PI3K/Akt signaling pathway-induced heme oxygenase-1 upregulation mediates the adaptive cytoprotection of hydrogen peroxide preconditioning against oxidative injury in PC12 cells

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Abstract. Both the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and heme oxygenase-1 (HO-1) create a survival signal against oxidative stress-induced injuries. Although we have demonstrated that hydrogen peroxide (H_2O_2) preconditioning confers adaptive cytoprotection against oxidative stress-induced injury in PC12 cells, it remains unknown whether these defense systems are involved in the protective effect of H_2O_2 preconditioning. In the current study, PC12 cells were preconditioned with 100 μM H_2O_2 for 90 min, followed by 24 h recovery and subsequent exposure to 300 μM H_2O_2 for further 12 h. The findings showed that preconditioning with 100 μM H_2O_2 upregulated HO-1 expression. Zinc protoporphyrin IX (ZnPP), a selective inhibitor of HO-1, at a concentration of 15 μM, significantly attenuated H_2O_2 preconditioning-elicited cytotoxicity, apoptosis, oxidative stress and mitochondrial membrane potential ($\Delta\Psi$m) loss in PC12 cells. In addition, H_2O_2 preconditioning enhanced phosphorylation of Akt. Treatment with 25 μM LY294002, a selective inhibitor of PI3K, for 20 min before H_2O_2 preconditioning blocked not only H_2O_2 preconditioning-induced HO-1 induction, but also the protective effect of H_2O_2 preconditioning against cytotoxicity. The present study provides novel evidence for the effect of preconditioning with H_2O_2 on the induction of HO-1, which contributes to the adaptive cytoprotection of H_2O_2 preconditioning against oxidative stress-induced cellular injury via a PI3K/Akt-dependent mechanism in PC12 cells.

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Introduction

It is well known that ischemic preconditioning (IPC) has adaptive cardioprotective effect (1). To date, this concept has been extended to preconditioning induced by non-ischemic stress, such as temperature (2), hypoxia (3,4), anesthetic (5,6) and reactive oxygen species (ROS) (7-9).

Recently, we have demonstrated that hydrogen peroxide (H_2O_2) preconditioning protects PC12 cells against apoptosis induced by oxidative stress (10-13). This cytoprotection by H_2O_2 preconditioning is associated with blockade of the decrease in the expression of Bcl-2 and generation of ROS (10), as well as overexpression of inducible nitric oxide synhase (iNOS) and cycloxygenase-2 (COX-2) (11), activation of the Janus tyrosine kinases (JAK)-signal transducer activator of transcription (STAT) pathway (12) and the transcription factor, nuclear factor-κB (NF-κB) (13) . These findings suggest that the molecular mechanisms responsible for H_2O_2 preconditioning-elicited adaptive cytoprotection may be complex and related to multiple genes and signaling pathways.

Inducible heme oxygenase-1 (HO-1), also known as HSP32 (heat shock protein of 32 kDa), is a stress response protein, which is response to multiple oxidative insults, such as heme, UV light, heavy metal, glutathione depletion and H_2O_2. This enzyme catalyzes the stepwise degradation of heme to release free iron and equimolar concentrations of carbon monoxide (CO) and the linear tetrapyrrol biliverdin, which is converted to bilirubin by the enzyme biliverdin reductase (14). Increasing evidence has demonstrated the potent antioxidant activity of the heme-derived metabolites produced by HO-1 catalysis (biliverdin and bilirubin) and the cytoprotective effects of CO on vascular endothelium and neuronal cells (14-17). In addition, the HO-1-deficient mice exhibit a serious damage of iron metabolism, resulting in liver and kidney oxidative insult and inflammation (18). Cells from mice with a target deletion of HO-1 are much more sensitive to apoptosis induced by serum deprivation, an effect that is significantly attenuated by overexpression of HO-1 (19). HO-1 induction in the brain also reduces stroke-related ischemic injury and might

contribute to the main neuroprotective effect of statins (20). A recent study has demonstrated that induction of HO-1 is involved in the neuroprotection of chondroitin sulfate against oxidative stress (21). Therefore, it is now widely accepted that induction of HO-1 expression represents an adaptive response that enhances cell resistance to noxious stimuli, including oxidative stress. Interestingly, the previous studies have shown that hyperbaric oxygen (HBO; i.e. exposure to pure oxygen under high ambient pressure) pretreatment confers an adaptive protection against H_2O_2-induced DNA damage in blood cells (22). This protection is associated with HO-1 induction (23). However, whether HO-1 is implicated in the adaptive cytoprotective effect of H_2O_2 preconditioning in neuronal cells is unclear.

