

Original Article

Tempol protect against hypoxia induced oxidative stress in PC12 cells

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Abstract: Hypoxia induces cellular oxidative stress that is associated with neurodegenerative diseases. Tempol (4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) is a membrane-permeable free radical scavenger with unique antioxidant properties. This study aims to investigate the potential neuroprotective effect of Tempol against hypoxia-induced oxidative stress in PC12 cell and to explore the underlying mechanisms. Treatment of PC12 cells with hypoxia reduced viability and increased LDH leakage. Tempol significantly attenuated PC12 cells damage induced by hypoxia with characteristics of increased the cell viability and reduced LDH release. In addition, Tempol suppressed the hypoxia induced oxidative stress by quenching ROS, inhibiting lipid peroxidation, normalizing the activity of SOD and CAT as well as downregulating HIF-1 α and VEGF expression. Furthermore, Tempol inhibited hypoxia induced cell apoptosis and the expression of caspase-3. Our present study indicated that Tempol has protective effect against damage induced by hypoxia in PC12 cell through its antioxidant and anti-apoptotic activities.

Keywords: Tempol, hypoxia, PC12 cells, neuroprotective effect, oxidative stress, apoptosis

Introduction

Hypoxia, defined as deficiency of oxygen reaching the tissues of the body, often occurs in physiologic conditions such as high altitude and in pathological conditions including ischemia, stroke, and neurodegenerative diseases [1]. Brain is particularly sensitive to hypoxia, compared with other organs, due to its high oxygen consumption, high content of iron and peroxidizable fatty acids as well as limited antioxidant capacity [2, 3]. Hypoxia resulted in the increased production of reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($\cdot OH$), and peroxynitrite ($ONOO\cdot$). Excessive ROS not only directly and non-specifically oxidize biological macromolecules such as DNA, proteins, and lipids, but also damage cells by activating a redox signaling cascade that ultimately leads to cell death [4, 5]. Therefore, antioxidant with capacity of scavenging ROS, such as vitamin C [6], edaravone [7], N-acetyl cysteine [8], and curcumin [9], could be used to inhibit oxidative stress and apoptosis induced by hypoxia. However, the application of these antioxidants is limited due

to its poor biocompatibility and weak cell membranes penetrate ability.

Tempol (4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl, **Figure 1**) is a membrane-permeable free radical scavenger with unique antioxidant properties and readily crosses the blood brain barrier [10]. Tempol degrades $O_2^{\cdot-}$ via a superoxide dismutase mimetic action, thereby preventing the formation of peroxynitrite. Furthermore, Tempol can suppress the formation of the $\cdot OH$ by inhibiting Fenton's reaction catalyzed by transition metals [11]. Many studies have indicated that Tempol exhibits excellent neuroprotective effect [12-17]. For example, Tempol has been shown to protect PC12 cells against cytotoxicity caused by cocaine and 1-methyl-4-phenylpyridinium ion [14, 15]. In addition, Tempol attenuates ischemia-reperfusion injury in a rat model of transient focal cerebral ischemia [16]. Recently, Tempol provides neuroprotection for chronic sleep-deprivation induced memory impairment [17]. However, the potential neuroprotective effects of Tempol against hypoxia-induced neurotoxicity and the mechanisms of protection remain to be clari-

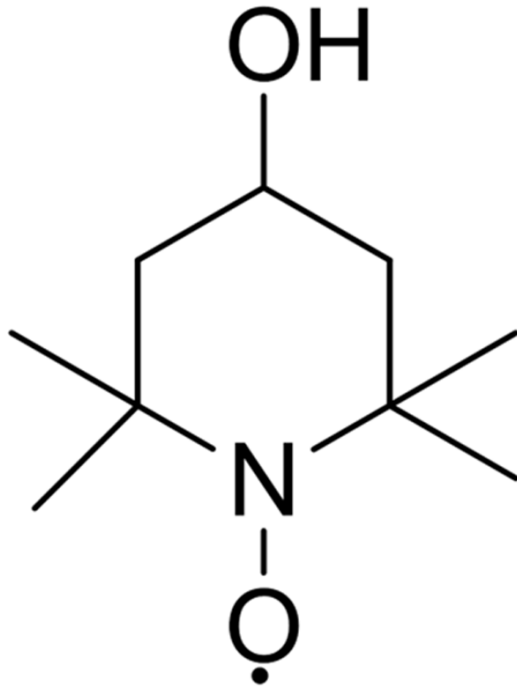


Figure 1. Chemical structure of Tempol.

fied. Therefore, here we investigated the effects of Tempol on hypoxia-induced oxidant stress in PC12 cell as well as its mechanisms.

