Pioglitazone protects PC12 cells against oxidative stress injury: An *in vitro* study of its antiapoptotic effects via the PPARγ pathway

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Abstract. To the best of our knowledge, the role of peroxisome proliferator-activated receptor γ (PPARγ) in oxidative stress-induced PC12 cell damage is unknown. Using a PC12 cell model with H_2O_2 treatment, the present study investigated the expression levels of apoptosis-related genes and neuronal apoptosis after oxidative stress injury. The present study further investigated the protective effect and mechanism of pioglitazone, a PPARγ agonist. PC12 cells treated with H_2O_2 were used as a model of oxidative stress injury. An MTT assay and flow cytometry were used to detect the effect of H_2O_2 on PC12 cell viability and the protective effect of pioglitazone. A TUNEL assay was used to detect neuronal apoptosis. The expression levels of PPARγ, Bax, Bcl-2 and caspase-3 were examined by reverse transcription-quantitative PCR and western blotting. H_2O_2 reduced PC12 cell viability in a dose- and time-dependent manner. H_2O_2 significantly upregulated the protein expression levels of Bax and the cleaved caspase-3/caspase-3 ratio ($P<0.01$), decreased the protein expression levels of Bcl-2 ($P<0.01$), and increased the apoptosis rate of PC12 cells. Pioglitazone significantly reduced the protein expression levels of Bax and the cleaved caspase-3/caspase-3 ratio ($P<0.01$), increased the expression levels of Bcl-2 ($P<0.01$), decreased the Bax/Bcl-2 expression ratio ($P<0.01$) and increased the viability of H_2O_2-damaged PC12 cells in a dose-dependent manner. Treatment with the PPARγ antagonist GW9662 or PPARγ small interfering RNA counteracted the protective effect of pioglitazone on PC12 cells to different extents ($P<0.01$). Therefore, the present study reported the role of PPARγ in protecting PC12 cells against oxidative stress injury, which may lead to novel therapeutic approaches for neurodegenerative diseases.

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Introduction

Neurodegenerative diseases resulting from the progressive loss of structure and/or function of neurons contribute to different paralysis degrees and loss of cognition (1). Progressive neuronal loss is a prominent pathological feature of neurodegenerative diseases, in which oxidative stress serves a vital role in neuronal apoptosis, and is difficult to restore (2). Neurons contain polyunsaturated fatty acids, which are sensitive to free radicals. Polyunsaturated fatty acids are easily attacked by free radicals, and neurons have a low content of antioxidant enzymes. Therefore, the antioxidant capacity of neurons is reduced (3). Together, these factors promote the sensitivity of neurons to oxidative stress injury (3,4). Methods to effectively reduce oxidative stress injury in neurons have attracted increasing attention. PC12 cells, which have the properties of neurosecretory cells and neurons, along with high stability, homogeneity and a high degree of differentiation, are currently widely used in the study of nerve cell function, differentiation, development and death as a cell model (5).

It is well known that apoptosis is a tightly regulated process, which involves changes in the expression of a distinct set of genes (6). Bax and Bcl-2 are major genes responsible for regulating apoptosis. Bax is a member of the Bcl-2 family, and promotes apoptosis, while Bcl-2 blocks cell death (7). The Bax/Bcl-2 ratio is a widely used parameter to determine cell susceptibility to apoptosis. Caspase-3 is the most important executing protease in the process of apoptosis (8). Cleaved caspase-3 is the activated form of caspase-3 (9). Previous studies have confirmed that H_2O_2 can induce PC12 cell injury (10) and the expression and activation of the apoptosis-related gene caspase-3 (11).

Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-activated nuclear receptor that regulates glucose and

lipid metabolism, endothelial function and inflammation (12). Thiazolidinediones (TZDs) are ligands that are known to bind to and activate nuclear PPARγ and are currently used as insulin sensitizers in type 2 diabetes (13). Our previous study has demonstrated that PPARγ agonists TZDs could protect the neuronal microenvironment and preserve nerve cells in the hippocampi of spontaneously hypertensive rats (SHRs) via antioxidative and antiapoptotic pathways (14). However, the underlying mechanism needs to be further studied. In the present study, a model of oxidative stress damage was generated in PC12 cells using H_2O_2 to observe whether pioglitazone had a neuroprotective effect and to determine the underlying mechanism.

Materials and methods

Cell culture and preconditioning protocols. PC12 cells were obtained from the Institute of Neurobiology, School of Medicine, Xi'an Jiaotong University (Xi'an, China). The cells were plated at a density of $3x10^5$ cells/well in 6-well plates and maintained in DMEM/F12 supplemented with 10% heat-inactivated foetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific, Inc.) at 37˚C under an atmosphere of 5% CO_2 and 95% air. The culture medium was changed three times per week. PC12 cells were preconditioned by 0, 6, 12, 24 or 36 h of exposure to different concentrations (0, 25, 50, 100, 200 and 400 μmol/l) of H_2O_2 at 37˚C. Experiments were performed at least three times.

Transfection experiments. A total of three alternative siRNA sequences targeting PPARγ were used to know down PPARγ protein expression. The following groups were used: NC group, siRNA-NC (non-targeting) group, PPARγ-siRNA 1 group, PPARγ-siRNA 2 group and PPARγ-siRNA 3 group. PPARγ-siRNAs and negative control siRNA were designed and synthesized by Shanghai GenePharma Co., Ltd. First, PC12 cells were seeded in 24-well plates at an optimized concentration of $1x10^5$ cells/well, 24 h before transfection. On the following day, when cell confluence had reached 60-70%, they were transfected with PPARγ-siRNA 1 (10 μM; 10 μl/well), PPARγ-siRNA 2 (10 μM; 10 μl/well), PPARγ-siRNA 3 (10 μM; 10 μl/well) or siRNA-NC (10 μM; 10 μl/well) using Lipofectamine® 2000 (cat. no. 11668019; Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 2 days according to the manufacturer's protocol. The sequence with the best inhibition rate, as determined by PPARγ protein expression analysis 48 h after transfection, was selected. The sequences are shown in Table I. Following 48 h of transfection, the cells were collected for subsequent experiments.

MTT assay

Experiment 1: Effect of H_2O_2 on the viability of PC12 cells. The viability of cultured cells was measured using an MTT assay (15). First, PC12 cells were preconditioned by 0, 6, 12, 24 or 36 h of exposure to different concentrations (0, 25, 50, 100, 200 and 400 μmol/l) of H_2O_2 at 37˚C. Subsequently, cells were incubated with MTT solution (0.5 mg/ml in PBS) at 37˚C for 4 h. The MTT solution was removed from the plate and the plate was dried. DMSO (100 μl) was added to each well to dissolve the formazan crystals before the optical density was measured at 570 nm. The results are presented as the percentage of MTT reduction, and the absorbance of the control cells was set as 100%. Experiments were performed at least three times.

Experiment 2: Protective effects of pioglitazone on PC12 cells with H_2O_2-induced injury. In this experiment, PC12 cells were divided into five groups: The control, H_2O_2 (100 μmol/l H_2O_2), low-concentration pioglitazone ($1x10^{-7}$ mol/l; cat. no. 111025-46-8; Alexis Biochemicals; Enzo Life Sciences), medium-concentration pioglitazone ($1x10^{-6}$ mol/l) and high-concentration pioglitazone ($1x10^{-5}$ mol/l) groups. Pioglitazone at different concentrations was utilized to precondition PC12 cells for a 1-h period at 37˚C, whereas the H_2O_2 group cells were instead treated with 0.9% saline; all the PC12 cells of H_2O_2 and piglitazone groups were then treated with 100 μM H_2O_2 for a 24 h period at 37˚C. For the control group, PC12 cells were treated with cell culture medium for 25 h at 37˚C. The viability of the cultured cells was measured by an MTT assay as aforementioned. Experiments were performed at least three times.

