



rhEPO Upregulates the PPAR γ Pathway in Long-term Cultured Primary Nerve Cells via PI3K/Akt to Delay Cell Senescence

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Abstract

Previous studies have confirmed that both recombinant human erythropoietin (rhEPO) and peroxisome proliferator-activated receptors γ (PPAR γ) activator pioglitazone can protect senescent nerve cells, and their mechanisms involve enhancing cell antioxidant capacity and reducing cell apoptosis. However, whether the PPAR γ pathway is involved in the rhEPO anti-aging process in neuronal cells is still unclear. In this study, to explore the relationship between rhEPO and the PPAR γ pathway at the cellular level, primary nerve cells cultured for 22 days were used to simulate the natural aging process of nerve cells. Starting on the 11th day of culture, rhEPO, LY294002, and GW9662 were added for treatment. Immunochemical methods and SA- β -gal staining were used to observe the changes in cellular antioxidant capacity and the fraction of senescent cells. The results showed that PPAR γ blockade retarded the effect of rhEPO on the cellular antioxidant capacity and altered the fraction of senescent cells. It was confirmed that PPAR γ was involved in rhEPO's anti-aging process in neuronal cells. Real-time fluorescent quantitative RT-PCR, Western blotting, and immunofluorescence staining were used to observe the changes in PPAR γ pathway-related factors in nerve cells after rhEPO treatment. The results showed that rhEPO significantly upregulated the expression of PPAR γ coactivator-1 α (PGC-1 α), PPAR γ , and nuclear PPAR γ in cells but did not affect the level of phosphorylated PPAR γ protein, confirming that rhEPO has the ability to upregulate the PPAR γ pathway. PI3K/Akt and PPAR γ pathway blockade experiments were used to explore the relationships among rhEPO, PI3K/Akt, and PPAR γ . The results showed that after PPAR γ blockade, rhEPO had no significant effect on the PI3K/Akt pathway-related factor p-Akt, while after PI3K/Akt blockade, rhEPO's effects on PPAR γ -related factors (PGC-1 α , PPAR γ , and nuclear PPAR γ) were significantly decreased. It is suggested that rhEPO delays the PI3K/Akt pathway in the process of neuronal senescence, which is located upstream of PPAR γ regulation. In conclusion, this study confirmed that rhEPO can upregulate the expression of PGC-1 α and PPAR γ in cells and the level of PPAR γ protein in the nucleus to enhance the antioxidant capacity of cells and delay the senescence of nerve cells through the PI3K/Akt pathway. These findings will provide ideas for finding new targets for neuroprotection research and will also provide a theoretical basis and experimental evidence for rhEPO anti-aging research in neural cells.

Keywords Erythropoietin · Aging · Nerve cells · Peroxisome proliferator-activated receptors γ · PI3K/Akt pathway

Erythropoietin (EPO) is an important glycoprotein hormone in the body that is widely distributed in the central and peripheral nervous systems (Genc et al. 2004). Studies have shown that EPO has neuroprotective effects in various forms of nerve damage resulting from ischemia and hypoxia (Sirén

et al. 2001; Simon et al. 2019), exogenous toxic substances (Mammis et al. 2009), primary or secondary inflammatory reactions (Nagańska et al. 2010), and nervous system aging (Wang et al. 2017). The PI3K/Akt signaling pathway exerts a wide range of biological effects in the nervous system (Feng et al. 2021; Tang et al. 2019). A number of studies have found that EPO can exert its biological effects through the PI3K/Akt pathway under both basic and stress conditions (Shen et al. 2010; Si et al. 2019).

Peroxisome proliferator-activated receptor γ (PPAR γ) is a type of nuclear transcription factor activated by ligands and is a member of the nuclear receptor superfamily.

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Activated PPAR γ combines with peroxisome response elements (PPAR response elements (PPREs)) in the promoter region of the gene to initiate transcription of target genes and play a biological role (Berger and Moller 2002; Lehrke and Lazar 2005). Recent experiments have shown that the activation of the PPAR γ pathway has a significant protective effect on various acute and slow forms of damage in the central nervous system and may be a new target for the treatment of various diseases, such as ischemic cerebrovascular disease (Li et al. 2020), Alzheimer's disease (Khan et al. 2019), Parkinson's disease (Corona and Duchon 2015), and multiple sclerosis (Liu et al. 2020). Its mechanism involves regulating oxidative stress (Collino et al. 2006), inhibiting inflammation (Lecca et al. 2015), and reducing cell apoptosis (Kaundal and Sharma 2011). Our previous studies have shown that activation of the PPAR γ pathway can enhance the antioxidant capacity of cells, reduce cell apoptosis, and protect senescent long-term cultured primary nerve cells (Wang et al. 2019).

The mechanism involved in the neuroprotection of EPO is similar to the neuroprotection mechanism activated by PPAR γ , but it is still unclear whether there is a cross-connection between the two. Based on this, this study aimed to investigate and clarify the relationship between recombinant human erythropoietin (rhEPO) and the PPAR γ pathway at the cellular level by observing the changes in PPAR γ pathway-related factors and the fraction of senescent cells in the long-term culture of primary nerve cells after rhEPO treatment to find new targets for neural anti-aging research.

Materials and Methods

Long-term Culture of Primary Nerve Cells (Wang et al. 2017, 2019)

One-day-old SD rats (Animal Experiment Center of Wuhan Buffal Biotechnology Co., Ltd., No. 42000600012249) were killed by cervical dislocation. The cerebral cortex was digested with 0.125% trypsin, filtered, and centrifuged for 5 min (800 n/min), and Neurobasal (Invitrogen Gibco, New York, USA) + B27 medium (Invitrogen Gibco, New York, USA) was used to prepare the cell suspension. The cells were inoculated in culture wells treated with polylysine and cultivated at 37 °C at 5% CO₂ with a saturated humidity. Half of the medium was changed every 2 days for a total of 22 days of culture. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Xi'an Jiaotong University.

Experimental Grouping

For the aging control group, the cells were cultured for a total of 22 days, representing aging nerve cells. For the EPO group, rhEPO (100 U/ml, PeproTech, Rocky Hill, USA) was added to the cells on the 11th day (Wang et al. 2017). For the EPO + LY294002 group, LY294002 (the PI3K/Akt inhibitor) (5 μ M, Selleck, Houston, USA) and rhEPO (100 U/ml, PeproTech, Rocky Hill, USA) were added starting on the 11th day. For the LY294002 group, LY294002 (5 μ M, Selleck, Houston, USA) was added on the 11th day (Petrozziello et al. 2017). For the EPO + GW9662 group, GW9662 (the PPAR γ inhibitor) (1 μ M, Selleck, Houston, USA) and rhEPO (100 U/ml, PeproTech, Rocky Hill, USA) were added starting on the 11th day. For the GW9662 group, GW9662 (1 μ M, Selleck, Houston, USA) was added starting on the 11th day (Wang et al. 2019). For the pioglitazone group, pioglitazone (the PPAR γ activator) (1 μ M, Selleck, Houston, USA) was added starting on the 11th day (Wang et al. 2019).