Materials and methods

Reagents

Tempol, Dimethylsulfoxide (DMSO), thiazolyl blue tetrazolium bromide (MTT) and 2',7'-Dichlorofluorescein diacetate (DCFH-DA) were obtained from Sigma Chemical (St. Louis, MO, USA). Annexin V fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China).

The measurement kits for lactate dehydrogenase (LDH), malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and the BCA (bicinchoninic acid) protein assay kit were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Rabbit HIF-1 α , VEGF, caspase-3 and β -actin polyclonal primary antibodies were from Abcam (Cambridge Science Park, UK). Horseradish peroxidase-conjugated goat anti-rabbit IgG and anti-mouse IgG secondary antibody was purchased from ZSGB-BIO (Beijing, China). En-

hanced chemiluminescence (ECL) was purchased from Sigma Chemical (St. Louis, MO, USA).

Cell culture

PC12 cells line originated from Experimental Animal Center of Fudan University (Shanghai, China). Cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 80 mg/ml gentamicin. Cultures were grown at 37°C in a humidified atmosphere incubator with 95% air and 5% CO₂. The medium was changed every 2-3 days.

Cell viability assay

PC12 cells were seeded onto 96-well culture plates at 1.0 \times 10⁵ cells/well and incubated at 37°C for 24 h. The medium was removed and fresh DMEM/F12 with or without the appropriate dilution of Tempol was added into well. Tempol were dissolved in DMSO and added to the medium at a final concentration of 0.1, 0.33, 1, 3.3 and 10 μ mol/L, respectively. After 1h of incubation, the cells were cultured for 24 h in hypoxia incubator (5% CO₂ and 95% N₂). Control well was the non-treated cells cultured for 24 h in normoxic condition. After that, the cells were incubation with MTT solution (final concentration is 0.5 mg/ml) and 200 μ l serum-free medium for 4 h. Finally, 100 μ l of DMSO was added to dissolve the formazan. The absorbance at the test wavelength of 570 nm was measured using a microplate reader (Model 550, Bio-Rad Laboratories, Inc). Cell viability was reported as percentage of the non-hypoxia control considered as 100%.

Detection of LDH Leakage, MDA content and antioxidant enzyme activity

The PC12 cells were seeded in 90-mm culture dish at a density of 1 \times 10⁵/ml and incubated for 24 h. Cells were treated with Tempol in the same way as described above. At the end of experiment, 100 μ l of the culture supernatants were collected to a well, and LDH activity was detected at 450 nm by the commercial assay kits (Jiancheng Institute of Biotechnology, Nanjing, China). The LDH activity was expressed as U/mL. Then the PC12 cells were washed with ice-cold PBS, harvested by centrifugation at 1000 \times g for 5 min, pooled in 0.5 ml of cold

Neuroprotective effect of Tempol on hypoxia injury

PBS and homogenized. The homogenate was centrifuged at 8000×g for 15 min, and the supernatant was collected for MDA, SOD, and CAT assay. The total protein content was determined with the BCA protein assay kit. MDA contents, SOD and CAT activity were measured according to the direction of the assay kit (Jiancheng Institute of Biotechnology, Nanjing, China). The MDA results were expressed as nmol/mg protein. The SOD and CAT was expressed as U/mg protein.

Detection of ROS level

The ROS level was measured using the 2',7'-dichlorofluorescein diacetate (DCFH-DA) method. Briefly, at the end of experiment, the cells were washed with PBS and incubated with DCFH-DA at a final concentration of 10 mM for 1 h at 37°C in darkness. After the cells were washed twice with PBS to remove the extracellular DCFH-DA, the fluorescence intensity was measured using flow cytometer (FACScan, Becton Dickinson, USA) with an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The level of intracellular ROS was expressed as a percentage of non-hypoxia control.

Cell apoptosis test

Apoptosis of cells was evaluated using an FITC-Annexin V/propidium iodide (PI) apoptosis detection kit (Beyotime, Shanghai, China). PC12 cells were treated as previously described. After that, PC12 cells were harvested by centrifugation at 1000×g for 5 min, washed twice with cold PBS and adjusted to a concentration of 1×10^6 cells/ml. Then the cells were suspended in 195 μ L Annexin V-FITC binding buffer and incubated with 5 μ L Annexin V-FITC and 10 μ L of PI at 20-25°C for 30 min in the dark. Cells were analyzed on a flow cytometer (FACScan, Becton Dickinson, USA).