Experiment 3: Role of PPARγ in the protective effect of pioglitazone on PC12 cells with H_2O_2-induced injury. In this next experiment, PC12 cells were divided into five groups: The control, H_2O_2, pioglitazone + H_2O_2, pioglitazone + GW9662 + H_2O_2 and pioglitazone + PPARγ-siRNA 3 + H_2O_2 groups. In the H_2O_2 group, PC12 cells were treated with 100 μM H_2O_2 for 24 h at 37˚C. PC12 cells in the control group were treated with cell culture medium at 37˚C for 24 h. For the pioglitazone + H_2O_2 group, $1x10^{-5}$ mol/l pioglitazone was utilized to precondition PC12 cells for a 1-h period at 37˚C, and then cells were treated with 100 μM H_2O_2 for 24 h at 37˚C. The pioglitazone + GW9662+ H_2O_2 group was treated with GW9662 ($1x10^{-6}$ mol/l; cat. no. M6191; MilliporeSigma) for 1 h at 37˚C, followed by $1x10^{-5}$ mol/l pioglitazone for 1 h at 37˚C and H_2O_2 (100 μmol/l) for 24 h at 37˚C. For the pioglitazone + PPARγ-siRNA 3 + H_2O_2 group, after PPARγ-siRNA transfection, $1x10^{-5}$ mol/l pioglitazone was added to the cells for 1 h at 37˚C prior to treatment with H_2O_2 (100 μmol/l) for 24 h at 37˚C. The viability of cultured cells was measured by an MTT assay as aforementioned. Experiments were performed at least three times.

Measurement of malondialdehyde (MDA) content, and superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities. Based on the MTT assay results in Experiment 1, the desired H_2O_2 concentration, action time and cell plating density for modelling oxidative stress injury in PC12 cells were 100 μmol/l, 24 h and $1x10^5$ cells/ml, respectively. To verify the successful modelling of PC12 cells, the MDA content and SOD and GSH-Px activity were measured in the control and H_2O_2 groups. In the H_2O_2 group, PC12 cells were treated with 100 μM H_2O_2 for 24 h at 37˚C. PC12 cells in the control group were treated with cell culture medium at 37˚C for 24 h. Cells were collected and rinsed with PBS, and lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM PMSF and 1% Triton X-100 was then added for homogenization. The cell supernatants were collected. The content of MDA (cat. no. A003-1-2) and the activities of SOD (cat. no. A001-1-2) and GSH-Px (cat. no. A005-1-2) were all determined according to

Table I. PPARγ-siRNA sequences.

siRNA	Sequence (5'-3')
PPARγ-siRNA 1 (sense)	AGAUAAAGCUUCUGGAUUU
PPARγ-siRNA 1(antisense)	AAAUCCAGAAGCUUUAUCU
PPARγ-siRNA 2 (sense)	AGGAAAGACAACAGACAAA
PPARγ-siRNA 2 (antisense)	UUUGUCUGUUGUCUUUCCU
PPARγ-siRNA 3 (sense)	CCUCCCUGAUGAAUAAAGATT
PPARγ-siRNA 3 (antisense)	UCUUUAUUCAUCAGGGAGGTT
Negative control siRNA (sense)	UUCUCCGAACGUGUCACGUTT
Negative control siRNA (antisense)	ACGUGACACGUUCGGAGAATT

PPARγ, peroxisome proliferator-activated receptor γ; siRNA, small interfering RNA.

the instructions provided by Nanjing Jiancheng Bioengineering Institute as previously described (16). Briefly, MDA content was determined using the thiobarbituric acid method. The wavelength of colorimetry was 532 nm. SOD activity was determined using the hydroxylamine method and the wavelength of colorimetry was 550 nm. GSH-Px activity was determined using a colorimetric method and the wavelength of colorimetry was 412 nm. The optical density value was read using a microplate reader, and two parallel samples were used to ensure the accuracy of the experiment. Experiments were performed at least three times.

Detection of apoptotic cells by flow cytometry. Cells in the control, H_2O_2, pioglitazone + H_2O_2, pioglitazone + PPARγ-siRNA 3 + H_2O_2 and pioglitazone + GW9662 + H_2O_2 groups were used to detect apoptosis by annexin V-FITC and PI staining followed by flow cytometry (Beckman Coulter, Inc.). The treatment of each group was performed as described for the MTT assay experiment 3. The procedure described in the documentation of the annexin V-FITC/PI detection test kit (cat. no. C1062; Beyotime Institute of Biotechnology) was followed. Cells were resuspended at a concentration of $1x10^6$ cells/ml in 400 μl 1X binding buffer solution. Then, 5 μl annexin V-FITC and 10 μl PI were added to stain the cells for 15 min at room temperature in the dark. Then, the cell apoptosis was detected by flow cytometric analysis (NL-CLC 1L-3L; Cytek NL-CLC Full Spectrum Flow Cytometer; Shanghai Xiatai Biotechnology Co., Ltd.). The excitation wavelength was 488 nm and the emission wavelength was 530 nm. Green fluorescence of FITC and red fluorescence of PI were observed after excitation. Data were analysed using FlowJo (v10; FlowJo LLC). Experiments were performed at least three times.

Reverse transcription-quantitative PCR (RT-qPCR) of PPARγ in PC12 cells. RT-qPCR was performed for the NC, siRNA-NC, PPARγ-siRNA 1, PPARγ-siRNA 2 and PPARγ-siRNA 3 groups after the transfection experiments. RT-qPCR was then performed to analyse cells in the control, H_2O_2, pioglitazone + H_2O_2, pioglitazone + PPARγ-siRNA 3 + H_2O_2 and pioglitazone + GW9662 + H_2O_2 groups. The treatment of each group was performed as described for the MTT assay experiment 3. Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA samples were transcribed into cDNA using the PrimeScript RT Master Mix Kit (Takara Bio, Inc.) according to the manufacturer's instructions. The SYBR ExScript RT-PCR Kit (Takara Bio, Inc.) was used for RT-qPCR on the IQ Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). The primers used to amplify rat PPARγ (forward, 5'-GGAGCCTAAGTTTGAGTTTGCTGTG-3' and reverse, 5'-TGCAGCAGGTTGTCTTGGATG-3') and reverse, 5'-ACCACCCTGGTCTTGGATCC-3') and β-actin (forward, 5'-GGAGATTACTGCCCTGGCTCCTA-3' and reverse, 5'-GACTCATCGTACTCCTGCTTGCTG-3') were designed and synthesized by Takara Biotechnology Co., Ltd. Amplification was carried out with the following thermal cycling program: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 3 sec and 60˚C for 30 sec. Cycle threshold values were obtained with Bio-Rad iQ5 2.0 Standard Edition Optical System software (Bio-Rad Laboratories, Inc.). β-actin was used as the internal control. Relative quantification was performed by the comparative cycle threshold ($2^{-\Delta\Delta Cq}$) method (17), and the data are presented as the mean ± SD of three separate experiments performed with triplicate samples.