Recently, the role of phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway in transcriptional regulation has gained attention. PI3Ks and their downstream target Akt (also known as protein kinase B) are a conserved family of signal transduction enzymes which play important roles in suppressing apoptosis and in promoting cell growth and proliferation (21,24-26). Salinas *et al* (27) reported that the PI3K/Akt pathway participates in nerve growth factor (NGF)-elicited attenuation of the intracellular ROS by regulating the expression of HO-1. In addition, in human neuroblastoma SH-SY5Y cells subjected to oxidative stress, such as H_2O_2, PI3K/Akt-mediated induction of HO-1 contributes to the neuroprotective effect of chondroitin sulfate, an endogenous perineuronal net glycosaminoglycan (21). The participation of the survival pathway PI3K/Akt in the regulation of HO-1 has also described in other cellular context, including the response to endotoxin (28), arsenite (29) and carnosol (30).

In the present study, we analyzed the following questions: i) effects of H_2O_2 preconditioning on the expression of HO-1 and Akt; ii) roles of HO-1 and PI3K/Akt pathway in the protective effects of H_2O_2 preconditioning against oxidative stress injury; iii) regulatory effect of PI3k/Akt on the induction of HO-1 by H_2O_2 preconditioning. The findings of this study provide new evidence that H_2O_2 preconditioning protects PC12 cells against oxidative stress injury by inducing HO-1 via the PI3K/Akt signaling pathway.

Materials and methods

Materials. 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), propidium iodide (PI), RNase, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), rhodamine 123 (Rh123) and zinc protoporphyrin IX (ZnPP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 medium, horse serum and fetal bovine serum (FBS) were supplied by Gibco-BRL (Calsbad, CA, USA). HO-1 antibody was purchased from StressGen Biotech (Victoria, BC, Canada). Total (t)-Akt and phosphorylated (p)-Akt antibodies were from Cell Signaling Technology (Danvers, MA, USA). Ly294002 was supplied by Calbiochem (Schwalbach, Germany). Caspase-Glo 3/7 kit was purchased from Promega (Madison, WI, USA).

Cell culture and preconditioning protocols. The rat pheochromocytoma cell line, PC12 cell, was obtained from the Sun Yat-sen University Experimental Animal Center (Guangzhou, China). PC12 cells were grown in RPMI-1640 medium supplemented with 5% heat-inactivated horse serum and 10% FBS at 37˚C under an atmosphere of 5% CO_2 and 95% air.

PC12 cells were preconditioned with 100 μM H_2O_2 for 90 min, followed by 24 h recovery and subsequent exposure to 300 μM H_2O_2 for 12 h. HO-1 inhibitor (ZnPP) at 15 μM or PI3K inhibitor (Ly294002) at 25 μM was administered 20 min before preconditioning with 100 μM H_2O_2.

Determination of cell viability. Cell viability was determined by the conventional MTT reduction assay. The PC12 cells were plated at a density of $5x10^4$ cells/well in 96-well plates. After the indicated treatments, cells were co-incubated with MTT solution (a final concentration of 0.5 mg/ml) for 4 h. The medium was removed and 150 μl dimethyl sulphoxide (DMSO) was added to each well. The formazan dye crystal was solubilized for 15 min and absorbance was measured at 570 nm with a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The mean optical density (OD) in the indicated groups was used to calculate percentage of cell viability according to the formula below: percentage of cell viability = OD treatment group/OD control group x 100%. Experiments were preformed in triplicate.