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells with Trizol (Invitrogen) according to the manufacturer's protocol and was then reverse-transcribed to cDNA with PrimeScript®RT reagent Kit (Takana, Dalian, China). Real-time PCR was then performed on each sample with the double-stranded DNA dye SYBR Green PCR Mastermix Takana SYBR®Primix Ex TaqTMKit (Takana, Dalian, China) according to the manufacturer's

instructions. The following primers were used in this study: HIF-1 α forward, 5'-CCAGATTCAGATCAGCCAGCA-3'; HIF-1 α reverse, 5'-GCTGTCCACATCAAAGCGTATA-3'; VEGF forward, 5'-ACATTGGCTCACTTCCAGAAACA-3'; VEGF reverse, 5'-TGGTTGGAACCGGATCTTTA-3'; caspase-3 forward, 5'-AGACAGACAGTGGAACTGACGATG-3'; caspase-3 reverse, 5'-GGCGCAAAGTGACTGGATGA-3'; GAPDH forward, 5'-GCCACAGTCAAGGCTGAGAATG-3'; GAPDH reverse, 5'-ATGGTGGTGAAGACGCCAGTA-3'. PCR was performed on an Applied Biosystems 7300HT Fast Real-Time PCR instrument with a 10 min hot start at 95°C followed by 40 cycles of a three step thermocycling program: denaturation: 30 s at 95°C, annealing: 5 s at 95°C, and extension: 31 s at 60°C. GAPDH was used as an internal. Three replicate PCRs were performed for RT-PCR analysis. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

After treatment, PC12 cells were harvested, washed with ice-cold PBS twice, lysed with lysis buffer on ice for 30 min and centrifuged at 12,000×g for 30 min. The protein concentration of supernatant was evaluated with BCA protein assay kit. Aliquots of protein were separated by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and then electrotransferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA). After blocking (5% nonfat dry milk) for 2 h, the membranes were respectively incubated with primary antibodies of β -actin (1:1000 dilution, Abcam), HIF-1 α (1:500 dilution, Abcam), VEGF (1:500 dilution, Abcam) and caspase-3 (1:500 dilution, Abcam) at 4°C overnight. The membrane was then washed and incubated with horseradish peroxidase-conjugated anti-mouse (1:2000 dilution, ZSGB-BIO) as well as anti-rabbit (1:5000 dilution, ZSGB-BIO) IgG secondary antibodies for 1 h at room temperature. ECL western detection reagent was used for detecting the antigen antibody and visualized by ChemiDoc-It² 610 imaging system (UVP, LLC, Upland, CA, USA). The reactive proteins were quantified using Image-Pro Plus 6.0 (Media Cybernetics, Inc, Bethesda, MD, USA).

Statistical analysis

Results were expressed as Mean \pm SD from at least three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) following by

Neuroprotective effect of Tempol on hypoxia injury

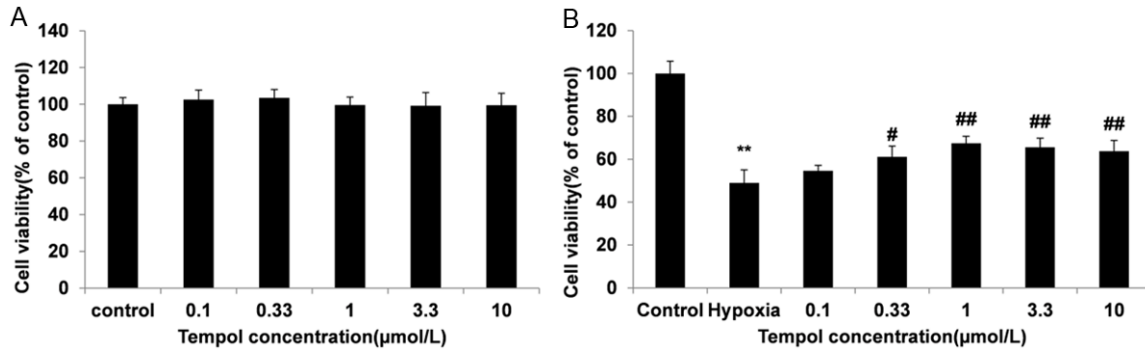


Figure 2. Tempol enhanced cells viability suppressed by hypoxia. (A) PC12 cells were pre-treated with tempol 1 h and then incubated for 24 h under normoxic condition (B) PC12 cells were pre-treated with Tempol for 1 h and then incubated for 24 under hypoxia condition. Data are presented as mean \pm SD of six independent experiments. *P < 0.05, **P < 0.01 compared to control group; #P < 0.05, ##P < 0.01 compared to hypoxia group.