Observations of the Antioxidant Capacity by Immunochemical Methods (Wang et al. 2017)

Superoxide dismutase (SOD) and glutathione (GSH) are important intracellular antioxidant factors. Malondialdehyde (MDA) is the final product of intracellular oxidation reactions. They represent the intracellular antioxidant levels. At the measured time points, RIPA cell lysis buffer (Beyotime, Shanghai, China, P0013B) was added to lyse the cells (400 μ l/bottle, shaken 30 min). The lysis buffer was centrifuged for 5 min (12,000 rpm, 4 °C), the precipitate was discarded, and the supernatant was retained. The protein concentration was determined by BCA method (Beyotime, Shanghai, China, P0010). The SOD activity and GSH and MDA contents were determined using SOD assay kits (Jiancheng Bioengineering, Nanjing, China, A001-1), GSH detection kits (Jiancheng Bioengineering, Nanjing, China, A006-2), and MDA assay kits (Jiancheng Bioengineering, Nanjing, China, A003-1).

Observation of the Ratio of Aged Cells by SA- β -gal Staining

Cover slips coated with cells were washed three times with PBS for 3 min each. The cells were fixed for 15 min with 4% paraformaldehyde, and the cover slip was washed again three times with PBS for 3 min each. Subsequently, 100 μ l of senescence-associated (SA)- β -gal staining solution (Nanjing KeyGen Biotech Co. Ltd., Nanjing, China) was added to each cover slip, which was then incubated in the dark at 37 °C in a CO₂ incubator for 10 h to allow the reaction to

proceed. The cover slip was then washed three times with PBS, the solution on the slip was absorbed with a piece of absorbent paper, and the slide was sealed with aqueous mounting medium. The cells were observed under a light microscope (200×), and cells in 5 fields were counted. The ratio of SA-β-gal-positive cells represented the ratio of aged cells.

Real-time Fluorescent Quantitative RT-PCR Detection of PPAR γ and PGC-1 α mRNA

Total RNA was extracted from the cells using TRIzol reagent (Aidlab, Beijing, China), and complementary DNA was then reverse-transcribed from 1 μ g of total RNA with a reverse transcription kit (Vazyme Biotech Co., Ltd., Nanjing, China) according to the manufacturer's instructions. PCR was performed using SYBR Green Master Mix (Vazyme Biotech Co., Ltd., Nanjing, China), and β -actin served as the internal control. The relative expression levels of PPAR γ and PGC-1 α were determined using the $2^{-\Delta\Delta C_t}$ method and are shown as fold changes compared to the controls. The primers were 5'-AGGGCGATCTTGACAGGAAA-3' (forward) and 5'-CGAAACTGGCACCCCTTGAAA-3' (reverse) for PPAR γ , 5'-CACTACAGACACCGCACACA-3' (forward) and 5'-CAGACT CCCGCTTCTCA TAC-3' (reverse) for PGC-1 α , and 5'-CACGATGGAGGGGCCGGACTCATC-3' (forward) and 5'-TAAAGACCTCTATGCCAACACAGT-3' (reverse) for β -actin. All primers were designed and synthesized by Tianyi Biotech (Wuhan, China). The cycling conditions included heat treatment at 95 °C for 10 min, one cycle of 95 °C for 1 min, and 40 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min, followed by one cycle at 72 °C for 10 min. The specificity of amplification was assessed by melting curve analysis and gel electrophoresis.

Western Blot Detection of Phosphorylated Akt(p-Akt), PPAR γ , PGC-1 α , and PPAR γ (p-PPAR γ)

The cells were lysed with RIPA lysate, the protein was extracted, and the protein concentration was measured with a DG-3022A microplate reader. After the electrophoresis gel was prepared, the prepared protein samples and marker were loaded into the wells with a micropipette, with the total protein content of each sample of 40 μ g. After loading the samples, the gels were electrophoresed at a constant voltage of 80 V until the bromophenol blue indicator dye formed a line at the junction of the spacer gel and the separation gel (p-Akt and PGC-1 α , 8% gel; PPAR γ and p-PPAR γ , 10% gel), after which the voltage was changed to 120 V until the bromophenol blue reached the bottom of the gel. The gel was removed, and the target band was excised according to the marker, followed by rinsing with distilled water and transfer to a membrane. For Western blot hybridization

after transfer, the following primary antibodies were used: rabbit anti-rat p-Akt antibody (1:1000, species: rabbit, CST, Boston, USA), rabbit anti-rat PGC-1 α antibody (1:1000, species: rabbit, Thermo Fisher Scientific, Waltham, USA), rabbit anti-rat PPAR γ antibody (1:1000, species: rabbit, Sanyin Biological Technology Co. Ltd., Wuhan, China), rabbit anti-rat p-PPAR γ (1:300, species: rabbit, Santa Cruz Biotechnology, Santa Cruz, USA), mouse anti-rat β -actin (1:200, species: Mouse, Boster Biological Technology Co. Ltd., Wuhan, China), and rabbit anti-rat Lamin B antibody (1:200, species: rabbit, Boster Biological Technology Co. Ltd, Wuhan, China). The primary antibodies were incubated with the membrane overnight at 4 °C. After washing, HRP goat anti-rabbit (1:5000, species: goat, Boster Biological Technology Co. Ltd., Wuhan, China) and HRP goat anti-mouse (1:5000, species: goat, Boster Biological Technology Co. Ltd., Wuhan, China) secondary antibodies were added. The ECL chemiluminescence method was used to detect protein bands, and the image was recorded by a gel imager. The BandScan system was used to analyze the film gray value.

PPAR γ Immunofluorescence Staining

After fixation with 4% paraformaldehyde for 15 min, immersion and washing were performed, and 0.5% Triton X-100 was used for permeabilization at room temperature for 20 min. Goat serum was used for blocking for 30 min. The primary antibody against PPAR γ (1:50, species: rabbit, Sanyin Biological Technology Co. Ltd., Wuhan, China) was added and incubated for 24 h at 4 °C in a humid box. Fluorescence (Cy3)-labeled goat anti-rabbit IgG (1:100, species: goat, Boster Biological Technology Co. Ltd., Wuhan, China) was added, and incubated at 37 °C for 1 h in a humid box. DAPI was added and incubated in the dark for 5 min, followed by mounting with mounting solution containing an anti-fluorescence quencher. The cells were observed under a laser scanning confocal microscope, and images were collected. CY3 exhibits red fluorescence with an excitation wavelength of 550 nm and an emission wavelength of 570 nm, and DAPI exhibits blue fluorescence with an excitation wavelength of 340 nm and an emission wavelength of 488 nm. IPP6.0 software was used to analyze the optical density of the immunofluorescence photos.

Statistical Analysis

All data were analyzed using SPSS (version 23.0) software. All experiments were performed in triplicate, and the data are presented as the mean \pm standard deviation (SD). Data were analyzed using one-way analysis of variance (ANOVA). The level of statistical significance was set at $P < 0.05$.