Flow cytometry analysis of apoptosis. After different treatments, PC12 cells were harvested and washed twice with phosphate buffer solution (PBS) and fixed with 70% ice-cold ethanol. After centrifugation, PC12 cells were adjusted to a concentration of $1x10^6$ cells/ml and then 0.5 ml RNase (1 mg/ml in PBS) was added to a 0.5 ml cell sample. After gentle mixing with 50 mg/l PI, mixed cells were filtered and incubated in the dark at 4˚C for 30 min before flow cytometric analysis. The PI fluorescence of individual nuclei was measured by a flow cytometer (Beckman-Coulter, Los Angeles, CA, USA). In the DNA histogram, the amplitude of the sub-G1 DNA peak, which is lower than the G1 DNA peak, represents the number of apoptotic cells.

Assay for caspase-3/-7 activity. PC12 cells were plated in 96-well plates at a density of $1x10^4$ cells/well. After the indicated treatments, caspases-3 and -7 activation were measured by caspase-Glo 3/7 assay (Promega) according to the manufacture's instructions. The assay provides a proluminescent caspase-3/-7 substrate which can be cleaved to aminoluciferin. The released aminoluciferin is a substrate which is consumed by luciferase, generating a luminescent signal. The signal is proportional to caspase-3/-7 activity. The experiment was performed at least three times with similar outcomes.

Measurement of intracellular ROS generation. Intracellular ROS levels were determined by fluorescent DCF derived from cell-permeable DCFH-DA. After treatment with indicated conditioned mediums, PC12 cells were incubated with 10 μM DCFH-DA solution at 37˚C for 30 min in the dark. DCF fluorescence was measured over the entire field of vision with a fluorescent microscope connected to an imaging system (BX50-FLA; Olympus, Tokyo, Japan). Mean fluorescence intensity (MFI) of DCF from 3 random fields was analyzed with ImageJ 1.41o software (National Institutes of Health (NIH), Bethesda, MD, USA).

Measurement of mitochondrial membrane potential (ΔΨm). ΔΨm was monitored by a fluorescent dye Rh123, a cell-permeable cationic dye that preferentially enters into mitochondria based on the highly negative ΔΨm. Depolarization of ΔΨm results in the loss of Rh123 from the mitochondria and a decrease in intracellular fluorescence. In the present study, Rh123 (100 mg/l) was added to cell cultures for 45 min at 37˚C and fluorescence was measured over the entire field of vision by using a fluorescence microscope connected to an imaging system (BX50-FLA; Olympus). MFI of Rh123 from 3 random fields was analyzed with ImageJ 1.41o software and the MFI was taken as an index of the level of ΔΨm.

Western blotting assay. At the end of the treatments, PC12 cells were harvested and re-suspended in ice-cold cell lysis solution and the homogenate was centrifuged at 10,000 x g for 15 min at 4˚C. After quantitated with the BCA protein assay kit (Kangchen Biotech, Shanghai, China), proteins were separated by 12% SDS-PAGE. The proteins in the gel were transferred into polyvinylidene difluoride (PVDF) membrane. After blocking with 5% fat-free dry milk in TBS-T for 1 h at room temperature, the membrane was incubated with the primary antibodies specific to HO-1 (1:1,000 dilution), t-Akt (1:1,000 dilution), p-Akt (1:1,000 dilution), or horseradish peroxidase (HRP)-conjugated β-actin (1:5,000 dilution) with gentle agitation at 37˚C overnight followed by further incubation with HRP-conjugated secondary antibodies (1:5,000 dilution; Wuhan Boster Biological Technology, Ltd., Wuhan, China) for 1.5 h at room temperature. The immunoreactive signals were visualized using an enhanced chemiluminescence (ECL) detection system (Applygen Technologies, Inc., Beijing, China). For quantifying the protein expression, the X-ray films were scanned and analyzed with ImageJ 1.41o software.

Data analysis and statistics. All data were presented as the mean ± SD. Differences between groups were analyzed by one-way analyses of variance (ANOVA) with SPSS 13.0 (SPSS, Inc.). P<0.05 was considered to indicate statistical significance.