Western blot analysis. PPARγ, Bax, Bcl-2, cleaved caspase-3 and caspase-3 protein expression was examined in the control, H_2O_2, pioglitazone + H_2O_2, pioglitazone + PPARγ-siRNA 3 + H_2O_2 and pioglitazone + GW9662 + H_2O_2 groups. The treatment of each group was performed as described for the MTT assay experiment 3. PC12 cells were harvested by scraping into ice-cold PBS and centrifuged at 12,000 x g for 8 min at 4˚C. Afterwards, RIPA cell lysis buffer (P0013B; Beyotime Institute of Biotechnology) was used for extraction of cellular protein using 1 mM PMSF. A BCA kit (P0010; Beyotime Institute of Biotechnology) was used to determine protein concentrations. Equal amounts of protein (20 μg/lane) from different samples were separated on 12% SDS polyacrylamide gels, transferred to PVDF membranes and blocked in 10% non-fat milk at room temperature for 2 h. The membranes were incubated at 4˚C overnight with rabbit polyclonal antibodies against PPARγ (1:400; cat. no. ab66343; Abcam), Bax (1:800; cat. no. bs2538; Bioworld Technology, Inc.), Bcl-2 (1:800; cat. no. bs1511; Bioworld Technology, Inc.), cleaved caspase-3 (1:1,000; cat. no. ab2302; Abcam), caspase-3

(1:800; cat. no. bs7004; Bioworld Technology, Inc.) and mouse monoclonal anti-β-actin (1:1,000; cat. no. sc-517582; Santa Cruz Biotechnology, Inc.). The membranes were washed with Tris-buffered saline containing Tween 20 (0.1%) three times and incubated with HRP-conjugated goat anti-rabbit IgG (1:5,000; cat. no. BS13278; Bioworld Technology, Inc.) and HRP-conjugated goat anti-mouse IgG (1:5,000; cat. no. BS12478; Bioworld Technology, Inc.) at room temperature for 2 h. After washing, protein bands were detected by incubation with chemiluminescent HRP substrate (SuperSignal West Pico; Thermo Fisher Scientific, Inc.) for 5 min at room temperature in the dark and exposure to X-ray film (FUJIFILM Wako Pure Chemical Corporation). Quantity One software 4.6.2 (Bio-Rad Laboratories, Inc.) was used to semi-quantify the band intensity, which was normalized to that of the loading control β-actin. The data are presented as the mean ± SD of three separate experiments performed with triplicate samples.

TUNEL assay. A DeadEnd™ Colorimetric TUNEL System (cat. no. G7130; Promega Corporation) was used to detect PC12 apoptosis in the control, H_2O_2, pioglitazone + H_2O_2, pioglitazone + PPARγ-siRNA 3 + H_2O_2, pioglitazone + GW9662 + H_2O_2 groups. The treatment of each group was performed as described for the MTT assay experiment 3. The coverslips were placed at the bottom of a 24-well plate. PC12 cells were inoculated into the 24-well plate and treated according to the grouping. Subsequently, the coverslips were fixed with 4% paraformaldehyde at room temperature (15-25˚C) for 30 min, incubated with protease K at room temperature for 20 min and washed three times with PBS. The coverslips were covered with equilibration buffer for 10 min and with rTdT incubation buffer (50 µl; containing TUNEL reagent) at 37˚C in the dark for 1 h. The coverslips were immersed in 2X saline-sodium citrate buffer for 15 min and rinsed with PBS. The coverslips were counterstained with DAPI (1:1,000; MilliporeSigma) for 5 min at room temperature, washed in PBS and mounted with 50% glycerin (IH0272; Beijing Legen Biotechnology Co., Ltd.). Fluorescence microscopy was carried out using an Olympus BX51 microscope (Olympus Corporation) with a mercury lamp power supply. Neurons with bright green nuclei were identified as TUNEL-positive neurons. The ratio of TUNEL-positive cells to DAPI-positive cells was used as the experimental indicator to calculate the apoptosis rate of PC12 cells. The number of TUNEL-positive cells was normalized to the number of DAPI-stained cells. A 20X objective lens was used to count apoptotic cells in nine random fields (3 samples in each group; three fields in each sample), and the observer was blinded to the treatment groups.

Statistical analysis. Statistical analysis was carried out using SPSS 16.0 software (SPSS, Inc.). Quantitative data are presented as the mean ± SD and are representative of three independent experiments. Unpaired Student's t-test and one-way ANOVA followed by Tukey's post hoc test were used to assess the significance of differences among groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PC12 cell apoptosis is induced by H_2O_2. To find the appropriate concentration of H_2O_2 for the PC12 cell damage model, different concentrations of H_2O_2 (25, 50, 100, 200 and 400 µmol/l) were used. PC12 cell viability was evaluated using an MTT assay after culture with H_2O_2 for 0, 6, 12, 24 and 36 h. The MTT assay results revealed that with the increase of the H_2O_2 concentration and incubation time, the viability of PC12 cells decreased gradually (Fig. 1A). To simulate the physiological conditions, the present study aimed to select a treatment method with slow action and resulting in a specific degree of damage. Conditions leading to a cell viability rate between 50 and 60% in the MTT assay were suitable for the apoptosis experiments. In the present experiment, treatment of PC12 cells with 100 µmol/l H_2O_2 for 24 h inhibited PC12 cell viability to 50-60% of that in the control group. Thus, the desired H_2O_2 concentration and action time were 100 µmol/l and 24 h, respectively.

Successful establishment of the PC12 cell model. The MDA content and the activities of SOD and GSH-Px were measured in the control and H_2O_2 groups to verify the successful establishment of the PC12 cell model (Fig. 2). After treatment with 100 µmol/l H_2O_2 for 24 h, the content of the active peroxidation product MDA in the H_2O_2 group was increased but the activities of SOD and GSH-Px were decreased compared with those in the control group, indicating that oxidative stress injury in PC12 cells was successfully modelled.

Effects of preconditioning with different concentrations of pioglitazone on PC12 cell viability reduced by H_2O_2. PC12 cells in the H_2O_2 group were treated with 100 µmol/l H_2O_2 for 24 h, while cells in the control group were treated with cell culture medium. The MTT assay results showed that H_2O_2 significantly reduced the viability of PC12 cells ($P<0.01$ vs. control group). Pioglitazone served a protective role in H_2O_2-treated PC12 cells in a concentration-dependent manner. The concentration at which pioglitazone exhibited its maximum effects was $1x10^{-5}$ mol/l, and $1x10^{-4}$ mol/l pioglitazone did not increased PC12 viability compared with $1x10^{-5}$ mol/l pioglitazone (data not shown). Therefore, $1x10^{-5}$ mol/l was selected as the concentration for the next experiment.

Pioglitazone increases PPARγ mRNA and protein expression. The RT-qPCR and western blotting results are shown in Fig. 3. PC12 cells were transfected with siRNA to knock down PPARγ protein expression. The following three groups were used: NC group, siRNA-NC group and PPARγ-siRNA group, divided into PPARγ-siRNA 1, PPARγ-siRNA 2 and PPARγ-siRNA 3. The gene knockdown rate in all groups was detected by RT-qPCR (Fig. 3A) and western blotting (Fig. 3B and C). The results demonstrated that PPARγ expression was decreased in PC12 cells transfected with PPARγ-siRNA compared with siRNA-NC, with PPARγ-siRNA 3 showing superior knockdown efficiency. Therefore, PPARγ-siRNA 3 was selected for subsequent experiments.

