal cord injury was revealed to promote the regeneration of damaged nerve cells (14). Our previous study (15) also suggested that PirB, which is induced by hypoxic-ischemic brain injury, inhibited nerve cell regeneration.

17 β -Estradiol (E2) is a neuroprotective agent (16,17), which is important in the development and function of the nervous system (18), particularly at the beginning of the neural precursor cell differentiation around the ventricle (19).

Correspondence to: Professor Jinlin Wu, Department of Pediatrics, West China Second University Hospital, Sichuan University, 20, Section 3, Renmin Nanlu, Chengdu, Sichuan 610041, P.R. China
E-mail: wujl_2000@sina.com

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Several hypoxia-ischemia and excitatory toxin models have demonstrated the neuroprotective effects of E2 on mature cells *in vivo* (20,21). In addition, E2 can also affect the apoptotic process at several different stages (21). During pregnancy, the E2 levels in the placenta exhibited an ~100-fold increase; however, they dropped rapidly within 24 h following the removal of the infant's umbilical cord. Premature babies experienced hormone withdrawal earlier compared with the full-term infants, which further increases the brain tissue damage by oxygen stimulation (22,23).

Despite previous reports suggesting that hyperoxia-induced apoptosis is crucial in brain damage (24), that hyperoxia induces immature or OPC apoptosis (25), that PirB inhibits nerve cell regeneration (26) and that E2 has neuroprotective effects (27), the association between hyperoxia, PirB and E2 remains to be elucidated. Therefore the aim of the present study was to investigate the effects of hyperoxia on OPCs, as well as the effects of E2 on OPC apoptosis.

Materials and methods

Animals. A total of 20 Sprague-Dawley rats, aged 2-days-old were purchased from the Laboratory Animal Center of Sichuan University (Sichuan, China) and used in the present study. Animal care, maintenance and surgery were performed in accordance with the regulations dictated by the Institutional Animal Care and Use Committee of Sichuan University. Rats were immediately sacrificed by decapitation under diethyl-ether (Sigma-Aldrich, St. Louis, MO, USA) anesthesia.

Primary cultures of OPCs. For the *in vitro* experiments, primary OPCs were isolated, as previously described (28). Following sacrifice, the rat's scalps and meninges were subsequently removed and the cortices were dissected, rinsed twice in ice-cold Hank's buffered salt solution (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and incubated at 37°C for 15 min with 0.01% trypsin and DNase (both Beyotime Institute of Biotechnology, Haimen, China). The tissue was subsequently triturated and filtered through a 40 μ m sterile cell strainer to remove insoluble debris. The cells were plated into poly-D-lysine coated T75 culture flasks, containing Dulbecco's modified Eagle's medium (DMEM) with 20% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), until the cells reached confluence (~10 days), by which time a bed of astrocytes had grown with a layer of OPCs on top. The flasks were subsequently agitated at 200 rpm for 1 h to dislodge dead cells and microglia. The media was changed and the flasks were agitated overnight at 200 rpm to dislodge the OPCs. The OPCs were collected and plated into poly-D, L-ornithine coated culture dishes with serum-free DMEM, supplemented with hormones and growth factors (10 nM of each platelet-derived growth factor- α and basic fibroblast growth factor). To induce differentiation, the growth factors were withdrawn from the medium and ciliary neurotrophic factor was added. To avoid spontaneous differentiation, the cells were not used beyond one passage. Cell viability was determined using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay, and the apoptosis rate was analyzed by flow cytometry.

Drug treatment. Stock solutions of E2 (10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} M) were prepared in dimethyl sulfoxide (DMSO). E2 was added to the culture medium for 0, 8, 16, 24 or 48 h, and subsequently the cells were collected and the mRNA expression of PirB was detected using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The concentration of E2 at which the PirB mRNA level dropped the most was selected as the appropriate concentration.