Results

Preconditioning with H_2O_2 upregulates expression of HO-1. To identify whether H_2O_2 preconditioning induces the expression of HO-1, PC12 cells were treated with 100 μM H_2O_2 for 90 min, and the samples were harvested at the indicated times (3, 6 and 9 h) after H_2O_2 preconditioning. The results of western blotting analysis (Fig. 1) showed that treatment with H_2O_2 induced a significant increase in HO-1 expression compared with the control group. Within 3-9 h after H_2O_2 preconditioning, there was a consistent increase in the expression of HO-1, which peaked at 6 h.

HO-1 contributes to the cytoprotection of H_2O_2 preconditioning against oxidative stress-induced injury. To confirm whether HO-1 is involved in the adaptive cytoprotection of H_2O_2 preconditioning, we first examined the role of HO-1 in the protective effect of H_2O_2 preconditioning against cytotoxicity induced by H_2O_2. As shown in Fig. 2A, exposure of PC12 cells to H_2O_2 at 300 μM for 12 h obviously attenuated cell viability (P<0.01). Preconditioning with 100 μM H_2O_2 inhibited the 300 μM H_2O_2-induced decrease in cell viability. Preconditioning with 100 μM H_2O_2 for 90 min alone did not markedly alter the viability. Importantly, this anti-cytotoxic effect of H_2O_2 preconditioning was blocked by treatment with 15 μM ZnPP for 20 min prior to preconditioning with H_2O_2, indicating that HO-1 mediates the adaptive cytoprotection of H_2O_2 preconditioning against cytotoxicity induced by oxidative stress.

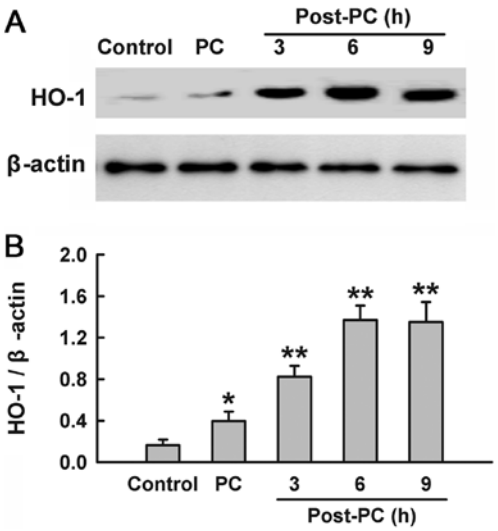

Figure 1. H_2O_2 preconditioning induced the expression of HO-1. (A) PC12 cells were pretreated with H_2O_2 at 100 μM for 90 min followed by culturing for the indicated time (i.e. 3, 6 and 9 h). Western blotting assay was used to detect HO-1 expression. (B) Densitometric analysis of HO-1 expression in (A). Data were shown as mean ± SD, n=3. *P<0.05; **P<0.01 vs. control group.

Secondarily, we detected the role of HO-1 in the cytoprotection of H_2O_2 preconditioning from H_2O_2-elicited apoptosis. Exposure to 300 μM H_2O_2 obviously elevated the caspases-3/-7 activation in PC12 cells (Fig. 2B). The increased activities of caspases-3 and -7 induced by H_2O_2 were inhibited by 100 μM H_2O_2 preconditioning. However, ZnPP at 15 μM blocked the protective effect of H_2O_2 preconditioning against the H_2O_2-induced caspases-3/-7 activation. In addition, the results of flow cytometric analysis (Fig. 2C and D) showed that exposure of cells to 300 μM H_2O_2 for 12 h obviously enhanced the percentage of apoptotic cells (P<0.01), which was reduced by preconditioning with H_2O_2. Preconditioning with 100 μM H_2O_2 alone had no significant effect on apoptosis. Notably, treatment with 15 μM ZnPP for 20 min before H_2O_2 preconditioning obviously abrogated the anti-apoptotic effect of H_2O_2 preconditioning. These results suggest that HO-1 is implicated in the anti-apoptotic effect of preconditioning with H_2O_2.

