Hydrogen peroxide preconditioning protects PC12 cells against apoptosis induced by oxidative stress

TANG Xiao-Qing1,2, CHEN Jing1, TANG Er-Hu, FENG Jian-Qiang1,*, CHEN Pei-Xi1

1Department of Physiology, Zhongshan Medical College, Sun Yat-sen University, Guangzhou 510080, China; 2Department of Physiology, Medical College of Nanhua University, Hengyang 421001, China

Abstract: Oxidative stress can induce significant cell death by apoptosis. We explore whether prior exposure to H 2O2 (H 2O2 preconditioning) protects PC12 cells against the apoptotic consequences of subsequent oxidative damages and what role the ATP-sensitive potassium (K ATP) channels play in the preconditioning protection. PC12 cells were preconditioned with 90 min exposure to H2O2 at 10 µmol/L, followed by 24-h recovery and subsequent exposures to different concentrations (20, 30, 50 and 100 µmol/L) of H2O2 for 24 h respectively. We used PI staining flow cytometry (FCM) to observe the apoptosis of PC12 cells. It was shown that 24-h exposures to H 2O2 at 20, 30, 50 and 100 µmol/L respectively induced substantial cell apoptosis, which was greatly prevented in the preconditioning cells, indicating that H2O2 preconditioning protected PC12 cells against apoptosis induced by H2O2. Administration of pinacidil (10 µmol/L), an K ATP channel activator, significantly attenuated the apoptosis of PC12 cells induced by H2O2 at 30 and 50 µmol/L for 24 h respectively. Glybenclamide (10 µmol/L), a KATP channel inhibitor, significantly suppressed or abolished the protective effects caused by the pinacidil but not by H2O2 preconditioning. However, when both H2O2 preconditioning and pinacidil were co-applied, their protection against the apoptosis of PC12 cells was much stronger than that of the individual one of them. These results suggest that H2O2 preconditioning protects PC12 cells against apoptosis and that the activation of K ATP channels is not involved in, but synergetically enhances adaptive protection of H2O2 preconditioning.

Key words: preconditioning; hydrogen peroxide; PC12 cells; apoptosis; ATP-sensitive potassium channel

过氧化氢预处理对抗氧化应激诱导的PC12细胞凋亡

唐小卿1,2, 陈 忍1, 唐二虎1, 冯鉴强1, 陈培熹1

1中山大学中山医学院生理学教研室, 广州 510080; 2南华大学医学院生理学教研室, 衡阳 421001

摘 要: 氧化应激可明显地诱导细胞凋亡。本研究旨在探讨H 2O2预处理能否对H 2O2诱导的PC12细胞凋亡产生保护作用及ATP敏感性K+ (ATP-sensitive potassium, K ATP)通道在其中的作用。采用PI染色流式细胞仪(flow cytometry, FCM)检测PC12细胞凋亡。结果表明, 经10 µmol/L H 2O2预处理90 min的PC12细胞，在20、30、50和100 µmol/L H 2O2作用24 h后，其细胞凋亡率明显下降，与未经H 2O2预处理的PC12细胞相比，差异极显著(P<0.01)，表明H 2O2预处理对H 2O2诱导PC12细胞凋亡具有保护作用。用10 µmol/L的K ATP通道激动剂pinacidil (Pin)可显著减少30和50 µmol/L H 2O2诱导的PC12细胞凋亡，10 µmol/L的K ATP通道拮抗剂glybenclamide (Gly)则显著地抑制甚至取消K ATP通道激动剂Pin对H 2O2诱导PC12细胞凋亡的保护作用，但并不影响H 2O2预处理对H 2O2诱导PC12细胞凋亡的保护作用，而当联合应用H 2O2预处理与Pin时，对PC12细胞凋亡的保护作用明显大于各自抗凋亡作用。提示K ATP通道开放不仅对H 2O2诱导PC12细胞凋亡具有保护作用，而且与H 2O2预处理一起产生抗PC12细胞凋亡的协同作用，但K ATP通道开放可能不参与H 2O2预处理的适应性保护作用。

关键词: 预处理; H 2O2; PC12细胞; 凋亡; ATP-敏感性钾通道

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Living systems have ability to regulate cell death pathway so as to protect themselves from stressful challenges. One example of such cytoprotection is ischemic preconditioning which was initially reported by Murry et al. in 1986 as a phenomenon whereby myocardium exposed to periods of brief ischemia develops protection against irreversible injury during a subsequent sustained ischemic episode. Ischemic preconditioning is now recognized to have important protective effects against ischemic damage in the heart, brain, skeletal muscle, liver, kidneys, and endothelium. Therefore, cytoprotection may be a universal protective mechanism in the living body.