Small interfering (si)RNA and transfection. PriB siRNA (5'-GTGTTTCAGTTGTTCCCTTGACATGA-3') and negative control (NC) siRNA were purchased from Shanghai Jima Biotechnology Co., Ltd. (Shanghai, China). The cells were plated at 50% confluence and transfected with 100 nM siRNA using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

OPC grouping. The OPCs were randomly divided into five groups: i) Normal culture condition plus DMSO treatment group (normal), where cells were cultured under normal conditions and treated with 1% DMSO for 48 h; ii) the hyperoxia plus DMSO treatment group (hyperoxia), where cells were first treated with 1% DMSO for 24 h under normal conditions and were subsequently cultured under hyperoxia conditions for 24 h; iii) the hyperoxia plus NC siRNA group (hyperoxia + NC), where the cells were first transfected with NC siRNA for 24 h under normal conditions and were subsequently cultured under hyperoxic conditions for 24 h; iv) the hyperoxia plus PirB siRNA group (hyperoxia + siRNA), where cells were first transfected with PirB siRNA for 24 h under normal conditions and were subsequently cultured under hyperoxic conditions for 24 h; v) the hyperoxia plus E2 group (hyperoxia + E2), where cells were first treated with E2 for 24 h under normal conditions and were subsequently cultured under hyperoxic conditions for 24 h. The hyperoxic groups were maintained at 37°C in a humidified air incubator containing 80% O₂, 5% CO₂ and 15% N₂. For the normal culture condition group, the cells were placed in a humidified incubator containing <21% O₂ and 5% CO₂ at 37°C. For the E2 treatment groups, the cells were cultured in culture medium supplemented with the appropriate concentration of E2.

RNA extraction and RT-qPCR. Cell pellets of 6×10^5 cells from each group were lysed using ice-cold TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. The RNA was reverse transcribed into cDNA using PrimeScript RT reagent kit with cDNA Eraser (Takara Bio, Inc., Dalian, China) in a 20 μ l reaction, according to the manufacturer's protocol. Equal quantities of cDNA were used as templates for RT-qPCR to detect the expression level of PirB expression relative to that of actin (endogenous control) using a Mx3000P Real-Time PCR system (Stratagene, La Jolla, CA, USA) and a SYBR Premix Ex TaqII PCR kit (Takara Bio, Inc.). Experiments were performed in duplicate and repeated three times. The fold induction of gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method.