Next, we also found involvement of HO-1 in H_2O_2 preconditioning-induced antioxidative stress and mitochondrial protection. As shown in Fig. 2E-H, preconditioning with 100 μM H_2O_2 considerably attenuated ROS generation (Fig. 2E and G) and a loss of ΔΨm (Fig. 2F and H) induced by 300 μM H_2O_2. However, these protective effects of H_2O_2 preconditioning were reversed by treatment with 15 μM ZnPP prior to H_2O_2 preconditioning. Alone, ZnPP did not affect ROS generation or ΔΨm.

Preconditioning with H_2O_2 enhances phosphorylation of Akt. Since Akt activation induces HO-1 expression, we explored

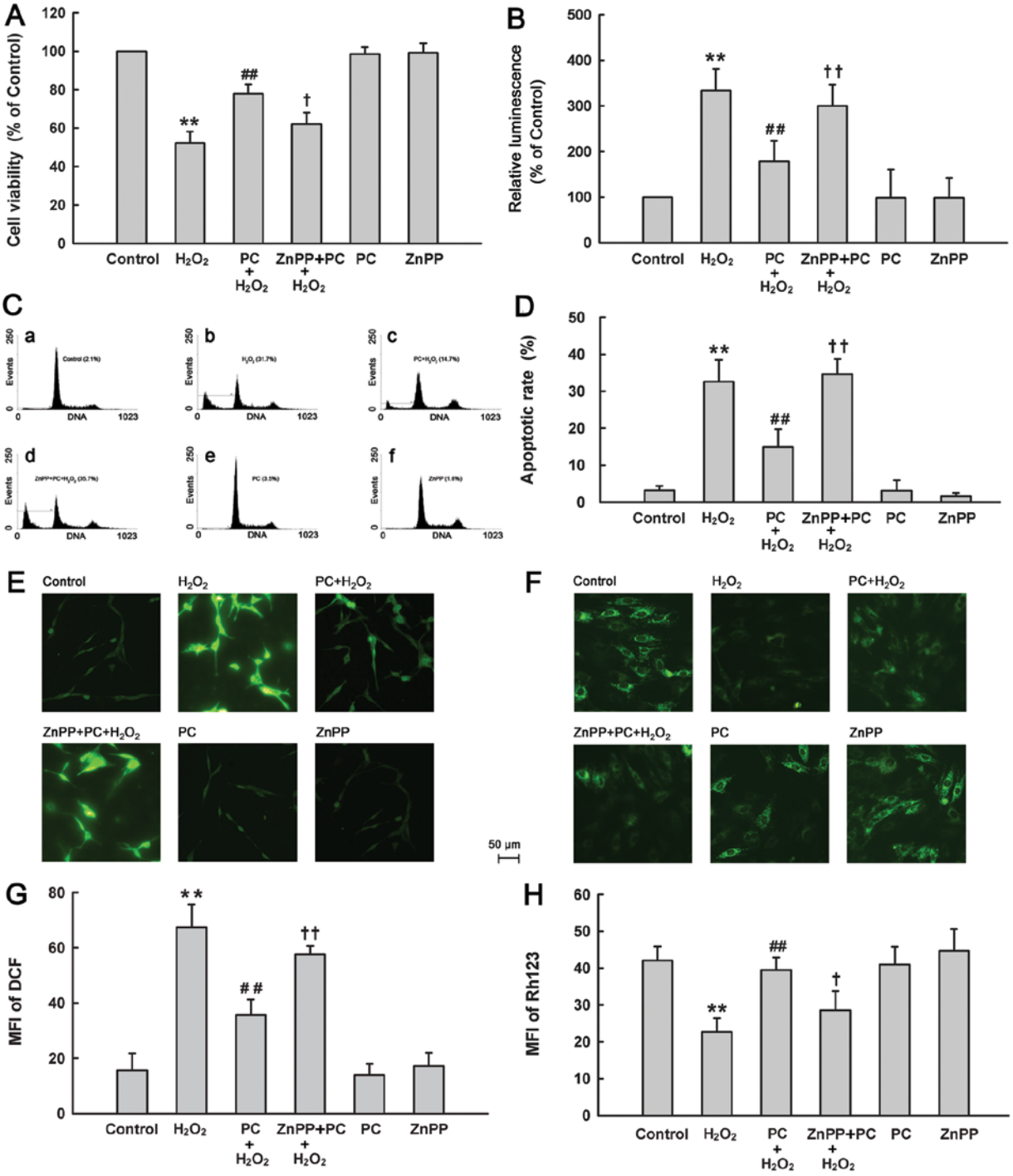

Figure 2. Effects of different treatments on cell injury of PC12 cells. After the indicated treatments, (A) cell viability, (B) activities of caspases-3/-7, (C and D) apoptosis, (E and G) ROS generation and (F and H) ΔΨm were evaluated. Control group, untreated PC12 cells. H_2O_2 group, cells were treated with 300 μM H_2O_2 for 12 h. PC+H_2O_2 group, cells were preconditioned with 100 μM H_2O_2 for 90 min before exposure to 300 μM H_2O_2 for 12 h. ZnPP+PC+H_2O_2 group, cells were treated with ZnPP (15 μM) for 20 min before H_2O_2 preconditioning, followed by exposure to 300 μM H_2O_2 for 12 h. PC group, PC12 cells were treated with 100 μM H_2O_2 for 90 min followed by a further 12 h culture. ZnPP group, PC12 cells were treated with 15 μM ZnPP for 20 min followed by a further 12 h culture. Data were presented as mean ± SD, n=3. **P<0.01 vs. control group; $^{\#}$P<0.05 vs. H_2O_2 group; $^{+}$P<0.05, $^{++}$P<0.01 vs. PC+H_2O_2 group.