Compared with the control group, H_2O_2 induced increases in PPARγ mRNA and protein expression ($P<0.01$). Pioglitazone pretreatment significantly increased PPARγ

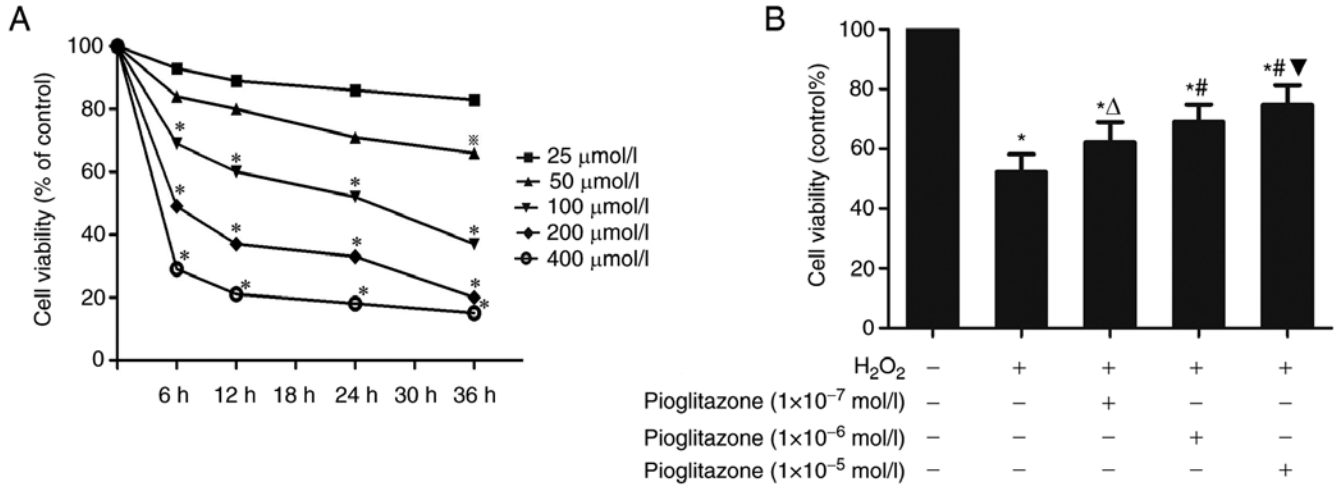

Figure 1. MTT assay results showing changes in the viability of PC12 cells. (A) PC12 cell viability decreased with increasing H_2O_2 concentration and in a time-dependent manner. Conditions leading to a cell viability rate between 50 and 60% in the MTT assay were suitable for the apoptosis experiments. In the present experiment, treatment of PC12 cells with 100 μmol/l H_2O_2 for 24 h inhibited PC12 cell viability to 50-60% of that in the control group. Thus, the appropriate H_2O_2 concentration and action time were 100 μmol/l and 24 h, respectively. (B) Changes in the viability of PC12 cells treated with H_2O_2 after preconditioning with pioglitazone at different concentrations. Control group, first group with no treatment; H_2O_2 group, second group with treatment with 100 μmol/l H_2O_2 for 24 h. *P<0.01, ※P<0.05 vs. control group; #P<0.01, △P<0.05 vs. H_2O_2 group; ▼P<0.05 vs. pioglitazone group ($1x10^{-7}$ mol/l).

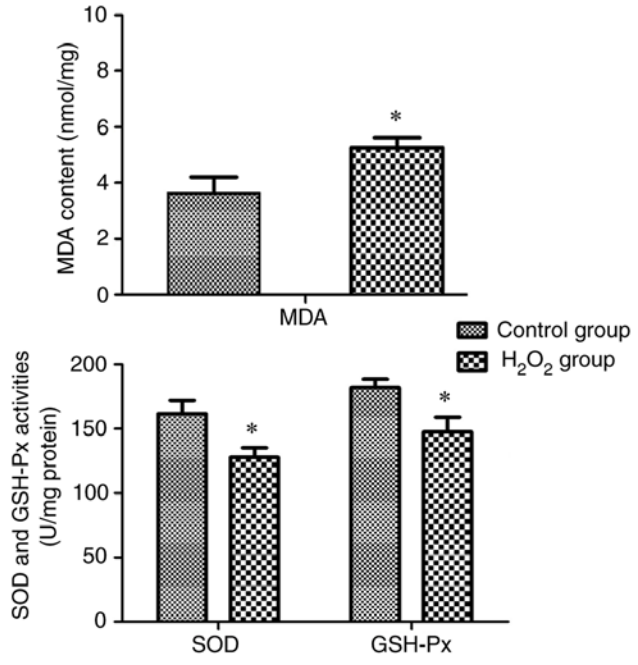

Figure 2. MDA content and SOD and GSH-Px activities in the control and H_2O_2 groups. *P<0.01 vs. control group. GSH-Px, glutathione peroxidase; MDA, malondialdehyde; SOD, superoxide dismutase; pro, protein.

mRNA and protein expression (P<0.01 vs. control and H_2O_2 groups). Pioglitazone treatment increased PPARγ mRNA and protein expression 4.36- and 4.4-fold, respectively, compared with the control group. Compared with pioglitazone treatment, treatment with PPARγ-siRNA 3 and the PPARγ antagonist GW9662 significantly reduced PPARγ mRNA and protein expression (P<0.01).

Neuroprotective effect of pioglitazone on H_2O_2-treated PC12 cells. To investigate the protective effect of PPARγ on H_2O_2-treated PC12 cells, an MTT assay, a TUNEL assay and flow cytometry were used to observe the neuroprotective effect of pioglitazone.

MTT assay results. As shown in Fig. 4, treatment with 100 μmol/l H_2O_2 significantly reduced PC12 cell viability to 56.8% of the value in the control group (P<0.01). Treatment with $1x10^{-5}$ mol/l pioglitazone increased PC12 cell viability to 80.2% of that in the control group, and there was a significant difference between the pioglitazone + H_2O_2 and H_2O_2 groups (P<0.01), suggesting piglitazone may exert a protective effect on oxidative stress-injured PC12 cells. However, the viability of cells in the pioglitazone + H_2O_2 group also differed from that of cells in the control group (P<0.01), suggesting that the protective effect of pioglitazone is insufficient to completely protect PC12 cells from oxidative damage. Pretreatment with either PPARγ-siRNA 3 or the PPARγ antagonist GW9662 reversed the neuroprotective effects of pioglitazone to similar degrees. The viability of PC12 cells treated with PPARγ-siRNA and GW9662 decreased to 59.6 and 59.1% of that in the pioglitazone +H_2O_2 group, respectively, with both showing a significant difference compared with the pioglitazone + H_2O_2 group (P<0.01), suggesting that pioglitazone exerts its neuroprotective effect through PPARγ activation.

Flow cytometry results. As shown in Fig. 5, PC12 cell apoptosis in the control group was 6.76% (Fig. 5A). The percentages in Fig. 5F are the averages for all three experimental repeats. After H_2O_2 treatment, the mean apoptosis increased significantly to 25.48% (P<0.01 vs. control group; Fig. 5B). Pioglitazone decreased the apoptosis of PC12 cells to 12.93% (Fig. 5C), which was significantly different from that in the H_2O_2 group and the control group (P<0.01 and P<0.05, respectively), suggesting that the neuroprotective effect of pioglitazone was insufficient to completely reverse the oxidative damage caused by H_2O_2. Pretreatment with PPARγ-siRNA 3 (Fig. 5D) and the PPARγ antagonist GW9662 (Fig. 5E) increased the apoptosis rates of PC12 cells to 23.14 and 21.51%, respectively, which both showing a significant difference compared with the pioglitazone + H_2O_2 group (P<0.01). This