Results

PPAR γ Participates in the Process by Which rhEPO Enhances the Antioxidant Capacity of Cells and Delays the Aging Process of Nerve Cells

Superoxide dismutase (SOD) and glutathione (GSH) are important intracellular antioxidant factors. Malondialdehyde (MDA) is the final product of intracellular oxidation reactions. They represent the intracellular antioxidant levels. To clarify whether PPAR γ is involved in the process by which rhEPO enhances the antioxidant capacity of long-term cultured primary nerve cells, immunochemical methods were used to determine the changes in cellular antioxidant factors SOD activity and GSH and MDA content after rhEPO and GW9662 treatment (Fig. 1). The results showed that the SOD activity ($P=0.0015$) and GSH content ($P=0.0003$) of the cells in the EPO group were significantly higher than those in the aging control group, while the MDA content was significantly reduced ($P=0.0005$). SOD activity ($P=0.0311$) and GSH content ($P=0.0036$) of the cells in the EPO + GW9662 group were significantly lower than those in the EPO group, while the MDA content increased significantly ($P=0.0103$), but each index still did not reach the corresponding index level of the aging control group (SOD: $P=0.0110$; GSH: $P=0.0033$; MDA: $P=0.0044$). SOD activity ($P=0.0029$) and GSH content ($P=0.0160$) in the GW9662 group were significantly lower than those of the aging control group, while the MDA content was significantly increased ($P=0.0051$). All indicators of the pioglitazone group were better than those of the aging

control group (SOD: $P=0.0193$; GSH: $P=0.0066$; MDA: $P=0.0013$), but worse than of the EPO group (SOD: $P=0.0100$; GSH: $P=0.0013$; MDA: $P=0.0321$).

To observe whether PPAR γ is involved in the process by which rhEPO reduces the fraction of senescent cells in long-term cultured primary neural cells, SA- β -gal staining was used to detect the changes in the fraction of senescent cells in each group after rhEPO and GW9662 treatment (Fig. 2). The results showed that the fraction of senescent cells in the EPO group was significantly lower than that in the aging control group ($P=0.0001$). The fraction of senescent cells in the EPO + GW9662 group was significantly higher than that in the EPO group ($P=0.0131$), but still lower than that in the aging control group ($P=0.0001$). The fraction of senescent cells in the GW9662 group was significantly higher than that in the EPO group ($P<0.0001$) and the EPO + GW9662 group ($P=0.0001$). The fraction of senescent cells in the pioglitazone group was lower than that in the aging control group ($P=0.0001$), but higher than that in the EPO group ($P=0.0058$).

Inhibition of PPAR γ Does Not Affect rhEPO Activation of the PI3K/Akt Pathway

Akt phosphorylation is a sign of PI3K/Akt pathway activation (He et al. 2021). To clarify whether PPAR γ affects rhEPO to regulate the activation of the PI3K/Akt pathway in the process of nerve cell aging, Western blotting was used to detect the expression of phosphorylated Akt (p-Akt) protein in nerve cells after PPAR γ inhibition (Fig. 3). The results showed that the p-Akt protein expression levels of the EPO group ($P=0.0015$) and EPO + GW9662 group ($P=0.0011$)

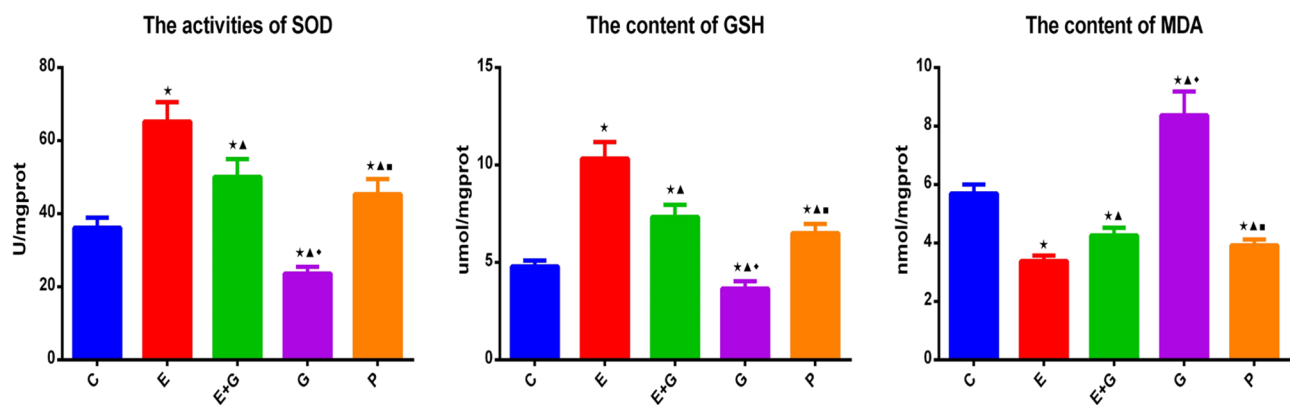


Fig. 1 Changes in SOD activity, GSH content, and MDA content under different treatment conditions. C: Aging control group; E: EPO group; E + G: EPO + GW9662 group; G: GW9662 group; P: pioglitazone group. Immunochemical methods were used to determine the cellular antioxidant enzyme SOD activity, GSH content, and cell peroxidation product MDA content. Data are shown as the mean \pm SD

values obtained from three separate experiments. Statistical analysis was performed by one-way ANOVA. The results show that the long-term cultured primary nerve cells had a significant increase in antioxidant capacity after rhEPO treatment, while the antioxidant capacity was significantly weakened after PPAR γ blocker GW9662 treatment. * $P<0.05$ vs. C; $\blacktriangle P<0.05$ vs. E; $\blacklozenge P<0.05$ vs. E + G