OPC cell viability measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The

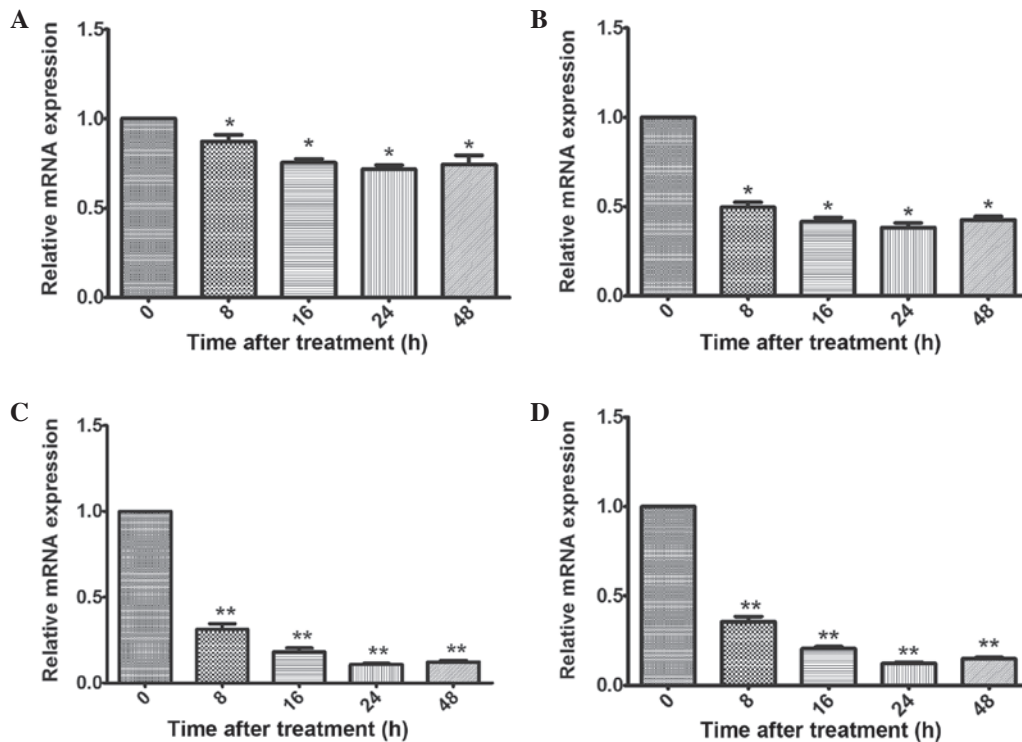


Figure 1. mRNA levels of paired immunoglobulin-like receptor B in oligodendrocyte precursor cells following different concentrations of E2 treatment. (A) 10^{-9} , (B) 10^{-8} , (C) 10^{-7} and (D) 10^{-6} E2 treatment at 0, 8, 16, 24 and 48 h (* $P < 0.05$, ** $P < 0.01$, compared with the 0 h treatment group). Fold changes in gene expression levels were calculated according to the $2^{-\Delta\Delta C_t}$ method. Data are presented as the mean \pm standard deviation of three independent measurements. E2, 17 β -Estradiol.

MTT assay is a laboratory test that measures changes in color for measuring the activity of an enzyme that reduces MTT (yellow color) to formazan (purple color). Following 48 h of incubation, 20 μ l (5 mg/ml) MTT reagent was added to each well and incubated for an additional 4 h. DMSO solution (200 μ l) was subsequently added to each well to solubilize the formazan crystals. The plates were read for optical density at 570 nm using a Multiskan MK3 plate reader (Thermo Fisher Scientific, Inc.). The cell survival rate was calculated based on the optical density of the cells.

Apoptosis analysis of OPCs using flow cytometry. Each OPC group was seeded into a 6-well plate, at a density of 10^4 cells/well. The treated cells were washed twice with cold phosphate-buffered saline and the cell pellet ($1-5 \times 10^5$) resuspended in binding buffer (Keygen Biotech Co., Ltd., Nanjing, China) at a concentration of 10^6 cells/ml. The cells were mixed with 10 μ l fluorescein isothiocyanate-conjugated annexin-V reagent and 10 μ l of 3 mM propidium iodide. Following incubation for 15 min at room temperature in the dark, flow cytometry was performed using a BD Accuri C6 FACScan analyzer (BD Pharmingen, San Diego, CA, USA).

Western blotting. The cells were lysed in radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (v/v) sodium deoxycholate and 0.1% sodium dodecyl sulfate (SDS)], supplemented with a mixture of protease and phosphatase inhibitors. The lysates were sonicated for 5 sec, centrifuged for 20 min at 12,000 \times g at 4°C and stored at -80°C. Equal concentrations of protein (50-60 μ g total protein) were