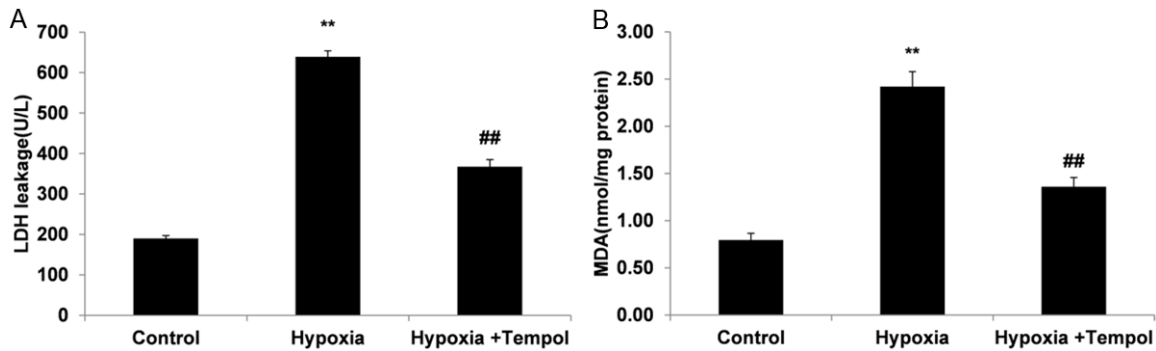


Figure 3. Tempol reduced the hypoxia-induced LDH leakage (A) and MDA content (B) in PC12 cells. Data were the mean \pm SD of three independent experiments. *P < 0.05, **P < 0.01 versus the control; #P < 0.05, ##P < 0.01 versus hypoxia group.

Student-Newman-Keuls post-hoc test. The values depicting P < 0.05 were considered as statistically significant.

Results

Tempol enhanced cells viability suppressed by hypoxia

Cells were treated with Tempol at concentrations between 0 and 1×10^{-5} mol/L. Tempol without hypoxia exposure did not show cytotoxicity or stimulation of the proliferation on PC12 cells (**Figure 2A**). Cell viability was decreased (P < 0.05) to 48.95% of control group after exposure hypoxia 24 h (**Figure 2B**). Pretreatment of cells with Tempol significantly (P < 0.05) elevated the cell viability with a conspicuous dose-response pattern (each P < 0.05). Concentrations exceeding 1×10^{-6} mol/L have no further cytoprotective effect (P > 0.05). Given this result, 1×10^{-6} mol/L was considered as the

optimal protection concentration for Tempol and was used in subsequent experiments.

Tempol reduced the LDH leakage induced by hypoxia

To verify the protective effect of Tempol, an LDH assay was performed. LDH is a stable cytoplasmic enzyme in all cells and will be rapidly released in a culture medium due to loss of cell membrane integrity. Thus, the increased LDH activity represented the degree of cell necrosis. As shown in **Figure 3A**, LDH activity in the culture supernatant significantly increased after exposure to hypoxia for 24 h. However, pretreated with Tempol reduced clearly the LDH activity in the culture supernatant.

Tempol inhibited hypoxia-induced lipid peroxidation and ROS generation

In order to measure oxidative stress induced by hypoxia, ROS level and MDA content were