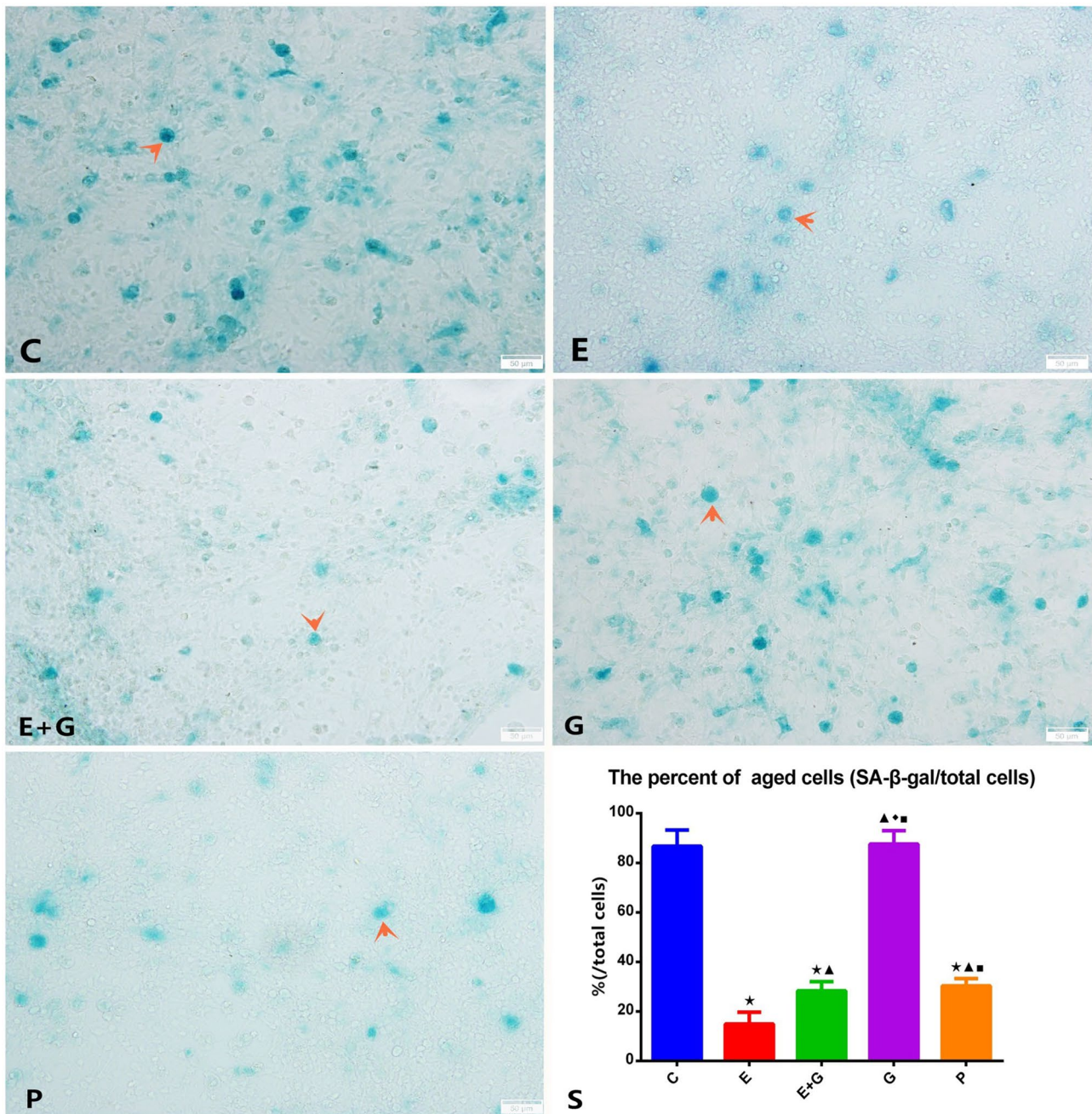


Fig. 2 Senescence of nerve cells in each group. C: Aging control group; E: EPO group; E+G: EPO+GW9662 group; G: GW9662 group; P: pioglitazone group. Senescent cells in each group were observed under an ordinary light microscope (200×magnification, scale=50 μm). SA-β-gal-positive cells that were stained blue represent senescent cells. Representative images of each group. S: Statistical analysis of the fraction of SA-β-gal-positive cells in each

group. Data are shown as the mean±S.D. values obtained from three separate experiments. Statistical analysis was performed by one-way ANOVA. The results showed that the fraction of senescent cells after EPO treatment was significantly lower than that in aging control group, while the fraction of senescent cells was significantly increased after GW9662 treatment. * $P < 0.05$ vs. C; ▲ $P < 0.05$ vs. E; ◆ $P < 0.05$ vs. E+G

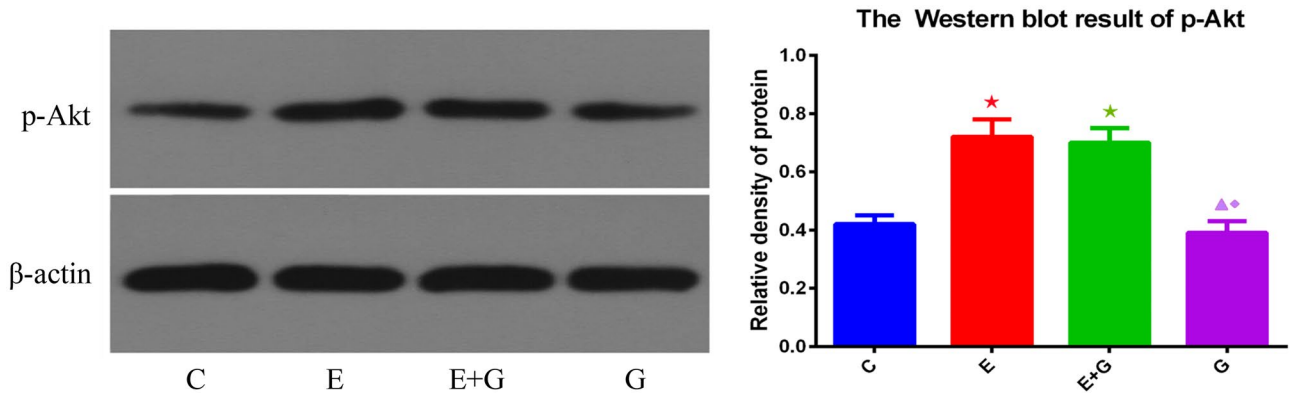


Fig. 3 The expression of p-Akt protein in primary cultured neural cells in each group. C: Aging control group; E: EPO group; E+G: EPO+GW9662 group; G: GW9662 group. Western blotting was used to observe the expression of p-Akt protein in nerve cells under different treatment conditions. Data are shown as the mean \pm SD values obtained from three separate experiments. Statistical analysis was

performed by one-way ANOVA. The results showed that the p-Akt protein of long-term cultured primary neural cells increased significantly after rhEPO treatment, and this effect did not decrease after GW9662 treatment. * $P < 0.05$ vs. C; ▲ $P < 0.05$ vs. E; ◆ $P < 0.05$ vs. E+G

were significantly higher than that of the aging control group. However, there was no significant difference between the EPO group and the EPO + GW9662 group ($P > 0.05$).

rhEPO Upregulates the Expression of PPAR γ and PGC-1 α mRNA in Long-term Cultured Primary Neural Cells Through the PI3K/Akt Pathway

PGC-1 α is an important transcriptional cofactor of PPAR γ and plays an important role in promoting the transcriptional activity of PPAR γ (Rowe et al. 2010). PGC-1 α and PPAR γ are key factors in the PPAR γ pathway. To clarify whether rhEPO upregulates the expression of PPAR γ and PGC-1 α mRNA in long-term cultured primary

neural cells through the PI3K/Akt pathway, real-time fluorescent RT-PCR was used to observe the expression of PPAR γ and PGC-1 α mRNA in nerve cells after rhEPO and LY294002 treatment (Fig. 4). The results showed that the mRNA expression of PPAR γ and PGC-1 α in the EPO group was significantly higher than that in the aging control group (PPAR γ : $P = 0.0002$; PGC-1 α : $P = 0.0003$), while the expression of PPAR γ and PGC-1 α mRNA in the EPO + LY294002 group was significantly lower than that in the EPO group (PPAR γ : $P = 0.0007$; PGC-1 α : $P = 0.0020$). The mRNA expression of PPAR γ and PGC-1 α in the LY294002 group was significantly lower than that in the aging control group (PPAR γ : $P = 0.008$; PGC-1 α : $P = 0.0011$).