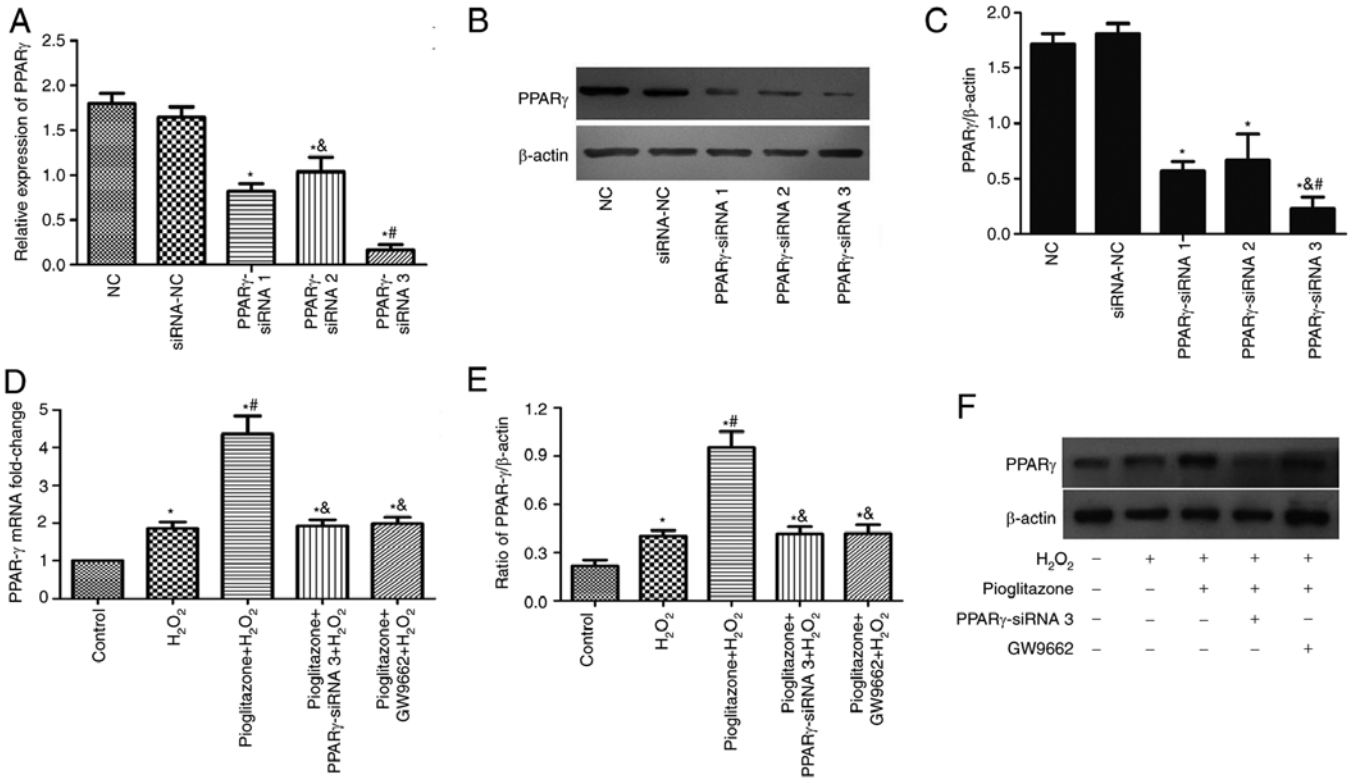

Figure 3. Effects of H_2O_2, pioglitazone, GW9662 and PPARγ-siRNA on PPARγ mRNA and protein expression. (A) PPARγ expression in PC12 cells transfected with PPARγ-siRNA detected by reverse transcription-quantitative PCR. (B) PPARγ expression in PC12 cells transfected with PPARγ-siRNA detected by western blotting and (C) semi-quantitative analysis of PPARγ protein expression. *P<0.01 vs. siRNA-NC group; &P<0.05 vs. PPARγ-siRNA 1 group; #P<0.01 vs. PPARγ-siRNA 2 group. (D) Effects of H_2O_2, pioglitazone, GW9662 and PPARγ-siRNA on PPARγ mRNA expression. (E) Effects of H_2O_2, pioglitazone, GW9662 and PPARγ-siRNA on PPARγ protein expression. (F) Representative western blot image showing PPARγ protein expression. *P<0.01 vs. control group; #P<0.01 vs. H_2O_2 group; &P<0.01 vs. pioglitazone + H_2O_2 group. NC, negative control; PPARγ, peroxisome proliferator-activated receptor γ; siRNA, small interfering RNA.

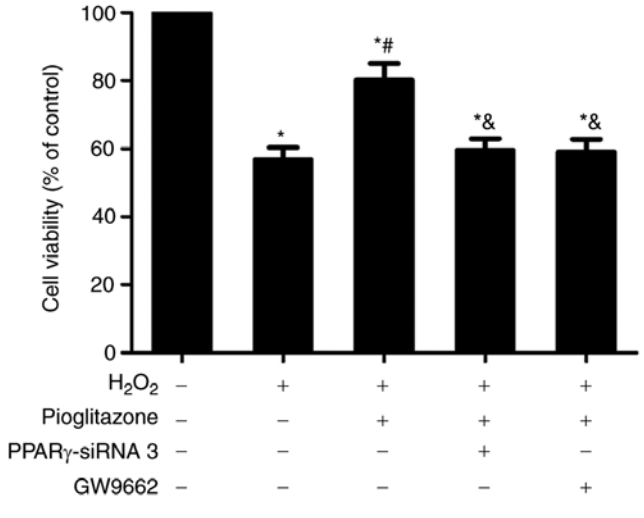

Figure 4. Effects of pioglitazone, PPARγ-siRNA 3 and GW9662 on the viability of H_2O_2-treated PC12 cells, as determined by an MTT assay. *P<0.01 vs. control group; #P<0.01 vs. H_2O_2 group; &P<0.01 vs. Pioglitazone + H_2O_2 group. PPARγ, peroxisome proliferator-activated receptor γ; siRNA, small interfering RNA.

result suggested that all types of PPARγ inhibition reversed the neuroprotective effect of pioglitazone to different degrees and that the neuroprotective effect of pioglitazone was mediated through the PPARγ activation pathway.

TUNEL assay results. As Fig. 6 shows, the control group showed an intact cell morphology, a low fluorescence intensity, larger nuclei, more DAPI-positive cells and fewer TUNEL-positive cells. The TUNEL/DAPI ratio was 5.20% (Fig. 6A). After treatment with H_2O_2 for 24 h, the number of DAPI-positive nuclei decreased, and high-intensity concentrated fluorescence appeared in the nuclei. The nuclei decreased in size and became condensed and fragmented, showing the characteristics of apoptotic cells (Fig. 6B). The mean TUNEL/DAPI ratio increased to 26.77% (P<0.01 vs. control group; Fig. 6F), suggesting that H_2O_2 promoted apoptosis in PC12 cells. Pioglitazone decreased the mean TUNEL/DAPI ratio to 11.90% (Fig. 6C), showing differences compared with the H_2O_2 group and the control group (P<0.01 and P<0.05, respectively), suggesting that pioglitazone could protect PC12 cells from H_2O_2-induced oxidative damage. However, the protective effect was insufficient to completely reverse the oxidative damage. Pretreatment with the PPARγ antagonist GW9662 and PPARγ-siRNA increased the mean TUNEL/DAPI ratio to 22.34% (Fig. 6D) and 23.27% (Fig. 6E), respectively, with significant differences compared with the pioglitazone + H_2O_2 group (P<0.01). This result suggested that pioglitazone exerts a neuroprotective effect through PPARγ activation.

Pioglitazone decreases the H_2O_2-induced increases in the Bax/Bcl-2 ratio and cleaved caspase-3/caspase-3 ratio. As shown in Fig. 7, after H_2O_2 treatment, the Bax and cleaved