Neuroprotective effect of Tempol on hypoxia injury

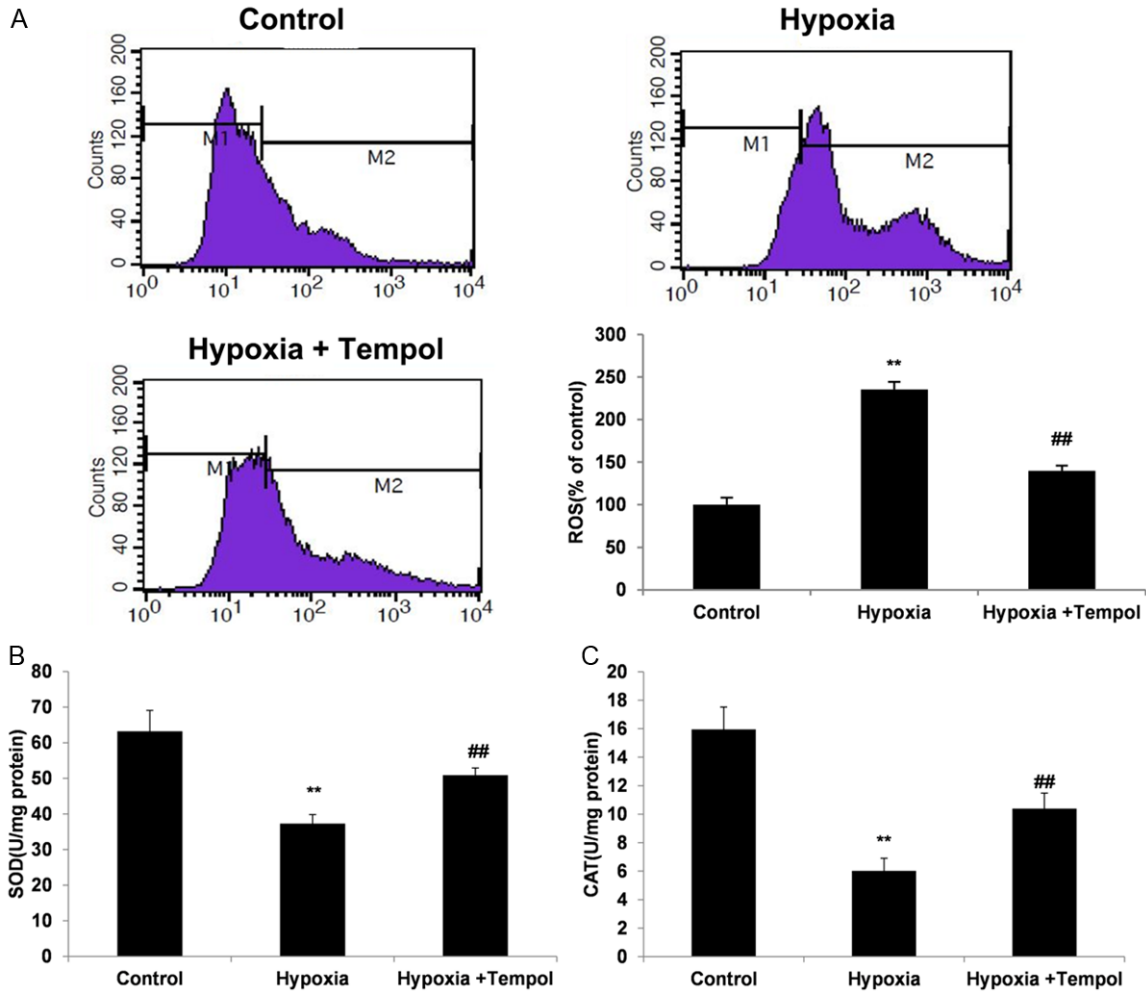


Figure 4. Tempol suppressed hypoxia-induced oxidative stress of PC12 cells. (A) ROS level (B) SOD activity (C) CAT activity. Data were the mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$ versus the control; # $P < 0.05$, ## $P < 0.01$ versus hypoxia group. ** $P < 0.01$ compared to control group; ## $P < 0.01$ compared to hypoxia group.

detected in PC12 cells. As shown in **Figures 3B** and **4A**, hypoxia induced increment in the ROS and MDA by two and three folds of control, respectively. However, the hypoxia-increased MDA and ROS production were significantly reduced ($P < 0.01$) by the pre-incubation with Tempol.

Tempol restored the activity of antioxidant enzymes suppressed by hypoxia

Endogenous antioxidant enzymes, such as SOD, CAT and GSH-Px, were always considered as the first defense line for oxidant stress and could reflect antioxidant ability indirectly. SOD has the ability to transform superoxide anions to hydrogen peroxide, which was decomposed to oxygen and water by CAT or GSH-Px [18]. As

shown in **Figure 4B** and **4C**, Hypoxia significantly ($P < 0.01$) inhibited SOD and CAT activities. However, these changes could be reversed by Tempol-pretreated. These results showed that Tempol improved the hypoxia-induced decrease in antioxidant capacity in PC12 cell.

Tempol suppressed cell apoptosis induced by hypoxia

To determine whether Tempol inhibits cell apoptosis induced by hypoxia, Annexin V-FITC/PI assay based on flow cytometry was used. As shown in **Figure 5**, hypoxia exposure increased the apoptosis rate from 5.11 to 11.93% compared to the control, which was declined to 6.87% by Tempol pretreatment.