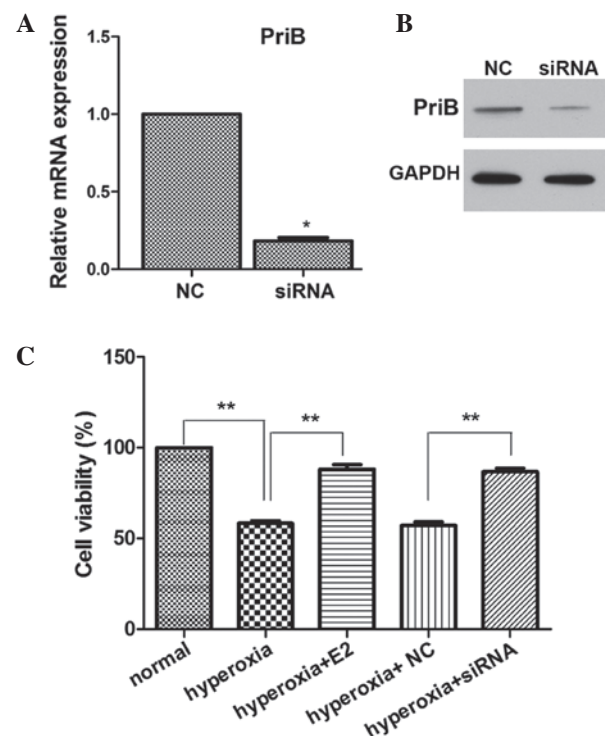


Figure 2. Effects of E2 treatment and PriB silencing on oligodendrocyte precursor cell viability under hyperoxia stimulation. (A) The mRNA level of PriB following PriB siRNA transfection was determined by reverse transcription-polymerase chain reaction. (B) The protein expression of PriB following PriB siRNA transfection was determined by western blotting. (C) Cell viability of oligodendrocyte precursor cells following different treatments was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. (* $P < 0.05$, ** $P < 0.05$). Data are presented as the mean \pm standard deviation of three independent measurements. PriB, paired immunoglobulin-like receptor B; E2, 17 β -Estradiol; si, small interfering RNA; NC, negative control.