the effect of H_2O_2 preconditioning on activation of Akt. Preconditioning with 100 μM H_2O_2 upregulated the expression of p-Akt at specific times (15, 30, 60, 90, 120 and 180 min after H_2O_2 preconditioning), compared with the control group (Fig. 3). Within 15-90 min after H_2O_2 preconditioning, the expression of p-Akt increased in a time-dependent manner, peaking at 90 min, and then gradually decreased at 120 and 180 min. However, H_2O_2 preconditioning had no effect on t-Akt expression.

The PI3K/Akt pathway modulates the induction of HO-1 induced by H_2O_2 preconditioning. Since both HO-1 and Akt were activated by H_2O_2 preconditioning, we explored the influence of PI3K/Akt pathway on the induction of HO-1 by preconditioning with H_2O_2. The expression of HO-1 was significantly upregulated by H_2O_2 preconditioning (Fig. 4). The H_2O_2 preconditioning-induced overexpression of HO-1 was blocked by treatment with Ly294002 (25 μM), a selective

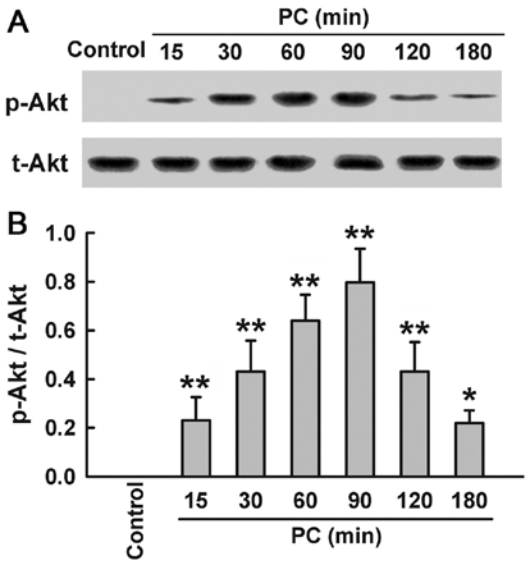

Figure 3. Effect of H_2O_2 preconditioning on Akt activation in PC12 cells. PC12 cells were preconditioned with 100 μM H_2O_2 for 90 min, the samples were harvested at the indicated time points after H_2O_2 preconditioning. (A) Expressions of t-Akt and p-Akt were analyzed by western blotting assay. (B) Densitometric analysis result from (A). Data were presented as mean ± SD, n=3. *P<0.05, **P<0.01 vs. control group.