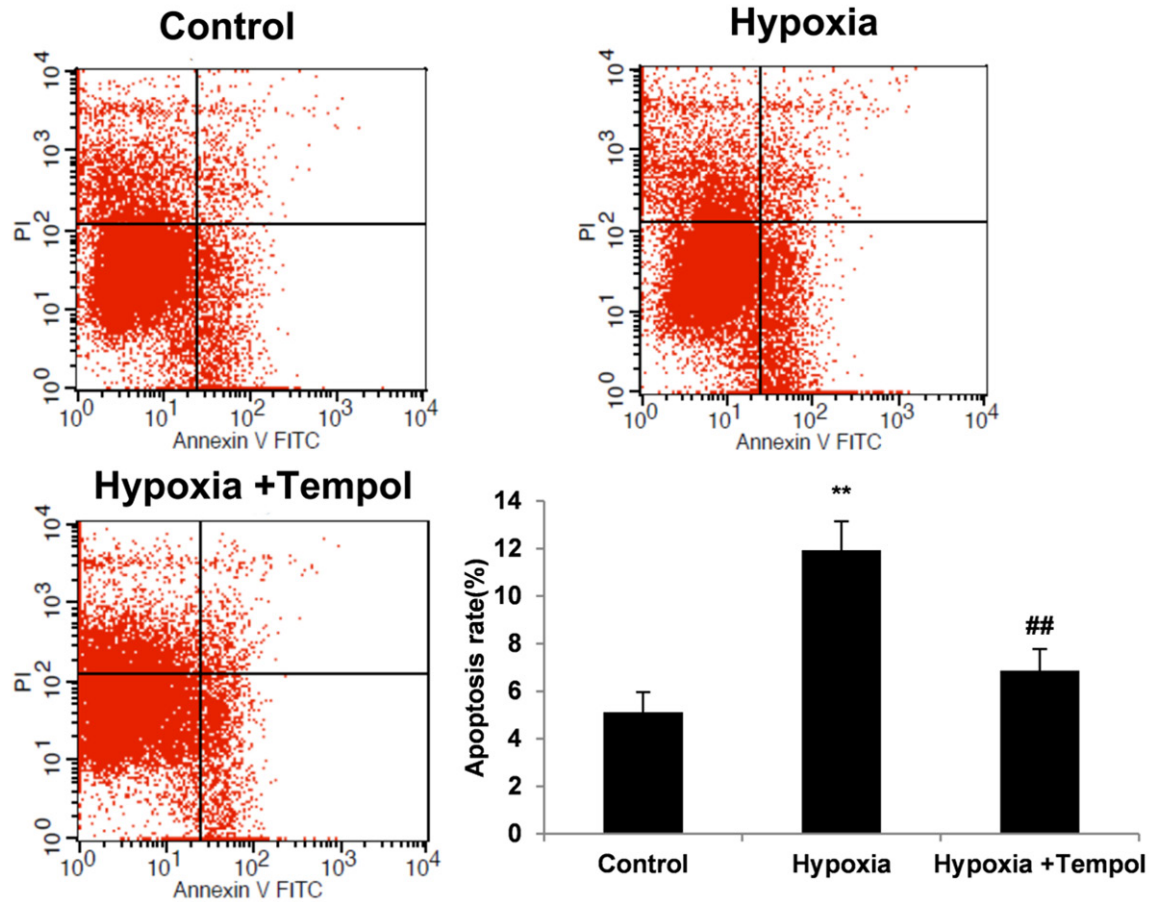


Figure 5. Tempol suppressed the hypoxia-induced apoptosis of PC12 cells. Data were the mean \pm SD of three independent experiments. *P < 0.05, **P < 0.01 versus the control; #P < 0.05, ##P < 0.01 versus hypoxia group.

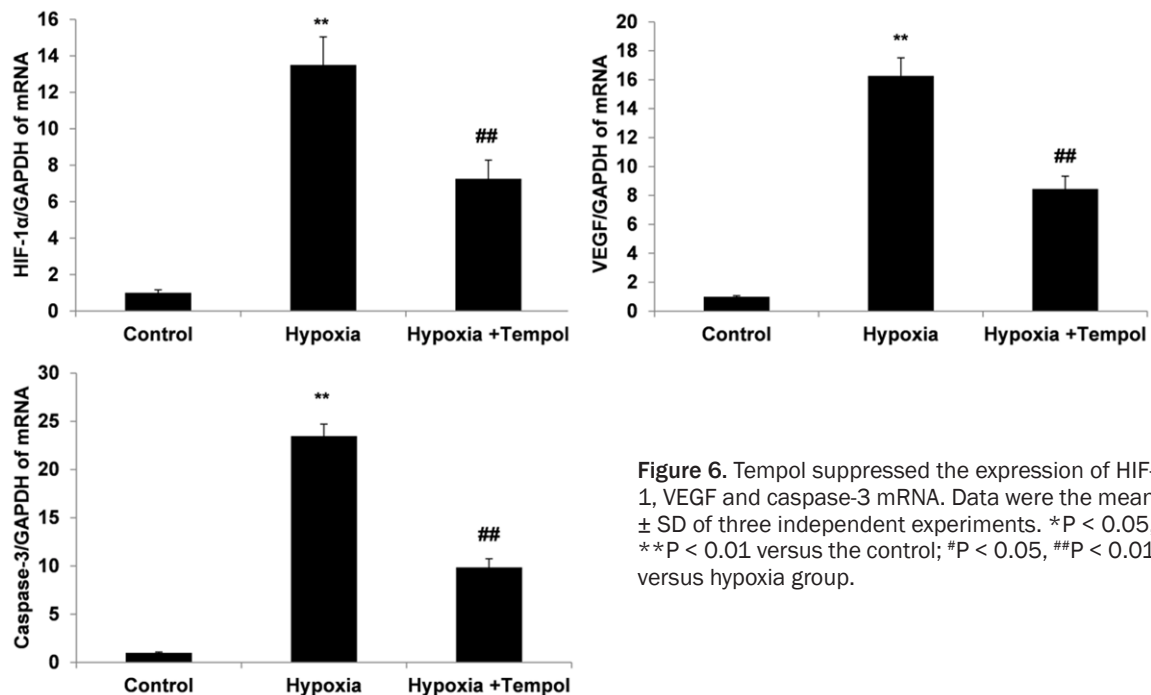


Figure 6. Tempol suppressed the expression of HIF-1, VEGF and caspase-3 mRNA. Data were the mean \pm SD of three independent experiments. *P < 0.05, **P < 0.01 versus the control; #P < 0.05, ##P < 0.01 versus hypoxia group.