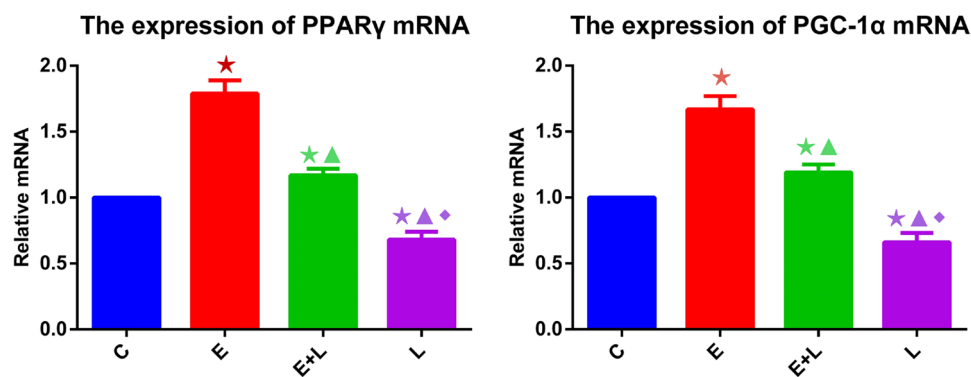


Fig. 4 The expression of PPAR γ and PGC-1 α mRNA in primary cultured neural cells in each group. C: Aging control group; E: EPO group; E+L: EPO+LY294002 group; L: LY294002 group. Real-time fluorescent RT-PCR was used to observe the expression of PPAR γ and PGC-1 α mRNA in long-term cultured primary nerve cells under different treatment conditions. Data are shown as the

mean \pm SD values obtained from three separate experiments. Statistical analysis was performed by one-way ANOVA. The results showed that the expression levels of PPAR γ and PGC-1 α mRNA increased significantly after rhEPO treatment, while the expression of both decreased significantly after treatment with LY294002. * $P < 0.05$ vs. C; ▲ $P < 0.05$ vs. E; ◆ $P < 0.05$ vs. E+L

rhEPO Upregulates the Protein Expression of PGC-1 α and PPAR γ in Long-term Cultured Primary Neural Cells Through the PI3K/Akt Pathway But Does Not Affect the Phosphorylation Level of the PPAR γ Protein

PGC-1 α protein plays an important role in promoting the transcriptional activity of PPAR γ . To determine whether rhEPO upregulates the expression of PGC-1 α in senescent long-term cultured primary neurons through the PI3K/Akt pathway, Western blotting was used to detect changes in the PGC-1 α protein (Fig. 5a) in nerve cells after rhEPO and LY294002 treatment. The results showed that the PGC-1 α protein level in the EPO group was significantly higher than that in the aging control group ($P=0.0409$), while the PGC-1 α protein level in the EPO + LY294002 group was significantly lower than that in the EPO group ($P=0.0213$). However, there was no significant difference compared with the aging control group ($P>0.05$). The protein level of PGC-1 α in the LY294002 group was significantly lower than that of the aging control group ($P=0.0006$).

The expression level of PPAR γ protein is an important indicator of PPAR γ pathway activation in cells (Berger and Moller 2002). To clarify whether rhEPO upregulates the expression of PPAR γ in long-term cultured primary neurons through the PI3K/Akt pathway, Western blotting was used to detect the expression of whole PPAR γ protein (w-PPAR γ) in nerve cells after rhEPO and LY294002 treatment (Fig. 5b). The results showed that the w-PPAR γ protein level of the EPO group was significantly higher than that of the aging control group ($P=0.0062$). The w-PPAR γ protein level of the EPO + LY294002 group was significantly lower than that of the EPO group ($P=0.0292$), but there was no significant difference with the aging control group ($P>0.05$). The w-PPAR γ protein level in the LY294002 group was significantly lower than that of the aging control group ($P=0.0080$).

Phosphorylation of PPAR γ is an important modification pathway of the PPAR γ protein (Montanari et al. 2020) and is one of the main factors affecting the transcriptional activity of the PPAR γ pathway. The results of Western blotting in this study showed that the levels of phosphorylated PPAR γ

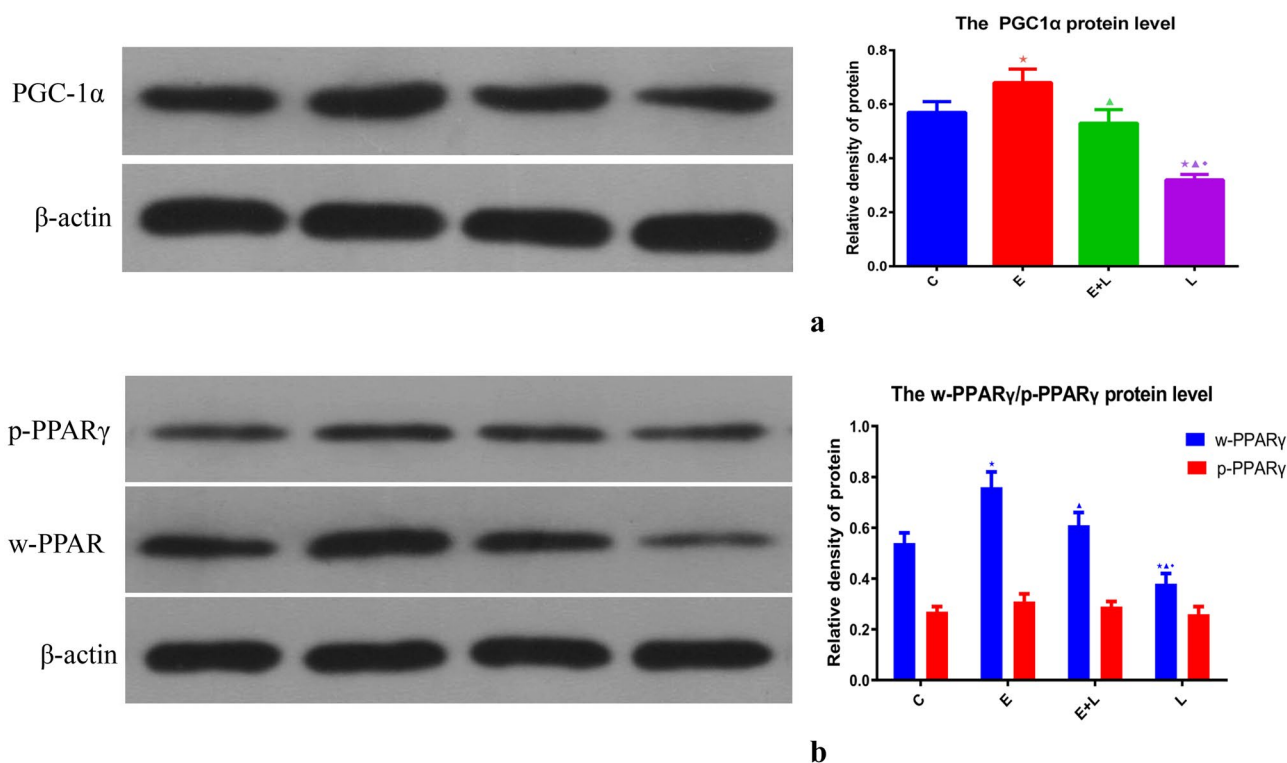


Fig. 5 The expression of PGC-1 α , whole PPAR γ (w-PPAR γ), and phosphorylated PPAR γ (p-PPAR γ) in primary cultured neural cells in each group. C: Aging control group; E: EPO group; E+L: EPO + LY294002 group; L: LY294002 group. **a** PGC-1 α expression. **b** w-PPAR γ and p-PPAR γ expression. Western blotting was used to observe the expression of PGC-1 α , w-PPAR γ , and p-PPAR γ in nerve cells under different intervention conditions. Data are shown as the mean \pm SD values obtained from three separate experiments. Sta-

