Protective Effect of Tempol Against Hypoxia-Induced Oxidative Stress and Apoptosis in H9c2 Cells

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Background: Hypoxia-induced oxidant stress and cardiomyocyte apoptosis are considered essential processes in the progression of heart failure. Tempol is a nitroxide compound that scavenges many reactive oxygen species (ROS) and has antioxidant and cardioprotective effects. This study aimed to investigate the protective effect of Tempol against hypoxia-induced oxidative stress and apoptosis in the H9c2 rat cardiomyoblast cell line, in addition to related mechanisms.

Material/Methods: H9c2 cells were pre-treated with Tempol, followed by hypoxia (37°C, 5% CO\textsubscript{2} and 95% N\textsubscript{2}) for 24 h. Cell viability was detected using MTT assay. ROS level was evaluated using DCFH-DA. Lactate dehydrogenase (LDH), creatinine kinase (CK), malondialdehyde (MDA), catalase (CAT), and superoxide dismutase (SOD) were evaluated using the relevant kits. Cell apoptosis was determined by Annexin V/7-AAD double labelling. The expression of apoptosis-related molecules was assessed with RT-PCR analysis and Western blotting.

Results: Tempol protected H9c2 cells against hypoxia-induced injury, with characteristics of increased cell viability and reduced LDH and CK release. Tempol also reduced oxidant stress by inhibiting ROS generation and lipid peroxidation, as well as enhancing antioxidant enzyme activity. Moreover, Tempol pretreatment upregulated the expression of Bcl-2 and downregulated the expression of Bax and caspase-3, thereby reducing hypoxia-induced apoptosis in H9c2 cells.

Conclusions: These results indicate that Tempol reduces the hypoxia-induced oxidant stress and apoptosis in H9c2 cells by scavenging free radicals and modulating the expression of apoptosis-related proteins.

MeSH Keywords: Apoptosis • Cell Hypoxia • Free Radical Scavengers • Oxidative Stress

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Background

Hypoxia, caused by an imbalance in the demand and supply of oxygen, often occurs in physiologic conditions such as high altitude and in pathological conditions including ischemia and cardiovascular difficulties [1]. The heart is very sensitive to hypoxia due to its high oxygen consumption. A sufficient supply of oxygen is essential for the proper functioning of cardiomyocytes [2]. Many studies indicated that hypoxia has adverse effects on myocardial cells [3–5], but the mechanism involved in the occurrence of hypoxia injury is far from fully understood. Cell hypoxia is associated with the generation of reactive oxygen species (ROS). Increased amounts of ROS such as $\text{O}_2^–$, $\text{H}_2\text{O}_2$, $\cdot\text{OH}$, and $\text{ONOO}^–$, generated via NADPH oxidase, mitochondrial electron transport, xanthine oxidase, and endothelial nitric oxide synthase (eNOS), can lead to apoptosis, resulting in lethal cell injuries [6]. Thus, focusing on the scavenging of ROS is a promising therapeutic target for hypoxia injury. Antioxidant compounds have been of great interest as potential therapies for hypoxia-induced oxidant stress. For example, Guaquiel et al. reported that the administration of the antioxidant vitamin C significantly improved hypoxia-mediated myocardial injury [7]. Recently, Geng et al. indicated that curcumin pretreatment protects against hypoxia-induced cardiac myocytes apoptosis [8]. However, these traditional antioxidants have poor biocompatibility and cannot readily penetrate the cell membrane, thereby limiting their applications.

Tempol (4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl, Figure 1) is a membrane-permeable free radical scavenger with unique antioxidant properties. Tempol can degrade superoxide radical in a superoxide dismutase (SOD) mimetic manner and suppresses the formation of the hydroxyl radical by inhibiting Fenton’s reaction [9]. All these features indicate that Tempol may have unique therapeutic potential for diseases and injuries related to oxidative stress [10]. For example, Tempol was shown to improve acute pancreatitis-mediated cardiac and pancreatic damage in rats [11]. Furthermore, Monti et al. showed that Tempol prevented the acute cardiac toxicity of Adriamycin in isolated rat hearts, an effect likely mediated by decreased ROS and Fe$^{2+}$ availability [12]. However, little information is available in the literature about Tempol’s effects on injury caused by hypoxia. The goal of the present study was to determine whether Tempol would prevent hypoxia-induced oxidant stress and apoptosis in H9c2 cells and the underlying mechanisms.