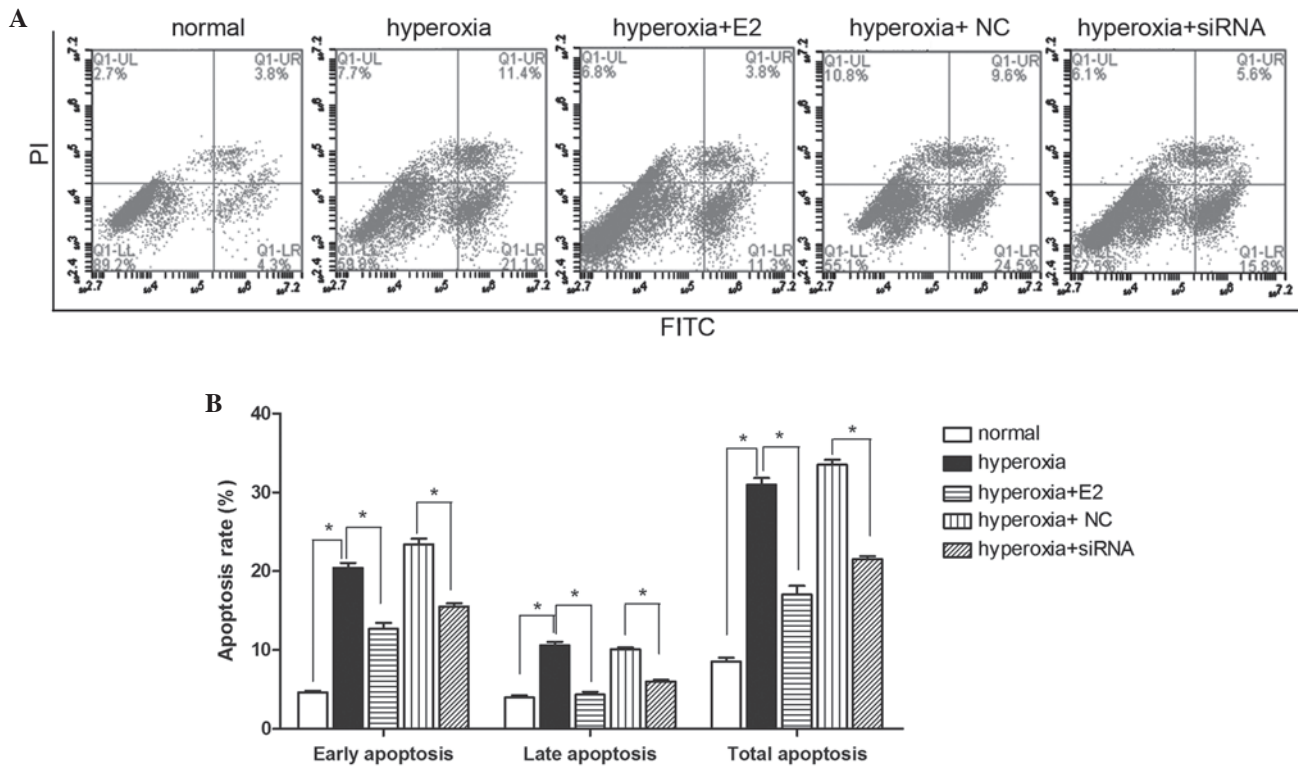


Figure 3. Effect of E2 treatment and PriB siRNA pre-treatment on oligodendrocyte precursor cell apoptosis under hyperoxia stimulation. (A) Flow cytometric analysis using annexin V-FITC/PI staining following the different treatments. (B) Graph showing the percentage of early, late and total apoptotic cells ($P < 0.05$). Data are presented as the mean \pm standard deviation of three independent measurements. Q1-LL, healthy cells; Q1-LR, early apoptotic cell; Q1-UL, necrotic cell; Q1-UR, late apoptotic cell; E2, 17 β -Estradiol; si, small interfering; FITC, fluorescein isothiocyanate; PI, propidium iodide; NC, negative control.

electrophoretically separated by 10% SDS-polyacrylamide gel (Beyotime Institute of Biotechnology) electrophoresis and transferred onto nitrocellulose membranes (Pall Life Sciences, Port Washington, NY, USA). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS), containing 0.01% Tween-20 (TBS-T) for 1 h at 37°C. The membranes were subsequently incubated with the following primary monoclonal antibodies overnight at 4°C: Rabbit anti-PriB (1:1,000; ta323298; OriGene Technologies, Inc., Beijing, China), mouse anti-Fas (1:2,000; 05-351), rabbit anti-caspase-3 (1:1,500; 04-439; both EMD Millipore, Billerica, MA, USA), rabbit anti-caspase-8 (1:1,000; ab119892; Abcam, Cambridge, MA, USA), mouse anti-phosphorylated (p)-Akt (1:2,500; MAB887) and mouse anti-Akt (1:2,000; MAB2055; both R&D Systems, Minneapolis, MN, USA). The membranes were subsequently washed three times with TBS-T, followed by a 2-3 h incubation with horseradish peroxidase-conjugated goat anti-rabbit (1:20,000; BA1055) or anti-mouse (1:25,000; BA1050; both BosterBio, Wuhan, China) immunoglobulin G secondary antibodies at 37°C. Following washing three times with TBS-T, the protein bands were visualized using Super Signal West Pico substrate (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The film was scanned and densitometric analysis was performed using Image Pro-Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA). The results of densitometric analysis were expressed as a relative ratio of the target protein to reference protein. The relative ratio of the target protein of the control group was arbitrarily presented as 1.

Statistical analysis. Statistical analysis was performed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). All results are presented as the mean \pm standard deviation. Student's t-test was used to determine the differences among the groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

E2 treatment suppresses the mRNA expression of PriB. To investigate the effect of E2 on OPCs, the mRNA level of PriB following treatment with different concentrations of E2 was first examined at different time-points. As shown in Fig. 1A, the lowest concentration of E2 (10^{-9} M) reduced the expression of PriB after 8 h, and the PriB expression began to gradually decrease at 16 h following treatment with E2. Compared with the 10^{-9} M treatment, the mRNA expression of PriB exhibited a clear decrease following treatment with 10^{-8} , 10^{-7} , 10^{-6} M E2 (Fig. 1B and C). The 10^{-7} and 10^{-6} M concentrations of E2 exhibited similar effects on the expression of PriB. Based on these results, the treatment with 10^{-7} M E2 for 24 h was selected for subsequent experiments.

E2 treatment and silencing of PriB increases OPC viability under hyperoxic stimulation. To further investigate the function of PriB on OPCs under hyperoxia stimulation, PriB siRNA was used to transfect OPCs, and the mRNA and expression levels of PriB were subsequently measured. As shown in Fig. 2A and B, PriB expression levels were successfully reduced. To investigate the role of E2 on OPCs