tistical analysis was performed by one-way ANOVA. The results showed that PGC-1 α and w-PPAR γ proteins in long-term cultured primary nerve cells increased significantly after rhEPO treatment, and their expression levels were significantly reduced after treatment with LY294002. There was no significant change in the level of w-PPAR γ under different interventions. $\star P<0.05$ vs. C; $\blacktriangle P<0.05$ vs. E; $\blacklozenge P<0.05$ vs. E+L

(p-PPAR γ) protein in nerve cells in each group were not significantly different ($P > 0.05$) (Fig. 5b).

rhEPO Increases the Level of PPAR γ Protein in the Nucleus of Long-term Cultured Primary Nerve Cells Through the PI3K/Akt Pathway

PPAR γ enters the nucleus to bind to PPREs, and thus, the level of PPAR γ protein in the nucleus represents the ability of PPAR γ to initiate gene transcription (Orzechowska-Jastrzab et al. 2019). To determine whether rhEPO can increase the PPAR γ protein level in the nucleus of long-term cultured primary nerve cells through the PI3K/Akt pathway, immunofluorescence staining was used to observe the distribution of PPAR γ in long-term cultured primary nerve cells after rhEPO and LY294002 treatment. The results showed that PPAR γ was distributed in the cytoplasm and nucleus of primary nerve cells in each group (Fig. 6a). Statistical analysis demonstrated that the PPAR γ expression intensity and nuclear-positive cell fraction of the EPO group were significantly higher than those of the aging control group (cellular PPAR γ expression intensity: $P = 0.0257$; nuclear-positive cell fraction: $P = 0.0080$), while the expression intensity of PPAR γ and the fraction of nuclear-positive cells in the EPO + LY294002 group were significantly lower than those in the EPO group (PPAR γ expression intensity of cells: $P = 0.0184$; fraction of nuclear-positive cells: $P = 0.0312$), but there was no significant difference with the aging control group. The expression intensity of PPAR γ and the fraction of nuclear-positive cells in the LY294002 group were significantly lower than those of the aging control group (the expression intensity of PPAR γ in cells: $P = 0.0424$; the fraction of nuclear-positive cells: $P = 0.0443$) (Fig. 6b).

To further observe the effect of rhEPO on the PPAR γ protein level in the nucleus of long-term cultured primary nerve cells through PI3K/Akt, Western blotting was used to detect the PPAR γ protein level in the nerve cell nucleus (n-PPAR γ) after rhEPO and LY294002 treatment (Fig. 6c). The results showed that the n-PPAR γ protein level of the EPO group was significantly higher than that of the aging control group ($P = 0.0044$), while the n-PPAR γ level of the EPO + LY294002 group was significantly lower than that of the EPO group ($P = 0.0022$), but there was no significant difference with the aging control group. The level of n-PPAR γ in the LY294002 group was significantly lower than that of the aging control group ($P = 0.0007$).

Discussion

PPAR γ is a nuclear transcription factor activated by ligands. Activated PPAR γ enters the nucleus and binds to PPREs under the combined action of coactivators, initiates

target gene transcription, and participates in various physiological activities of the body (Harada et al. 2015; Zhu et al. 2021; Shang et al. 2020; Álvarez-Almazán et al. 2017). Existing studies have shown that PPAR γ pathway activation has an important neuroprotective effect on a variety of central nervous system pathological changes and have proposed that PPAR γ may be a therapeutic target for neurodegenerative diseases (Li et al. 2020; Khan et al. 2019; Corona and Duchon 2015; Liu et al. 2020; Katsouri et al. 2012). Our previous studies have shown that after treatment with the PPAR γ inhibitor GW9662, the antioxidant capacity of long-term cultured primary nerve cells decreases and the apoptotic cell fraction increases, while after treatment with the PPAR γ agonist pioglitazone, the cell antioxidant capacity significantly increases and the apoptotic cell fraction decreases significantly. It is suggested that the PPAR γ pathway is involved in the protection from senescence and the long-term culture of primary nerve cells and that it is one of the protective pathways of aging nerve cells (Wang et al. 2019). Another study of ours showed that the mechanism by which rhEPO protects senescent nerve cells is the same as that of the PPAR γ pathway activator pioglitazone, which also involves the enhancement of intracellular antioxidant capacity (Wang et al. 2017). However, whether the PPAR γ pathway is involved in the anti-senescence process of rhEPO in neuronal cells has not yet been reported. This study showed that the antioxidant capacity of cells in the EPO + GW9662 group was significantly lower than that of the EPO group and that the fraction of senescent cells was significantly increased. It is suggested that the PPAR γ pathway is involved in the physiological process of rhEPO's enhancement of the antioxidant capacity of cells and protection against senescence in the long-term culture of primary nerve cells.

The transcriptional activity of PPAR γ is affected by the expression level of PPAR γ (Berger and Moller 2002), protein phosphorylation (Montanari et al. 2020), and the intensity of ligand stimulation (Santos et al. 2021). The level of PPAR γ in the nucleus (Orzechowska-Jastrzab et al. 2019) and transcription cofactors such as PGC-1 α (Zhang et al. 2018) are also critical. To further explore the effect of rhEPO on the PPAR γ pathway in long-term cultured primary nerve cells, this study compared the expression of the PPAR γ pathway-related factors PPAR γ and PGC-1 α after rhEPO treatment. Both RT-PCR and Western blotting results showed that rhEPO significantly enhanced the expression of the PPAR γ pathway-related factors PPAR γ and PGC-1 α in nerve cells and increased the level of PPAR γ in the nucleus but did not affect the phosphorylation of PPAR γ . This proves that rhEPO can significantly increase PPAR γ pathway activity in long-term cultured nerve cells, but the mechanism is still unclear.