Material and Methods

Reagents

Tempol, dimethylsulfoxide (DMSO), thiazoyl blue tetrazolium bromide (MTT), and 2’,7’-Dichlorofluorescin diacetate (DCFH-DA) were obtained from Sigma Chemical (St. Louis, MO, USA). Annexin V fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kits were purchased from Beyotime Institute of Biotechnology (Liansu, China)

Lactate dehydrogenase (LDH), malondialdehyde (MDA), superoxide dismutase (SOD), and catalase (CAT) and BCA protein assay kits were obtained from Nanjing Jiangcheng Co (Nanjing, China). Rabbit Bcl-2-associated X protein (Bax), B cell leukemia/lymphoma-2 (Bcl-2), and caspase-3 and β-actin 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). Enhanced chemiluminescence (ECL) kits were purchased from Sigma Chemical (St. Louis, MO, USA).

Cell culture

The H9c2 cells line originated from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin. Cultures were grown at 37°C in a humidified atmosphere incubator with 95% air and 5% CO$_2$. The medium was changed every 2–3 days.

Cell viability assay

H9c2 cells were seeded in 96-well plates at a density of 1.5×10$^4$ cells/well. After incubation for 24 h at 37°C in a CO$_2$ incubator, H9c2 cells were incubated with different concentrations of Tempol (0.1, 0.33, 1, 3.3, and 10 µmol/L) for 1 h except for the control cells receiving medium instead. Then, H9c2 cells were placed in a hypoxia incubator with 95% N$_2$ and 5% CO$_2$ for 24 h. Control cells were cultured for 24 h in normoxic condition. After that, the cells were washed with cold PBS, and then incubated in 100 µL with MTT solution (0.5 mg/ml in medium) for 4 h. Finally, the violet crystals were dissolved
with 100 µl DMSO. The absorbance was measured at 570 nm with a microplate reader (Model 550, Bio-Rad Laboratories, Inc). Cell viability is expressed as the percentage of control.

Detection of CK, LDH, antioxidant enzyme activity, and MDA content

The H9c2 cells were plated in a 90-mm dish at a density of 1x10^5 cell/ml and incubated for 24 h to stabilize under normoxia. Cells were treated with Tempol as described above. At the end of the experiment, 100 µl of the culture supernatant was collected into a well, and CK and LDH activities were determined using commercial assay kits (Nanjing Jiancheng Co, China). The activity of LDH and CK are expressed as U/ml. Then, the H9c2 cells were washed with ice-cold PBS, harvested by centrifugation at 1000 x g for 5 min, pooled in 0.5 ml of cold PBS, and homogenized. The homogenate was centrifuged at 8000 x g for 15 min, and the supernatant was collected for MDA, SOD, and CAT assay. The BCA protein assay kit was used to determine the total protein content. MDA contents, SOD, and CAT activity were measured using commercial assay kits according to the manufacturer’s protocol. The level of MDA is expressed as nmol/mg protein. The activity of SOD and CAT are expressed as U/mg protein.

Detection of ROS level

The ROS level was measured using the 2',7'-dichlorofluorescin diacetate (DCFH-DA) method. Briefly, at the end of the experiment, the cells were washed with PBS and incubated with DCFH-DA at a final concentration of 10 µM for 1 h at 37°C in the dark. After the cells were washed twice with PBS to remove the extracellular DCFH-DA, the fluorescence intensity was measured using a 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 is expressed as a percentage of control.

Cell apoptosis test

Apoptosis of cells was evaluated using an FITC-Annexin V/propidium iodide (PI) apoptosis detection kit (Beyotime, Shanghai, China). H9c2 cells were treated as previously described. After that, H9c2 cells were harvested by centrifugation at 1000 x g for 5 min, washed twice with cold PBS, and adjusted to a concentration of 1x10^6 cells/ml. Then, the cells were suspended in 195 µL Annexin V-FITC binding buffer and incubated with 5 µL Annexin V-FITC and 10 µL 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 then reverse-transcribed to cDNA using a 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 in the Takana SYBR®Premix Ex TaqTM kit (Takana, Dalian, China) according to the manufacturer’s instructions. The reaction conditions were 95°C for 10 min, 40 cycles of 95°C for 30 s, 95°C for 5 s, and 60°C for 30 s. GAPDH was used as an internal control. The following primers were used in this study:

Bax forward, 5'-TGCGGATGAACTGGACAAACA-3';
Bax reverse, 5'-GGGACGTCTGATCCACTGCA-3';
Bcl-2 forward, 5'-GGTGTGGGAAAGCTTTCAGC-3';
Bcl-2 reverse, 5'-GGTGTGGGAAAGCTTTCAGC-3';
Caspase-3 forward, 5'-AGAATGTTGTGGCTGAACA-3';
Caspase-3 reverse, 5'-GGCGAAAGTGACTGGATGA-3';
GAPDH forward, 5'-GCCACATGCAAGGTAGATG-3';
GAPDH reverse, 5'-ATGGTGTTGAAGAGCCAGTA-3'.

Three replicate PCRs were performed for RT-PCR analysis. Data were analyzed by the 2^-ΔΔCt method.

Western blot analysis

After treatment, H9c2 cells were harvested, washed twice with ice-cold PBS, lysed with lysis buffer on ice for 30 min, and centrifuged at 12 000 x g for 30 min. The protein concentration of supernatant was evaluated with the 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), Bax (1: 500 dilution, Abcam), Bcl-2 (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) and 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

Data are presented as mean ±SD from at least 3 independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls post hoc test. P<0.05 was considered as statistically significant.
Results

Cell proliferation

As shown in Figure 2A, various doses of Tempol without hypoxia did not show cytotoxicity of H9c2 cells. As shown in Figure 2B, cell viability was decreased (p<0.05) to 59.52% of control group viability after 24-h hypoxia exposure, suggesting that H9c2 cells were very sensitive to hypoxia-induced cell injury. However, pretreatment of cells with Tempol (0.1–10 µmol/L) significantly (p<0.05) elevated the cell viability, with a conspicuous dose-response pattern (each p<0.05). Concentrations exceeding 1

Figure 2. Effect of Tempol on H9c2 cells. (A) H9c2 cells were treated variable concentrations of Tempol for 24 h without hypoxia. (B) Pretreatment of H9c2 cells with variable concentration of Tempol for 1 h, then hypoxia exposure for 24 h. The results are expressed as percentage of the control group. Data are presented as mean ±SD (n=6). * p<0.05; ** p<0.01 versus the control; # p<0.05; ## p<0.01 versus hypoxia group.

Figure 3. Tempol inhibited hypoxia-induced cell membrane damage and oxidative stress of H9c2 cells. LDH leakage (A), CK leakage (B), ROS level (C), MDA content (D), SOD activity (E), and CAT activity (F). Pretreatment of H9c2 cells with 1 µmol/L Tempol for 1 h, then hypoxia exposure for 24 h. Data are presented as mean ±SD (n=6). ** p<0.01 compared to control group; ## p<0.01 compared to hypoxia group.
µmol/L have no further cytoprotective effect (p>0.05). Given this result, 1 µmol/L Tempol was used in subsequent experiments.

LDH and CK leakage

As shown in Figures 3A and 3B, compared to the control group, the activity of LDH and CK showed a noticeable increase in the hypoxia group (p<0.01). Tempol significantly decreased the LDH and CK release induced by hypoxia (p<0.01).

ROS level and MDA content

As shown in Figures 3C and 3D, the treatment of H9c2 cells with hypoxia increased levels of ROS and MDA by 5- and 4-fold that of control, respectively. However, the hypoxia-increased MDA and ROS production were significantly reduced (p<0.01) by pre-incubation with Tempol.

Antioxidant enzyme activity

As shown in Figures 3E and 3F, hypoxia significantly (p<0.01) inhibited the activities of SOD and CAT, whereas Tempol pre-treatment reversed these changes.
Cell apoptosis

As shown in Figure 4A, hypoxia exposure increased the apoptosis rate from 6.79% to 12.60% compared to the control, which was decreased to 7.54% by Tempol pretreatment.

Bax, Bcl-2, and Caspase-3 mRNA expression

As shown in Figure 4B, hypoxia increased mRNA expression of Bax and caspase-3 and decreased mRNA expression of Bcl-2 (p<0.01). Pretreatment with Tempol down-regulated the level of mRNA expressions of Bax and caspase-3, and up-regulated the expression of Bcl-2.