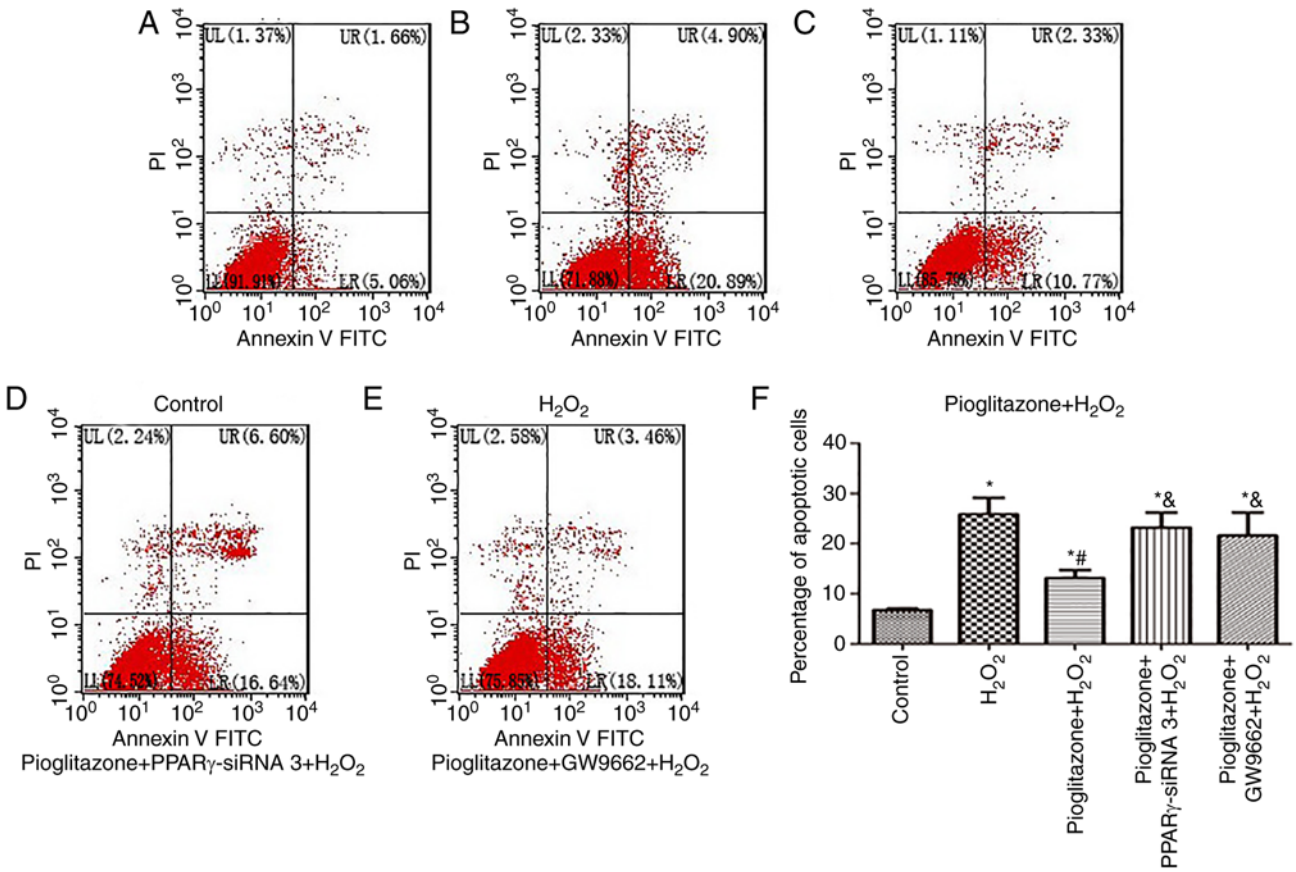

Figure 5. Effect of pioglitazone on apoptosis in H_2O_2-treated PC12 cells, as determined by flow cytometry. (A) Control group. (B) H_2O_2 group. (C) Pioglitazone + H_2O_2 group. (D) Pioglitazone + PPARγ-siRNA 3 + H_2O_2 group. (E) Pioglitazone + GW9662 + H_2O_2 group. (F) Analysis of the flow cytometry results. *P<0.01, ※P<0.05 vs. control group; #P<0.01 vs. H_2O_2 group; &P<0.01 vs. Pioglitazone + H_2O_2 group. PPARγ, peroxisome proliferator-activated receptor γ; siRNA, small interfering RNA; UL, upper left; UR, upper right; LL, lower left; LR, lower right.

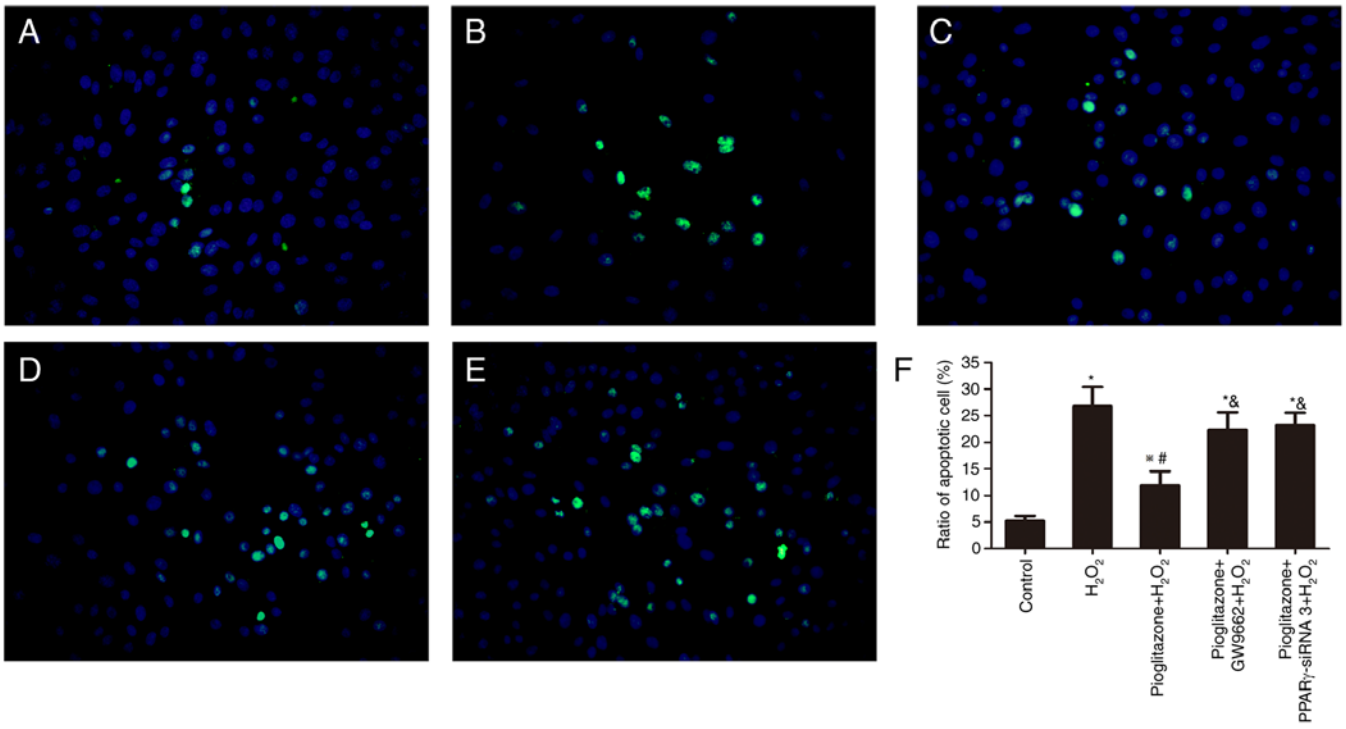

Figure 6. Effect of pioglitazone on apoptosis in H_2O_2-treated PC12 cells, as determined by a TUNEL assay (magnification, x400; scale bar, 50 μm). (A) Control group. (B) H_2O_2 group. (C) Pioglitazone + H_2O_2 group. (D) Pioglitazone + GW9662 + H_2O_2 group. (E) Pioglitazone + PPARγ-siRNA 3 + H_2O_2 group. (F) Analysis of the TUNEL assay results. *P<0.01, ※P<0.05 vs. control group; #P<0.01 vs. H_2O_2 group; &P<0.01 vs. Pioglitazone + H_2O_2 group. PPARγ, peroxisome proliferator-activated receptor γ; siRNA, small interfering RNA.