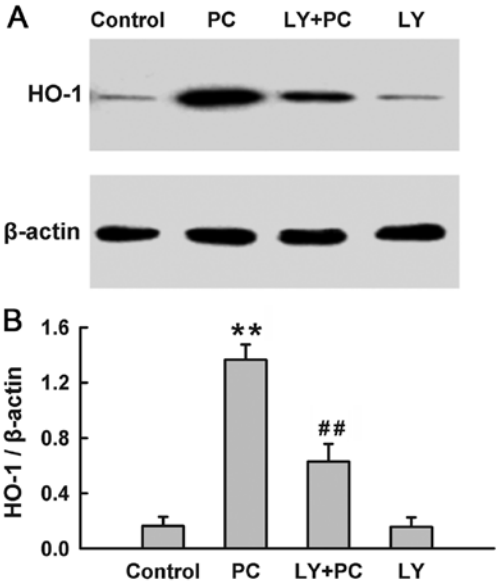

Figure 4. Role of PI3K/Akt pathway in HO-1 induction by H_2O_2 preconditioning. (A) After the indicated treatments, western blotting assay was applied to detect HO-1 expression. Control group, normal PC12 cells. PC group, PC12 cells were pretreated with 100 μM H_2O_2 for 90 min. LY+PC group, PC12 cells were treated with 25 μM Ly294002 (a selective inhibitor of PI3K/Akt pathway, LY) for 20 min before H_2O_2 preconditioning. LY group, PC12 cells were treated with Ly294002 (25 μM) for 20 min. The samples were harvested at 6 h after H_2O_2 preconditioning. (B) Densitometric analysis for the changes in expression of HO-1 in (A). Data were shown as mean ± SD, n=3. **P<0.01 vs. control group; $^{\#\#}$P<0.01 vs. PC group.

inhibitor of PI3K/Akt, which was administered for 20 min before H_2O_2 preconditioning. Alone, Ly294002 did not alter the basal expression of HO-1. These findings suggest that the H_2O_2 preconditioning-induced overexpression of HO-1 is dependent on the activation of the PI3K/Akt pathway.

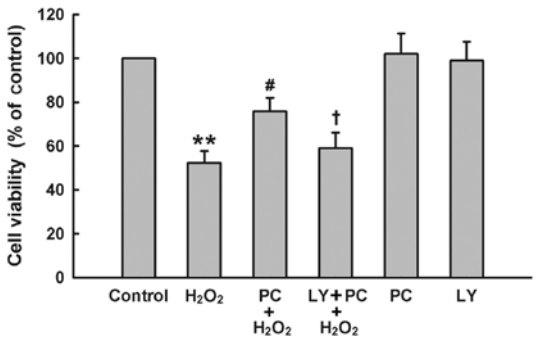

Figure 5. The PI3K/Akt pathway mediates the anti-cytotoxic effect of H_2O_2 preconditioning. After different treatments, the MTT reduction assay was used to assess cell viability. Control group, normal cells. H_2O_2 group, cells were treated with 300 μM H_2O_2 for 12 h. PC+H_2O_2 group, cells were preconditioned with 100 μM H_2O_2 for 90 min before exposure to 300 μM H_2O_2 for 12 h. LY+PC+H_2O_2 group, cells were treated with Ly294002 (25 μM) for 20 min before H_2O_2 preconditioning, followed by exposure to 300 μM H_2O_2 for 12 h. PC group, PC12 cells were preconditioned with 100 μM H_2O_2 for 90 min followed by a further 12 h culture. LY group, cells were treated with 25 μM Ly294002 for 20 min alone followed by a further 12 h culture. Data were shown as mean ± SD, n=3. **P<0.01 vs. control group; $^{\#}$P<0.05 vs. H_2O_2 group; †P<0.05 vs. PC+H_2O_2 group.