Neuroprotective effect of Tempol on hypoxia injury

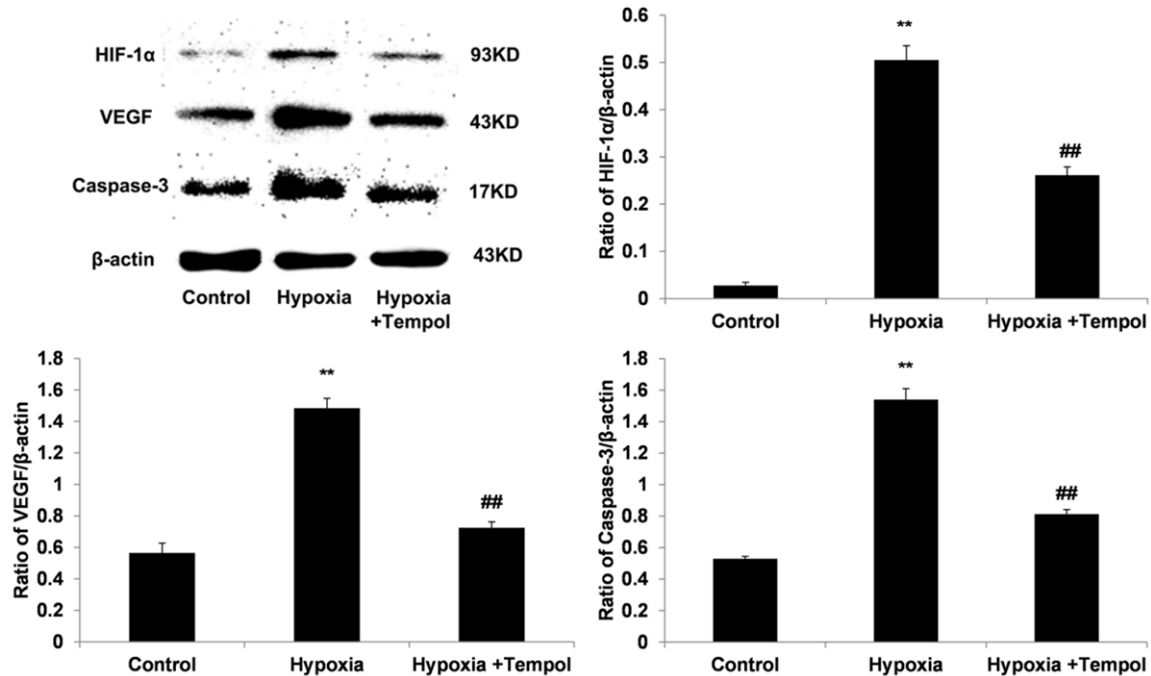


Figure 7. Tempol suppressed the expression of HIF-1, VEGF and caspase-3 protein. Data were the mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$ versus the control; # $P < 0.05$, ## $P < 0.01$ versus hypoxia group.

Tempol suppressed the expression of HIF-1, VEGF and Caspase-3 mRNA and protein

To investigate the underlying mechanism of Tempol against hypoxia induced cells injury, the expression of mRNAs related to hypoxia and apoptosis were detected by quantitative RT-PCR. As shown in **Figure 6**, compared with the control group, the relative quantitative expression of HIF-1, VEGF and Caspase-3 mRNA in hypoxia groups significantly increased ($P < 0.01$). Pretreatment of Tempol decreased the mRNA expression of HIF-1, VEGF and Caspase-3 in PC12 cell under hypoxia.

The expression of protein related to hypoxia and apoptosis were further confirmed by west blot. As shown in **Figure 7**, hypoxia exposure significantly upregulated the expression of HIF-1, VEGF and Caspase-3 ($P < 0.01$). Pretreatment of Tempol downregulated remarkably the expression of HIF-1, VEGF and Caspase-3, compared with hypoxia group ($P < 0.01$).