Recently, the concept has been extended to preconditioning triggered by non-ischemic stress, such as some chemical medicines, hypoxia, and reactive oxygen radicals.

Oxidative stress is known to be involved in neuronal damage mediated through reactive oxygen species (ROS) such as free radicals. Several studies suggest that free radicals are associated with the pathophysiology of some neurodegenerative diseases, such as Alzheimer’s disease, Parkinson’s disease (PD) and multiple sclerosis. On the other hand, recent studies have reported that brief exposure to exogenous oxygen species induced beneficial effects against subsequent ischemia/reperfusion injury.

In some cells, ATP-sensitive potassium (K\textsubscript{ATP}) channels exist in both the surface membrane and the inner mitochondrial membrane. The activation of mitochondrial K\textsubscript{ATP} channels can protect cardiac myocytes against ischemia.

The mitochondrial K\textsubscript{ATP} channel opener diazoxide protected cardiac myocytes against apoptosis induced by hydrogen peroxide (H\textsubscript{2}O\textsubscript{2})\textsuperscript{13}. These findings indicate that the prevention of apoptosis may be an important mechanism of K\textsubscript{ATP}-related cardiac protection, but less is known about the roles of K\textsubscript{ATP} channels in other cells.

In the present study, we explored whether H\textsubscript{2}O\textsubscript{2} preconditioning protected against H\textsubscript{2}O\textsubscript{2}-induced apoptosis of PC12 cells and the roles of K\textsubscript{ATP} channels in this adaptive protection.

1 MATERIALS AND METHODS

1.1 Materials
Glybenclamide (Gly, a K\textsubscript{ATP} channel inhibitor), pinacidil (Pin, a K\textsubscript{ATP} channel activator), propidium iodide (PI) and RNase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). RPMI-1640 medium, horse serum and fetal bovine serum were supplied by GibocoBRL (Grand Island, NY, USA).

1.2 Cell culture and preconditioning methods
PC12 cells, obtained from Sun Yat-sen University Experimental Animal Center, were maintained routinely in DMEM supplement with 10% heat-inactivated horse serum and 5% fetal bovine serum at 37 ºC in a humidified atmosphere of 5% CO\textsubscript{2}/95% air.

PC12 cells were preconditioned with 90-min exposure to 10 µmol/L H\textsubscript{2}O\textsubscript{2}, followed by 24-h recovery and subsequent exposure to different concentrations (20, 30, 50, and 100 µmol/L) of H\textsubscript{2}O\textsubscript{2} for 24 h.

1.3 Flow cytometry (FCM) analysis — measurement of apoptosis
The treated PC12 cells were digested with trypsin (2.5 g/L) and were centrifuged at 1 000 r/min for 10 min. The supernatant aliquot was taken away. The cells were washed with PBS 2 times and fixed by 70% ethanol. The cells were then centrifuged at 1 000 r/min for 10 min, washed with PBS 2 times and adjusted to a concentration of 1×10\textsuperscript{6} cells/ml. To a 0.5 ml cell sample 0.5 ml RNase (1 mg/ml in PBS) was added. Followed by gentle mixing with propidium iodide (PI, at terminal concentration of 50 mg/L), the mixed cells were filtered and incubated in the dark at 4 ºC for 30 min before the flow-cytometric analysis. The PI fluorescence of individual nuclei was measured using a flow cytometer (Beckman-Coulter Co., USA). All the data of DNA labeling were analyzed by the research software matched with FCM. In the DNA histogram, the amplitude of sub-G1 DNA peak, which is lower than the G1 DNA peak represents the number of apoptotic cells.

1.4 Statistical analysis
Data are expressed as mean±SD. The significance of inter-group difference was evaluated by one-way analysis of variance (ANOVA: Scheffe’s test for post hoc comparisons). The level of significance was accepted at P<0.05.