Bax, Bcl-2, and Caspase-3 protein expression

As shown in Figure 4C, compared with the control group, hypoxia exposure significantly upregulated the Bax/Bcl-2 ratio and the expression of caspase-3 (p<0.05). However, Tempol pretreatment significantly decreased (p<0.05) the ratio of Bax/Bcl-2 and the expression of caspase-3. These results suggest that Tempol exposure has a protective effect on hypoxia-induced apoptosis in H9c2 cells.

Discussion

Hypoxia-induced oxidative stress and the resulting cell apoptosis are central in the pathogenesis of heart failure [13–15]. H9c2 cells are rat embryonic myoblast cells and retain several characters similar to cardiac cells and have been extensively used in vitro models to study cardiac ischemia/hypoxia [16–18]. In this study, we observed that H9c2 cells were highly sensitive to hypoxia-induced injury in terms of loss of cell viability. Pretreatment with Tempol significantly increased cell survival. The protective effect of Tempol was also confirmed by LDH and CK leakage. LDH and CK are released into the culture supernatant due to loss of cell membrane integrity [19]. An increase in LDH and CK activity in the culture supernatant indicates an increased number of necrotic and terminal apoptotic cells. We observed that Tempol significantly reduced hypoxia-induced LDH and CK leakage.

Hypoxia stimulates overproduction of ROS, which may attack the membrane lipoproteins and polyunsaturated fatty acids and induce lipid peroxides [6]. MDA, a product of lipid oxidation, is a reliable biomarker of oxidative stress [20]. In the present study, the production of ROS and MDA increased in H9c2 cells that underwent hypoxia injury, while the Tempol treatment reversed these changes. This finding suggests Tempol protects cells from hypoxia injury by reducing the generation of ROS and oxidant stress.

In normal condition, ROS are produced by oxidative phosphorylation and maintained at normal levels by the innate antioxidant system, including antioxidant enzymes (e.g., SOD, CAT, and GSH-Px) and non-enzyme antioxidant enzymes (e.g., GSH) [21]. SOD catalyzes the reduction of superoxide radicals to $\text{H}_2\text{O}_2$, which decomposes to $\text{H}_2\text{O}$ by CAT or GSH-Px [22]. Under hypoxia condition, the innate antioxidant system would be depleted [23,24], which was consistent with our present results. However, Tempol administration was found to significantly prevent the depletion of innate antioxidant status during hypoxia by maintaining antioxidant enzyme activity.

Apoptosis of cardiomyocytes has been identified as an essential process in the progression of heart failure. Growing evidence indicates that hypoxia can induce the accumulation of ROS, which involves the triggering of cells to apoptosis [1,25,26]. In this study, we confirmed that hypoxia exposure significantly increased the apoptosis rate of H9c2 cell, while Tempol prevented hypoxia-induced apoptosis. To further examine whether the mitochondrial anti-apoptotic pathway is involved in the effect of Tempol against hypoxia injury, variations of Bcl-2, Bax, and Caspase-3 were detected by RT-PCR and Western blotting. Bcl-2 and Bax are 2 important members of the Bcl-2 family, which play a key role in cell apoptosis. Bcl-2 has an anti-apoptotic role, while Bax has a pro-apoptotic effect [27]. The relative ratio of Bcl-2/Bax serves as a rheostat to determining whether cells undergo apoptosis. Bax-Bax homodimer acts as an apoptosis inducer, while Bcl-2-Bax heterodimer evokes a survival signal for the cells [28]. Caspase-3 is known to be involved in the final execution phase of apoptosis [29]. In the present study, RT-PCR results showed that hypoxia significantly increased Bax and caspase-3 mRNA expression and decreased Bcl-2 mRNA expression. We also observed that hypoxia enhanced the Bax/Bcl-2 ratio and activated caspase-3 protein expression. In contrast, Tempol reversed these phenotypes.

Conclusions

In summary, pretreatment with Tempol effectively alleviated oxidative stress through restoring CAT and SOD activities, as well as decreasing LDH and CK release and MDA level and ROS generation in H9c2 cells caused by hypoxia. Tempol also enhanced the cell viability, downregulated the Bax/Bcl-2 ratio, and reduced caspase-3 protein expression. These results indicate that Tempol reduces hypoxia-induced oxidant stress and apoptosis in H9c2 cell by scavenging free radicals and modulating the expression of apoptosis-related protein.

Conflict of interests

The authors declare that they have no competing interests.
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