caspase-3/caspase-3 ratio increased by 3.95- and 3.11-fold, respectively (both P<0.01 vs. control group; Fig. 7A and D), while Bcl-2 protein expression was significantly reduced (P<0.01 vs. control group; Fig. 7B). The Bax/Bcl-2 ratio in the H_2O_2 group was significantly higher than that in the control group (P<0.01; Fig. 7C). Pioglitazone reduced the mean protein levels of Bax and cleaved caspase-3/caspase-3 ratio to 46.91 and 59.38% of the value in the H_2O_2 group, respectively (P<0.01), and increased the mean expression levels of Bcl-2 by 1.53-fold (P<0.01) compared with those in the H_2O_2 group, thus significantly reducing the Bax/Bcl-2 ratio (P<0.01). Compared with the pioglitazone + H_2O_2 group, the pioglitazone + GW9662 + H_2O_2 and pioglitazone + PPARγ-siRNA 3 + H_2O_2 groups showed increased Bax protein levels and cleaved caspase-3/caspase-3 ratio levels, reduced Bcl-2 expression and an increased Bax/Bcl-2 ratio (P<0.01). However, no significant differences between the pioglitazone + GW9662 + H_2O_2 and pioglitazone + PPARγ-siRNA 3 + H_2O_2 groups were observed. This result suggested that the PPARγ agonist pioglitazone can increase the expression levels of the antiapoptotic protein Bcl-2 and decrease the expression levels of the proapoptotic proteins Bax and the cleaved caspase-3/caspase-3 ratio, thus protecting PC12 cells from H_2O_2-induced oxidative damage. Pioglitazone mainly exerts its antiapoptotic effect through the PPARγ pathway (18).

Discussion

Our previous study demonstrated that SHRs exhibited an age-dependent increase in TUNEL-positive cells in the CA1 subfield of the hippocampus, which was accompanied by increased expression of oxidative stress markers and reduced mRNA and protein expression levels of PPARγ (19). PPARγ agonist rosiglitazone can exert neuroprotective effects through antioxidative and antiapoptotic pathways independent of blood pressure control (14). However, the limitation of our previous study was that PPARγ was not inhibited in animal experiments (14). In the present study, to verify the role of PPARγ activation in protection against oxidative stress injury, PC12 cells were treated with H_2O_2 as a cellular model of oxidative stress injury. The PPARγ antagonist GW9662 and PPARγ-siRNA were used to block PPARγ expression in PC12 cells. Pioglitazone exerted an antiapoptotic effect and promoted the survival of PC12 cells with oxidative stress injury. This effect was mediated through PPARγ activation.

PC12 is a tumour cell line isolated from a rat adrenal pheochromocytoma. Differentiated PC12 cells have typical neuronal characteristics in terms of morphology and function and are widely used as a cell model to study the apoptosis and differentiation of nerve cells (20). H_2O_2 is considered to be the major precursor of reactive oxygen species (ROS) and is widely used to induce oxidative stress injury (5). Exogenous H_2O_2 easily enter cells through the cell membrane, resulting in the production of large amounts of ROS (21). Thus, H_2O_2 is commonly used to simulate cellular peroxidative damage *in vitro* (22). There have been a number of studies on PC12 cell apoptosis induced by H_2O_2, and the conclusions were consistent (23,24). However, the effective concentrations and action times were different. In general, low and moderate concentrations (50-500 μmol/l) of H_2O_2 could induce oxidative stress, while high concentrations could rapidly cause cell necrosis. To determine the appropriate concentration and working time of H_2O_2, viability of PC12 cells was analysed using an MTT assay. The results showed that cell viability was decreased in PC12 cells following treatment with 100 μmol/l H_2O_2 for 24 h.

ROS act on the unsaturated lipids in cell membranes, causing peroxidation of membrane lipids and leading to cell damage and the formation of lipid peroxidation products (25). MDA is an important ROS metabolite in cells and is a good indicator of the degree of tissue peroxidation (26). SOD and GSH-Px are two important antioxidant enzymes in living organisms (27). In the present study, H_2O_2 induced an increase in the content of the active peroxidation product MDA and decreases in the SOD and GSH-Px activities. This result indicated the successful establishment of the oxidative stress injury model in PC12 cells.

The pathways by which H_2O_2 induces apoptosis differ among cell types. Dumont *et al* (28) reported that H_2O_2-induced apoptosis in T cells mainly depended on mitochondrial ROS and NF-κB activation. H_2O_2 promoted apoptosis by activating caspase-3 in HL-60 cells (29). Kitamura *et al* (30) found that Bcl-2 and Bax expression did not increase significantly after treatment with H_2O_2 but mediated apoptosis by increasing P53 expression. H_2O_2 induced apoptosis in hepatoblastoma cells not only by upregulating the expression of P53 but also by decreasing the protein levels of Bcl-2 and Bax (31). In the present study, H_2O_2 upregulated the expression levels of Bax and caspase-3 and downregulated the expression levels of Bcl-2. It was suggested that the mechanism by which H_2O_2 induced apoptosis in PC12 cells may involve increasing the levels of proapoptotic proteins and decreasing those of anti-apoptotic proteins, thus changing the apoptotic environment in PC12 cells. This is consistent with recent research showing that H_2O_2 could increase the Bax and cleaved caspase-3 protein levels in PC12 cells (32).

The process of apoptosis in neurons is similar to that in other cells. After recognition of death signals, apoptosis-promoting proteins such as Bax and BH3 interacting domain death agonist are translocated to the outer mitochondrial membrane and interact with antiapoptotic proteins such as Bcl-2, which abolishes the apoptosis-inhibiting effect of antiapoptotic proteins, increases the permeability of the mitochondrial membrane, releases cytochrome c into the cytoplasm and activates caspase-3, which eventually leads to apoptosis (33). The Bax/Bcl-2 ratio serves a decisive role in determining whether apoptosis is initiated in cells; thus, the Bax/Bcl-2 expression ratio is often used to evaluate the degree of apoptosis (34). The present results showed that H_2O_2 increased the protein levels of the proapoptotic factors Bax and cleaved caspase-3 and reduced the expression levels of the antiapoptotic protein Bcl-2, thus increasing the Bax/Bcl-2 ratio, which activated caspase-3 and induced apoptosis. The results of the TUNEL assay also confirmed these findings.

PPARγ is a ligand-activated nuclear transcription factor. PPARγ, when activated by its ligands, can bind to specific DNA response elements and regulate gene transcription and expression (35). Our previous study demonstrated that the PPARγ agonist rosiglitazone could upregulate PPARγ mRNA and protein expression in aged SHRs, while it reduced the expression of oxidative stress markers (inducible nitric oxide