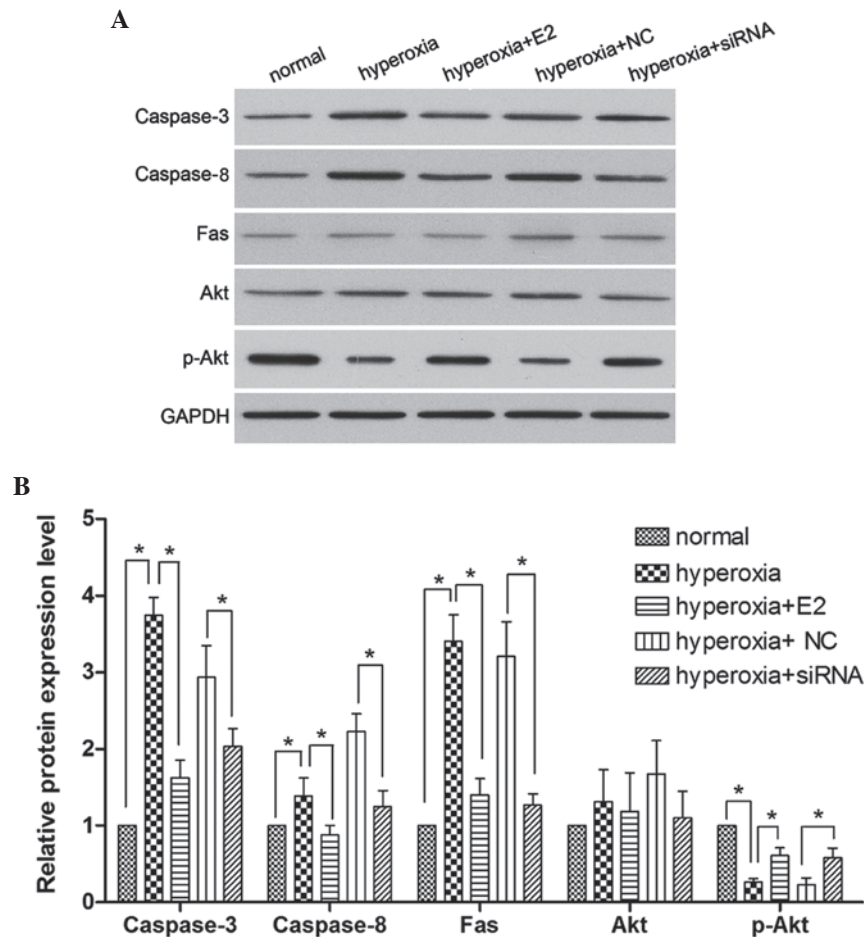


Figure 4. Effect of E2 treatment and PriB siRNA pre-treatment on the expression of caspases-3 and -8, Fas, p-Akt and Akt under hyperoxia stimulation. (A) Western blot analysis following the different treatments. (B) The protein expression levels of caspases-3 and -8, Fas, p-Akt, and Akt were quantified using a densitometer (* $P<0.05$). Data are presented as the mean \pm standard deviation of three independent measurements. E2, 17 β -Estradiol; si, small interfering; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NC, negative control; p-, phosphorylated.

under hyperoxia stimulation, the effect of E2 on cell viability was detected. As shown in Fig. 2C, hyperoxic stimulation significantly decreased cell viability, as compared with the normal culture controls. E2 pretreatment markedly increased cell viability, as compared with the hyperoxia group. PriB siRNA pretreatment also markedly increased cell viability, as compared with the negative control siRNA pretreatment group under hyperoxic stimulation (Fig. 2C).

E2 treatment and silencing of PriB decreases cell apoptosis under hyperoxic stimulation. To further investigate the role of E2 on OPCs under hyperoxia stimulation, the effect of E2 on cell apoptosis was examined. As shown in Fig. 3A and B, hyperoxic stimulation markedly increased both early and late apoptosis, as compared with the normal culture condition controls. E2 pretreatment notably decreased both early and late apoptosis compared with the hyperoxia group. Furthermore, PriB siRNA pretreatment also markedly decreased both early and late apoptosis compared with the negative control siRNA pretreatment group under hyperoxic stimulation (Fig. 3A and B).

E2 treatment and silencing of PirB significantly reduces the expression of caspases-3 and -8, Fas and Akt, however

induces p-Akt. To further investigate the role of E2 and PirB on OPC apoptosis, western blot analysis was performed to measure the protein expression of caspases-3 and -8, Fas, Akt and p-Akt. As shown in Fig. 4, hyperoxia stimulation significantly induced the expression of caspases-3 and -8, and Fas; however, hyperoxia stimulation significantly reduced p-Akt. E2 pretreatment significantly decreased the hyperoxia-induced expression of caspases-3 and -8, and Fas, and increased the expression of p-Akt. Furthermore, PriB siRNA pretreatment also significantly decreased the hyperoxia-induced expression of caspases-3 and -8, and Fas and hyperoxia-induced p-Akt reduction compared with the NC siRNA pretreatment group under hyperoxic stimulation. No significant difference was identified in the expression levels of Akt among all groups.