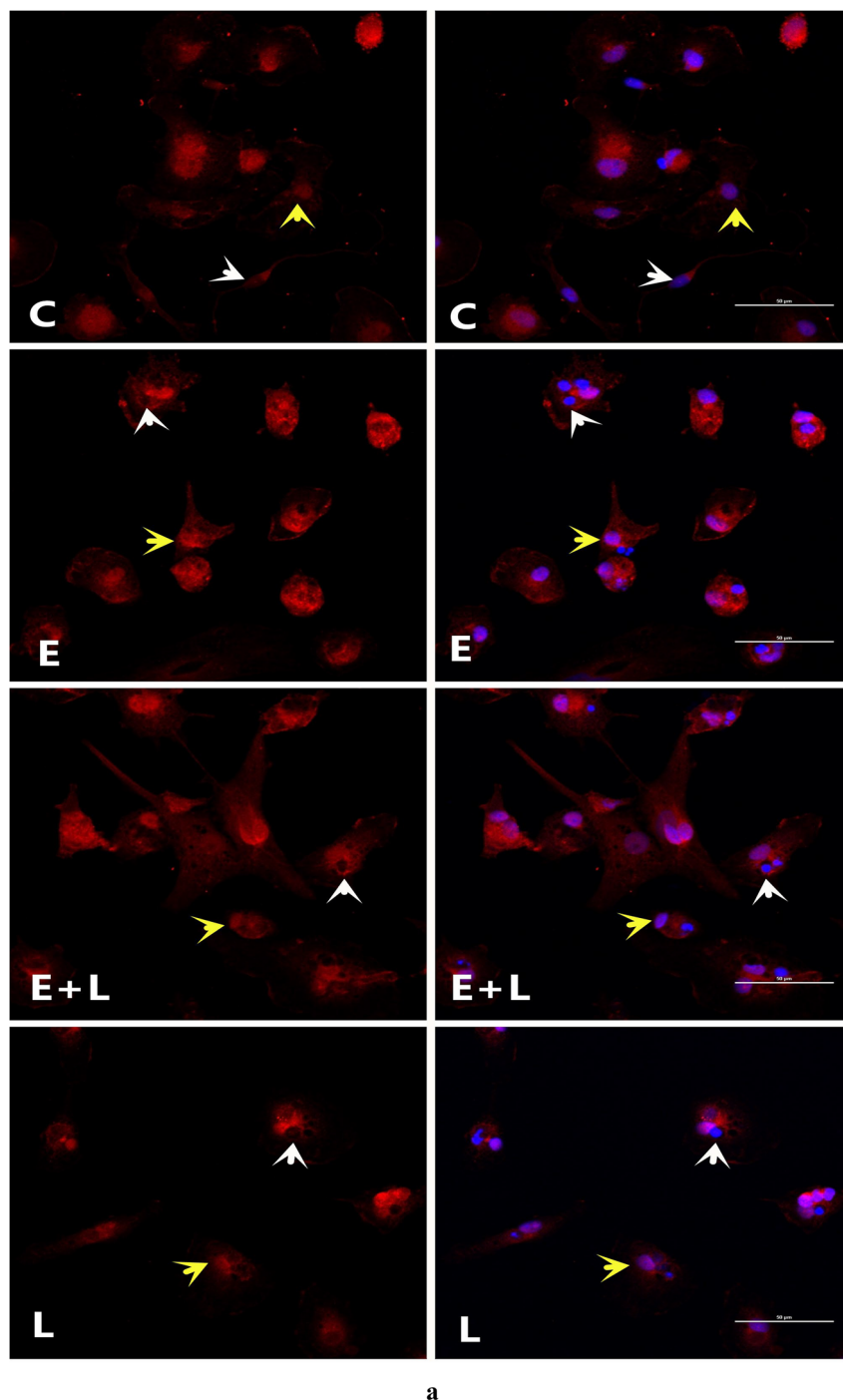


Fig. 6 The protein levels of PPAR γ in the nucleus (n-PPAR γ) of cultured primary nerve cells in each group. **C**: Aging control group; **E**: EPO group; **E+L**: EPO+LY294002 group; **L**: LY294002 group. **a** The results of the immunofluorescence staining showed the distribution of PPAR γ in cells of each group (confocal laser microscope, 600 \times magnification). The left image is PPAR γ immunofluorescence staining, and the right image is the merged image of DAPI nuclear staining and PPAR γ staining. The yellow arrow points to the PPAR γ nuclear staining-positive cells; the white arrow points to the PPAR γ nuclear staining-negative cells. Representative images of each group. **b** PPAR γ immunofluorescence staining intensity of cells in each group and a statistical graph of the fraction of PPAR γ nuclear staining-positive cells. The results showed that PPAR γ was distributed in

the cytoplasm and nucleus of long-term cultured primary nerve cells. After rhEPO treatment, the expression level of nerve cell PPAR γ and the fraction of nucleus-positive cells increased significantly, and this phenomenon was significantly reduced after LY294002 treatment. **c** The n-PPAR γ protein level of cells in each group. Western blotting was used to observe the expression of n-PPAR γ protein in nerve cells under different treatment conditions. The results showed that the n-PPAR γ level of nerve cells increased significantly after rhEPO treatment, and LY294002 treatment inhibited this phenomenon. Data are shown as the mean \pm SD values obtained from three separate experiments. Statistical analysis was performed by one-way ANOVA. * $P < 0.05$ vs. C; $\blacktriangle P < 0.05$ vs. E; $\blacklozenge P < 0.05$ vs. E+L