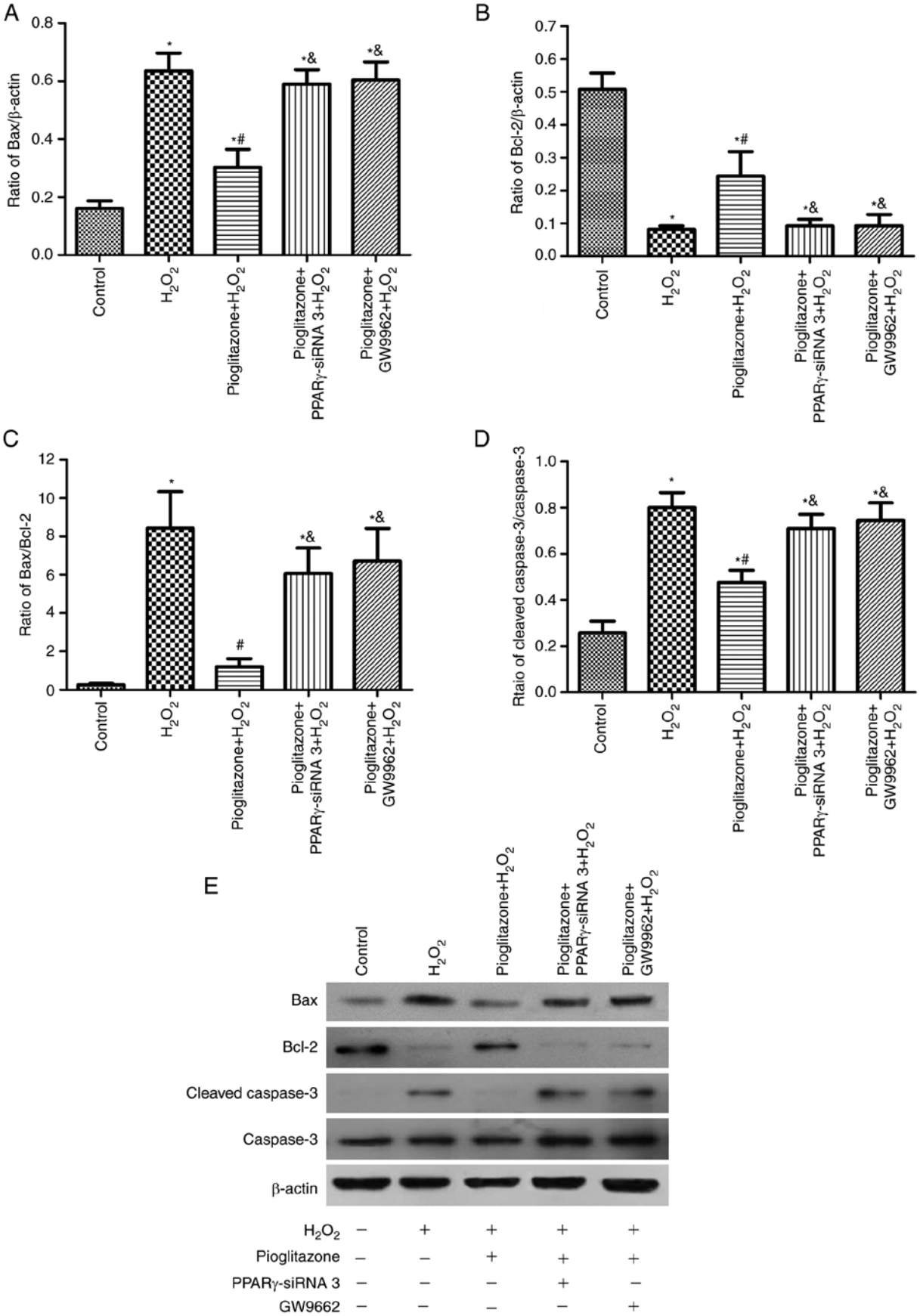

Figure 7. Effect of pioglitazone on the protein expression levels of Bax, Bcl-2 and cleaved caspase-3/caspase-3 ratio in PC12 cells. (A) Effects of H_2O_2, pioglitazone, GW9662 and PPARγ-siRNA 3 on Bax protein expression. (B) Effects of H_2O_2, piogitazone, GW9662 and PPARγ-siRNA 3 on Bcl-2 protein expression. (C) Effects of H_2O_2, piogitazone, GW9662 and PPARγ-siRNA 3 on the Bax/Bcl-2 ratio in PC12 cells. (D) Effects of H_2O_2, pioglitazone, GW9662 and PPARγ-siRNA 3 on the ratio of cleaved caspase-3/caspase-3. (E) Bax, Bcl-2, cleaved caspase-3 and caspase-3 expression in different groups detected by western blotting $^{*}P<0.01$ vs. control group; $^{\#}P<0.01$ vs. H_2O_2 group; $^{\&}P<0.01$ vs. pioglitazone + H_2O_2 group. PPARγ, peroxisome proliferator-activated receptor γ; siRNA, small interfering RNA.

synthase and NADPH oxidase subunit gp47^{phox}) and proapoptotic markers (Bax and caspase-3) (14). Fuenzalida *et al* (36) demonstrated that rosiglitazone upregulated Bcl-2 protein expression in neurons, induced mitochondrial stabilization, and prevented oxidative stress and apoptosis. These results indicated that the PPARγ agonist rosiglitazone may exert neuroprotective effects through antioxidative and antiapoptotic mechanisms. To confirm the protective effect of PPARγ agonists on PC12 cells, PC12 cells were incubated with different concentrations of pioglitazone before exposure to H_2O_2 for 1 h. The MTT assay results showed that pioglitazone concentration-dependently increased the survival rate of PC12 cells. The results of flow cytometric analysis and the TUNEL assay also confirmed the conclusion of the MTT assay. There were fewer early and late apoptotic cells in the pioglitazone + H_2O_2 group than in the H_2O_2 group, and the apoptosis rate was considerably lower in the pioglitazone + H_2O_2 group. RT-qPCR and western blot analyses confirmed that pioglitazone significantly increased PPARγ expression in PC12 cells to a level 4.4-fold higher than that in the control group. This confirmed a previous report that neuroprotective concentrations of pioglitazone can induce a 5-fold increase in PPARγ expression, thereby maintaining responsiveness of cortical neurons by increasing the expression of its receptors (37).

In addition, H_2O_2 induced an increase in PPARγ expression, which may be a compensatory protective mechanism for the cells against oxidative stress injury (38). Western blotting was used to evaluate the protein expression levels of Bax, Bcl-2 and cleaved caspase-3/caspase-3 ratio. The results demonstrated that 100 μmol/l H_2O_2 increased the protein expression levels of Bax and cleaved caspase-3/caspase-3 ratio, decreased Bcl-2 protein expression and increased the ratio of Bax to Bcl-2. Pioglitazone downregulated the protein expression levels of Bax and cleaved caspase-3/caspase-3 ratio, upregulated Bcl-2 protein expression, thus reducing the ratio of Bax to Bcl-2. These results suggested that pioglitazone could attenuate the H_2O_2-induced proapoptotic environment in PC12 cells. To further explore whether rosiglitazone can serve a role in the activation of PPARγ, the PPARγ antagonist GW9662 and PPARγ-siRNA were used to block PPARγ expression in PC12 cells. The results demonstrated that pretreatment with GW9662 significantly increased the Bax/Bcl-2 ratio and cleaved caspase-3/caspase-3 ratio in PC12 cells compared with those in the pioglitazone + H_2O_2 group. PPARγ-siRNA had the same effects. Therefore, GW9662 and PPARγ-siRNA could offset the protective effect of pioglitazone on PC12 cells with H_2O_2-induced injury.

In conclusion, in the present study, a model of neuronal apoptosis induced by oxidative stress was established *in vitro*, and the neuroprotective effect of pioglitazone was studied. The results showed that pioglitazone increased antioxidant activity in PC12 cells with H_2O_2-induced injury, increased the expression levels of Bcl-2, and decreased the protein levels of Bax and cleaved caspase-3, thus ameliorating the proapoptotic environment and reducing the apoptosis rate of PC12 cells. Treatment with a PPARγ antagonist or PPARγ-siRNA inhibited the protective effect of pioglitazone on PC12 cells with H_2O_2-induced injury, suggesting that pioglitazone could protect PC12 cells against oxidative stress injury through PPARγ activation. In conclusion, pioglitazone exerted an antiapoptotic effect and promoted the survival of PC12 cells in the presence of oxidative stress injury. This effect occurred through PPARγ activation. Therefore, the present study suggested that PPARγ activation might have intervention potential in neurodegenerative disorders. The limitation of the present study was that primary neuronal cultures, which represent neuronal properties better than PC12 cells, were not used. In addition, ROS were not directly detected before and after H_2O_2 treatment, which would be an improved approach to support the successful establishment of the PC12 cell model.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YalL, LX and XN conceived and designed the experiments. LL, BH, LX and JL performed all experiments. YanL, BH, JL and ZY collected experimental data and performed the statistical analysis. YalL and LX wrote the paper, which was revised and polished by XN. JL and YanL confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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