Discussion

It has been shown that extremely low birth weight remains an important risk factor for neurodevelopmental impairment at 18 months of age (29,30). Oxygen is one of the most widely used treatments in Neonatal Intensive Care Units, and previous studies have shown that neonatal exposure to chronic hyperoxia leads to reduced hippocampal size in adult mice (3). Although recent findings suggest that neonatal rats, which

have been exposed to supraphysiological concentrations of oxygen are at high risk for neurodevelopmental impairment in adults (6,7), they do not rule out the possibility that hyperoxia may promote apoptosis in OPCs. Certain previous studies have demonstrated that neuroactive steroids, including E2, can protect neurons from various harmful compounds (31,32). PirB is a receptor expressed on myeloid cells and is known to have the ability to inhibit nerve cell regeneration (14,15). The aim of the present study was to investigate the association between hyperoxia, PirB and E2 treatment.

In the present study, OPCs were first treated with various concentrations of E2 at different exposure durations, and the ones during which the pirB mRNA expression reached its lowest level were selected as the dosage and exposure durations of E2 for the following assays. The results demonstrated that the optimum concentration and exposure duration were 10^{-7} M and 24 h, respectively. Subsequently, the effect of E2 treatment on OPC cell viability was analyzed using an MTT assay. Hyperoxia was revealed to significantly decrease the cell viability of OPCs, which was reversed by E2 treatment. Based on the knowledge that E2 treatment decreases the expression of PirB, the role of PirB in the hyperoxia-induced decrease in OPC cell viability was investigated next. PirB was successfully silenced by siRNA and this silencing was revealed to abolish the hyperoxia-induced decrease in OPC cell viability, indicating that E2 treatment and PirB silencing can protect OPCs against hyperoxia-induced cell damage.

The effects of E2 treatment and PirB silencing on OL apoptosis was also analyzed using flow cytometry. Hyperoxia was found to significantly increase the apoptosis of OPCs, while E2 treatment was shown to partially reverse it. Furthermore, the silencing of PirB was also found to abolish the hyperoxia-induced apoptosis of OPCs, indicating that E2 treatment or silencing of PirB can protect OPCs against hyperoxia-induced apoptosis, which was consistent with previous studies (8,23,33,34).

In order to further investigate how hyperoxia induces OPC apoptosis, western blot analysis was performed to measure the protein expression levels of Akt, caspases-3 and -8, Fas and p-Akt. GAPDH was used as the loading control. As shown in Fig. 4, the expression levels of caspases-3 and -8, and Fas, increased significantly following oxygen treatment, whereas the expression of p-Akt significantly decreased. E2 treatment partially reversed the hyperoxia-induced upregulation of caspases-3 and -8, and Fas, as well as the downregulation of p-Akt. Furthermore, the silencing of PirB was also found to reverse the hyperoxia-induced upregulation of caspases-3 and -8, and Fas, as well as the downregulation of p-Akt. Based on the present and previous results (11-14), it was concluded that hyperoxia resulted in the upregulation of caspases-3 and -8, and Fas and the downregulation of p-Akt, leading to cell damage and apoptosis.

Hyperoxia was found to increase the apoptosis and decrease the survival rate of OPCs. OPC apoptosis was induced by hyperoxia via the upregulation of caspases-3 and -8, and Fas. E2 treatment significantly downregulated the expression of PirB. The downregulation of PirB, either by E2-treatment or PirB silencing, markedly decreased hyperoxia-induced apoptosis, increased cell viability, and decreased the expression of caspases-3 and -8, and Fas in OPCs, indicating that E2 can

protect against hyperoxia-induced apoptosis, predominantly via the downregulation of PirB in OPCs.

Acknowledgements

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