Discussion

Hypoxia-mediated oxidative stress plays a pivotal role in the central nervous system in pathological situations, such as stroke and ischemia

[19]. Rat pheochromocytoma (PC12) cells, with many properties similar to neurons, have been widely used as a model for the in vitro study of hypoxia/ischemia induced neuron damage [20-22]. In the present study, we examined the neuroprotective effect of Tempol against hypoxia-induced neurotoxicity in PC12 cells. We observed that PC12 cells exhibited a marked decrease in cell viability after hypoxia 24 h. Pretreatment with Tempol significantly improved cell viability. It has been shown that LDH is reliable markers of cell necrosis [23]. Therefore, the protective effect of Tempol was further confirmed by the LDH leakage. We found that Tempol significant suppressed the LDH leakage caused by hypoxia.

ROS is a by-product of cell aerobic metabolism and functions as secondary messengers to regulate cell growth, survival and proliferation. But overproduced ROS has hazardous effects on the cell structure and function [24]. The mechanisms of hypoxia induced damage are very complicated. Oxidative stress resulting from imbalance between ROS production and elimination play a critical role in hypoxia injury [25]. In this work, hypoxia significantly increased ROS generation in PC12 cell, which was significantly reduced by Tempol treatment. MDA, an

end product of lipid peroxidation, has been widely used for determining the level of oxidative stress and indirectly reflects the cell injury under conditions of excessive ROS production [26]. Compared with control group, hypoxia significantly increased the MDA level in PC12 cell. In contrast, MDA levels were significantly decreased by the treatment of Tempol.

It is known that cell has an innate antioxidant system including SOD, CAT and GSH-Px and GSH which inhibit the formation of ROS and keep ROS at a basal non-toxic level [27]. SOD catalyzes the reduction of superoxide radicals to H_2O_2 , which is detoxified by CAT or GSH-Px [28]. Previous studies have shown that hypoxia induced ROS accumulation is associated with the depletion of innate antioxidant system [29]. In according with previous study, we also found that the activity of antioxidant enzymes in PC12 cell under hypoxia was significantly reduced. However, Tempol administration was found to significantly maintain the innate antioxidant status during hypoxia. These findings suggested that the protective effect of Tempol might be associated with the inhibition of intracellular ROS production.

Growing evidence indicates that hypoxia induce the accumulation of ROS, which involves the triggering of cells to apoptosis [4, 30]. Caspase-3, an essential member of the caspase family, is considered as the ultimate mediator of cell apoptosis [31, 32]. In the present study, we confirmed that hypoxia exposure significantly increased the apoptosis rate and unregulated caspase-3 mRNA and protein expression, while Tempol could prevent hypoxia-induced apoptosis and activation of caspase-3.

Tempol could suppress the oxidant stress and apoptosis also supported by the fact that Tempol could reverse the increase of expression of HIF-1 and VEGF induced by hypoxia. HIF-1 is a transcriptional factor that regulates multiple gene expression in response to hypoxic conditions. It is a heterodimer composed of a constitutively expressed β subunit and an oxygen-regulated α subunit [33]. Under normal conditions, HIF-1 α is continuously synthesized and degraded. In contrast, under hypoxia, HIF-1 α degradation is inhibited. HIF-1 α dimerized with HIF-1 β in nucleus and bind to hypoxia responsive elements. Then, multiple target genes, including vascular endothelial growth factor (VEGF), will be activated [34]. A number

of studies have demonstrated that HIF-1 α accumulated following by hypoxia, but the beneficial and detrimental effects of HIF-1 α elevation is still elusive. Lopez-Hernandez et al revealed that unregulated HIF-1 α protects neurons from death during mild hypoxia [35]. Yin et al reported that overexpression of HIF-1 α attenuated the hypoxia induced apoptosis of the PC12 cells [36]. Nevertheless, increased HIF-1 α indicates that the cells are in a challenged state as ROS is necessary for stabilizing HIF-1 α under hypoxia [37, 38]. In addition, HIF-1 α can also trigger cell apoptosis during hypoxia [39, 40]. 2-methoxyestradiol has protective effect against hypoxia-ischemia-induced neonatal brain damage by decreasing HIF-1 α expression and subsequently down-regulating VEGF levels [41]. One possible explanation for the discrepancies between these studies is that HIF-1 α may have a dual effect that depends on the severity and time of the hypoxia. In the present study, we found that a clear up-regulation of HIF-1 α and VEGF was induced by 24 h of hypoxia. The up-regulation was reversed by the Tempol. These results indicated that Tempol reduced hypoxia induced injury on PC12 cell, likely by inhibition of HIF-1 α and downstream targets VEGF, which lead to the decrease in oxidant stress and cell apoptosis.

In summary, our finding indicated that Tempol protected PC12 cell against hypoxia-induced injury by ameliorating ROS generation and apoptosis. However, further studies are necessary to confirm the protective effect of Tempol on animal test.

Acknowledgements

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Disclosure of conflict of interest

None.

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Neuroprotective effect of Tempol on hypoxia injury

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