2 RESULTS

2.1 H\textsubscript{2}O\textsubscript{2}-induced apoptosis in PC12 cells
We used flow cytometric analysis of cells after PI staining for evaluating the influences of different concentrations of H\textsubscript{2}O\textsubscript{2} on PC12 cells apoptosis. As shown in Fig.1, exposing PC12 cells to 10 µmol/L H\textsubscript{2}O\textsubscript{2} for 24 h, the percentage of PC12 cells apoptosis was 3.8%, compared with the control (3.7%, Fig.1A), there is no significant difference in percentage of apoptosis (P<0.05), indicating that 10 µmol/L H\textsubscript{2}O\textsubscript{2} did not obviously damage PC12 cells. However, when the doses of H\textsubscript{2}O\textsubscript{2} were increased to 20, 30, 50, and 100 µmol/L, the percentages of apoptosis significantly enhanced...
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2.2 Influence of H2O2 preconditioning on PC12 cells apoptosis induced by H2O2

To investigate the effect of oxidative preconditioning on H2O2-induced PC12 cells apoptosis, the preconditioning was performed in PC12 cells cultures by an incubation with 10 µmol/L H2O2 (non-lethal stress) for 90 min followed by 24 h of recovery. Fig.2 shows that oxidative preconditioning (10 µmol/L H2O2) can significantly reduce the percentages of PC12 cells apoptosis induced by different doses (20, 30, 50, and 100 µmol/L) of H2O2 respectively ($P<0.01$), indicating that non-lethal oxidative stress can prevent apoptosis induced by subsequent H2O2 exposure in cultured PC12 cells.

2.3 Effects of pinacidil and glybenclamide on H2O2-induced apoptosis in PC12 cells

It was shown that after PC12 cells were incubated with 10 µmol/L pinacidil (the surface $K_{ATP}$ channel agonist) or 10 µmol/L glybenclamide ($K_{ATP}$ channel blocker) for 24 h, the percentage of apoptosis was 4.3% and 3.6%, respectively, compared with the control group (3.7%), no significant differences in percentage of apoptosis were found among the three groups. However, as shown in Fig.3, after PC12 cells were treated with 10 µmol/L pinacidil and H2O2 at 30 or 50 µmol/L for 24 h, the percentage of PC12 cell apoptosis was significantly reduced, suggesting that $K_{ATP}$ channel agonist pinacidil protected PC12 cells against apoptosis induced by H2O2, but the inhibition of pinacidil on the apoptosis was blocked by 10 µmol/L glybenclamide (Fig.3).

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Fig.1. Apoptosis of PC12 cells induced by H2O2 (n=5). A: Control. B: 10 µmol/L H2O2. C: 20 µmol/L H2O2. D: 30 µmol/L H2O2. E: 50 µmol/L H2O2. F: 100 µmol/L H2O2.

Fig.2. Effect of H2O2 preconditioning on apoptosis of PC12 cells induced by H2O2 (n=3). *$P<0.01$ vs no-preconditioned.
2.4 Effect of glybenclamide on cytoprotection of H$_2$O$_2$ preconditioning

As shown in Fig.4, H$_2$O$_2$ preconditioning affords protection against H$_2$O$_2$-induced apoptosis, significantly reducing the percentages of PC12 cells apoptosis induced by H$_2$O$_2$ at 30 and 50 µmol/L. After H$_2$O$_2$ preconditioning and 30 min before treating with H$_2$O$_2$ at 30 or 50 µmol/L, incubation with 10 µmol/L glybenclamide did not change the percentage of apoptosis ($P>0.05$), indicating that K$_{ATP}$ channel blocker glybenclamide did not affect the adaptive cytoprotection of H$_2$O$_2$ preconditioning and that activation of K$_{ATP}$ channel may not participate in the protection of H$_2$O$_2$ preconditioning, at least in PC12 cells.

2.5 Interaction between H$_2$O$_2$ preconditioning and pinacidil on PC12 cells apoptosis induced by H$_2$O$_2$

To test whether H$_2$O$_2$ preconditioning and pinacidil synergistically protect PC12 cells against the apoptosis, after the preconditioning with 10 µmol/L H$_2$O$_2$ for 90 min followed by 24 h of recovery, PC12 cells were incubated with 10 µmol/L pinacidil and 50 µmol/L H$_2$O$_2$ for 24 h. As shown in Fig.5, when H$_2$O$_2$ preconditioning or pinacidil acted alone on the H$_2$O$_2$-induced apoptosis, the percentage of apoptosis was 22.5% and 30.9% respectively. However, while both H$_2$O$_2$ preconditioning and pinacidil synergically acted on the apoptosis, the percentage of apoptosis decreased to 16.2%, compared with H$_2$O$_2$ preconditioning group (22.5%) or pinacidil group (30.9%) respectively ($P<0.05$). The above data suggest that the interaction between H$_2$O$_2$ preconditioning and pinacidil on PC12 cells apoptosis induced by H$_2$O$_2$ is synergistic.