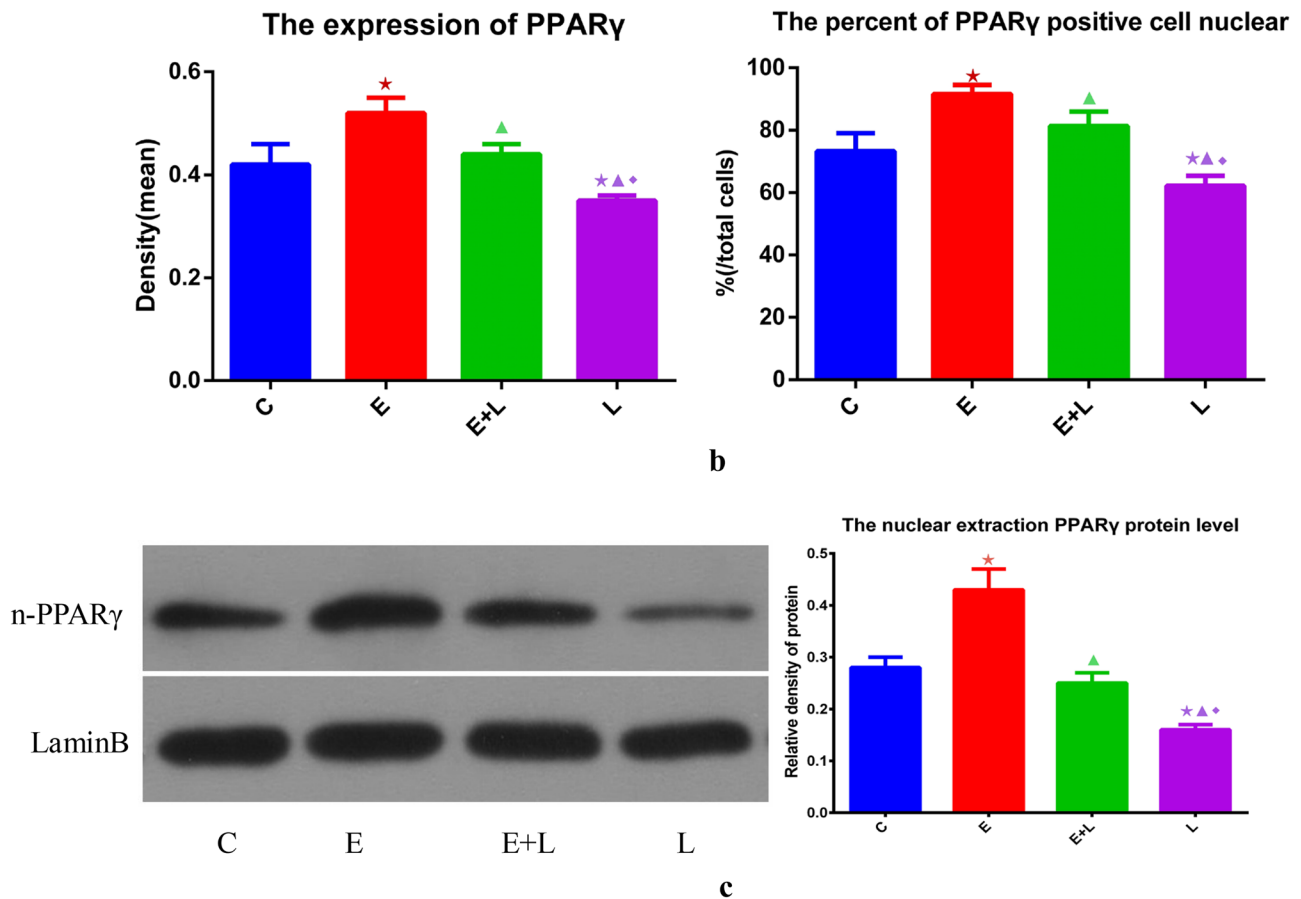


Fig. 6 (continued)

The PI3K/Akt pathway is one of the key pathways by which EPO protects nerve cells (Shen et al. 2010; Si et al. 2019; Ma et al. 2018), but it is not clear whether there is a connection between the PI3K/Akt pathway and the PPAR γ pathway in the process by which rhEPO protects senescent nerve cells. Previous studies have shown that the PI3K/Akt pathway is the downstream pathway of PPAR γ in most cases and that it can affect the activity of the PI3K/Akt pathway by regulating the phosphorylation level of Akt protein (Li et al. 2014, 2018; Lv et al. 2019). Based on this, we speculate that the PI3K/Akt pathway may also be a downstream pathway of PPAR γ in the process by which EPO protects senescent nerve cells. To verify this hypothesis, in this study, we first compared and observed the changes in the PI3K/Akt pathway-related protein p-Akt after rhEPO treatment while inhibiting the PPAR γ pathway. The results showed that there was no significant change in the protein expression of p-Akt between the EPO + GW9662 group and the EPO group. This suggests that the PI3K/Akt pathway is not located downstream of the PPAR γ pathway in the process of rhEPO's protection from senescence in the long-term culture of primary nerve cells, and its relationship with the PPAR γ

pathway is different from what has been demonstrated in previous studies.

The PI3K/Akt pathway affects the expression of a variety of proteins in cells (Xie et al. 2019; Xu et al. 2020), and PPAR γ pathway-related factors are regulated by a variety of proteins (Villapol 2018; Vallée and Lecarpentier 2018; Chen et al. 2019). Since the PI3K/Akt pathway is not downstream of rhEPO's activation of the PPAR γ pathway for protection from senescence in long-term cultured primary nerve cells, is it upstream of the factors that regulate the PPAR γ pathway? To solve this problem, we observed changes in the protein and mRNA levels of the PPAR γ pathway-related factors PPAR γ and PGC-1 α in long-term cultured primary nerve cells while inhibiting the PI3K/Akt pathway after rhEPO treatment. The results showed that the PGC-1 α , w-PPAR γ , and n-PPAR γ protein and mRNA levels in the EPO + LY294002 group were significantly lower than those in the EPO group, while the p-PPAR γ protein level did not change significantly. This result confirms that the PI3K/Akt pathway is upstream of the PPAR γ pathway in the process by which rhEPO protects nerve cells from senescence. This shows that rhEPO can upregulate the expression of PGC-1 α

and PPAR γ in cells and the level of PPAR γ protein in the nucleus by activating the PI3K/Akt pathway, and play a role in protecting senescent nerve cells. However, its specific regulatory molecular mechanism is still unclear and needs further study.

At the same time, we also found an interesting phenomenon in the study: the PGC-1 α and w-PPAR γ proteins in the EPO + LY294002 group were significantly lower than those in the EPO group, and there was no significant difference compared with the aging control group, but in the EPO + LY294002 group, the mRNA levels of PGC-1 α and PPAR γ were significantly lower than those of the EPO group, although they were still higher than those of the aging control group. This difference in protein and mRNA levels suggests that PI3K/Akt pathway inhibition at the protein level can almost completely block the effect of rhEPO on the PPAR γ pathway of nerve cells, but it does not achieve a complete blocking effect at the mRNA level. Analysis suggests that the reason for this phenomenon may be that PI3K/Akt pathway regulation of PPAR γ -related factors not only affects gene transcription but may also affect the translation and modification of related proteins by regulating the activity of certain modified enzymes. In addition, this study also found that the protein and mRNA levels of w-PPAR γ , n-PPAR γ , and PGC-1 α in the LY294002 group of nerve cells in which the PI3K/Akt pathway was inhibited were significantly lower than those in the aging control group. It is suggested that even without rhEPO treatment, the PI3K/Akt pathway may be one of the important upstream pathways that regulates the activity of the PPAR γ pathway in the process of neuronal senescence. This is the direction of our further research in the future.

In this study, by first observing the effects of rhEPO treatment in combination with a PPAR γ inhibitor to hinder changes in cell antioxidant capacity and the fraction of senescent cells, it was confirmed that PPAR γ participates in rhEPO's anti-aging process in neuronal cells. Furthermore, by observing the changes in PPAR γ pathway-related factors in long-term cultured primary nerve cells after rhEPO treatment, it was confirmed that rhEPO has the ability to upregulate the cellular PPAR γ pathway. Finally, PPAR γ and PI3K/Akt pathway blockade experiments confirmed that rhEPO delays the process of neuronal senescence and that the PI3K/Akt pathway is located upstream of PPAR γ regulation. In conclusion, this study confirms that rhEPO can upregulate the expression of PGC-1 α and PPAR γ in cells and the level of PPAR γ protein in the nucleus through the PI3K/Akt pathway, enhance cell antioxidant capacity, and delay the senescence of primary neurons in long-term culture, but its specific regulatory molecules are unclear, and further research is needed.

Author Contribution Huqing Wang, Xiaorui Yu, and Haiqin Wu designed the study. Ming Chen and Tao Zhang participated in several steps of the experiment: long-term culture of primary nerve cells and observations of the antioxidant capacity by immunochemical methods. Zhen Gao and Yu Gong participated in the SA- β -gal staining, immunofluorescence staining, RT-PCR, and Western blot. Huqing Wang participated in the whole process of the experiment and wrote the manuscript. Xiaorui Yu and Haiqin Wu revised the manuscript.

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Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics Approval and Consent to Participate This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Xi'an Jiaotong University.

Consent for Publication No conflict of interest exists in the submission of this manuscript, and this manuscript is approved by all authors for publication.

Competing Interests The authors declare no competing interests.

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