3 DISCUSSION

The PC12 cells line derived from a rat pheochromocytoma has been used as a model system for the study of neurons, because PC12 cells are similar to neurons in their structure and functions. The results of present study show that H$_2$O$_2$ induces PC12 cells apoptosis. This is consistent with previous reports using other cell models such as motoneurons[14]. The neuron apoptosis is now recognized to be a major factor in the pathogenesis of some neurodegenerative diseases[10,11]. Recent studies have shown that oxidative stress induced neuron apoptosis[14,16]. Therefore, it is significant to explore the mechanisms of neuronal apoptosis induced by oxidative stress for future therapeutic research related to neurodegenerative diseases.

Our studies also showed that brief periods of oxidative stress can prevent apoptosis induced by subsequent H$_2$O$_2$ exposure in PC12 cells. Vanden Hoek et al. have reported that preconditioning of chick cardiomyocytes with H$_2$O$_2$ decreased cell death induced by hypoxia, although the
mechanism of cell death was not determined[7]. Oxidative preconditioning may be a somewhat paradoxical cellular mechanism and is analogous to other phenomena such as ischemic preconditioning[6], thermal preconditioning[8] and chemical preconditioning[9]. All these studies have shown that a repeated and non-lethal stress is able to afford protection against a prolonged and severe stress.

Recent several results demonstrated that the activation of mitochondria K<sub>ATP</sub> channels can protect cardiac myocytes against ischemia and the apoptosis induced by H<sub>2</sub>O<sub>2</sub>[12-13]. Nakagawa et al. also reported that ATP-dependent potassium channel mediated neuroprotection by chemical preconditioning with 3-mitropropionic acid in gerbil hippocampus[20]. Our data showed that the surface K<sub>ATP</sub> channel agonist pinacidil protected PC12 cells against apoptosis induced by H<sub>2</sub>O<sub>2</sub> and this protective effect was abolished by the K<sub>ATP</sub> channel blocker glibenclamide, indicating that opening of surface K<sub>ATP</sub> channels was protective against PC12 cell apoptosis induced by H<sub>2</sub>O<sub>2</sub>. Teshima et al. also demonstrated that mitochondrial K<sub>ATP</sub> channel activation afforded protection against H<sub>2</sub>O<sub>2</sub>-induced cerebellar granular neurons apoptosis[21]. This is consistent with our data.

Several studies have shown that H<sub>2</sub>O<sub>2</sub> can promote the opening of K<sub>ATP</sub> channels[22-23]. To determine whether the anti-apoptotic effects of oxidative preconditioning are mediated by K<sub>ATP</sub> channels or not, we observed the role of K<sub>ATP</sub> channel blocker glibenclamide in oxidative preconditioning protection against PC12 cells apoptosis induced by H<sub>2</sub>O<sub>2</sub>. Our results showed that glibenclamide did not attenuate the protective effects of oxidative preconditioning in PC12 cells. However, we observed that the activation of K<sub>ATP</sub> channels can synergistically enhance the protection of H<sub>2</sub>O<sub>2</sub>-preconditioning against the PC12 cells apoptosis. Thus it is necessary to further investigate the mechanisms associated with the synergistic effect of H<sub>2</sub>O<sub>2</sub> preconditioning and activation of K<sub>ATP</sub> channels on H<sub>2</sub>O<sub>2</sub>-induced apoptosis in PC12 cells.

In summary, according to our results, H<sub>2</sub>O<sub>2</sub> preconditioning protects PC12 cells against apoptosis induced by H<sub>2</sub>O<sub>2</sub>. Opening of surface K<sub>ATP</sub> channels is protective against PC12 cells apoptosis induced by H<sub>2</sub>O<sub>2</sub> and is not related to adaptive protection of H<sub>2</sub>O<sub>2</sub> preconditioning, but synergetically enhances the protection of H<sub>2</sub>O<sub>2</sub> preconditioning.

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