The PI3K/Akt pathway mediates the cytoprotective effect of H_2O_2 preconditioning against oxidative stress-induced cytotoxicity. To further demonstrate the role of PI3K/Akt pathway in the cytoprotection of H_2O_2 preconditioning against oxidative stress, PC12 cells were treated with Ly294002 (25 μM) for 20 min prior to H_2O_2 preconditioning. The results of Fig. 5 showed that H_2O_2 preconditioning protected PC12 cells against H_2O_2-induced cytotoxicity, evidenced by an increase in cell viability. Treatment of cells with Ly294002 at 25 μM significantly blocked the anti-cytotoxic effect of H_2O_2 preconditioning. Ly294002 alone had no effect on cell viability in PC12 cells. These findings indicate that the PI3K/Akt pathway participates in the protection of H_2O_2 preconditioning against H_2O_2-induced cytotoxicity in PC12 cells.

Discussion

Based on our previous studies (10-13), this study further demonstrates that PC12 cells have intrinsic mechanisms that respond to a brief exposure to oxidative stress by enhancing cellular resistance to the induction of oxidative injuries by subsequent sustained oxidative exposure. Here, we provide new evidence for a key mechanism that the PI3K/Akt-HO-1 pathway plays a critical role in the adaptive cytoprotective effect of oxidative (H_2O_2) preconditioning against oxidative stress injuries in PC12 cells. This is strongly supported by the findings that i) H_2O_2 preconditioning enhanced the expression of HO-1; ii) inhibition of HO-1 by ZnPP blocked the cytoprotection of H_2O_2 preconditioning against oxidative injuries, evidenced by the decreases in cell viability and $\Delta\Psi$m, and increases in apoptotic cells, ROS generation as well as caspases-3 and -7 activities; iii) the expression of p-Akt was upregulated by H_2O_2 preconditioning; iv) Ly294002, a selective inhibitor of PI3K, attenuated H_2O_2 preconditioning-induced overexpression of HO-1, indicating the regulatory effect of the PI3K/Akt pathway on the expression of HO-1; v) Ly294002 blocked the protective effect of H_2O_2 preconditioning against

oxidative stress-elicited cytotoxicity, suggesting the involvement of the PI3K/Akt pathway in the adaptive cytoprotection of preconditioning with H_2O_2.

HO is the rate-limiting enzyme of microsomal heme degradation. Three isoforms of HO, HO-1, HO-2 and HO-3, have been characterized. It has been shown that both HO-2 and HO-3 are constitutively expressed whereas HO-1 is an inducible isoform with low basal expression (14). HO-2 functions as a physiologic regulator of cellular function and HO-3 appears to have only low enzyme activity, whereas HO-1 plays a critical role in modulating tissue responses to injury in pathophysiologic states (21,27,31). HO-1 is induced by a variety of cell- and species-dependent stress factors including oxidative stress (27,31,32). Increasing evidence reveals that HO-1 has antioxidant (14,21,27,33), anti-apoptotic (19,32), and cytoprotective effects, including neuroprotection (14,20,21,33). Therefore, the role of HO-1 in adaptive cytoprotection has been investigated.

In human proximal tubular (HK-2) cells, HO-1 is involved in the protective effect of oxidant preconditioning against lethal oxidant injury (8). In human lymphocytes, HO-1 mediates the adaptive cytoprotection of HBO preconditioning (22). In addition, cardiac ischemic preconditioning fails to occur in HO-1 knockout mice, suggesting an important role of HO-1 in mediating tissue protection by ischemic preconditioning. HO-1 also contributes to the cardioprotection of H_2O_2 preconditioning from oxidative stress in rat neonatal cardiomyocytes (9). However, whether HO-1 is implicated in the neuroprotective effect of H_2O_2 preconditioning against oxidative stress injury remains unknown. In the present study, we found that preconditioning with H_2O_2 upregulated the expression of HO-1 in PC12 cells. Inhibition of HO-1 by ZnPP significantly blocked the adaptive cytoprotection of H_2O_2 preconditioning against oxidative stress injuries, characterized by increases in cytotoxicity, apoptotic cells, activities of caspases-3/-7, ROS generation and a loss of MMP, suggesting that HO-1 contributes to the anti-cytotoxic, anti-apoptotic and antioxidative effects as well as mitochondrial improvement induced by H_2O_2 preconditioning. Our findings are comparable with those previous studies (8,9,22). This study and others (8,22) reveal that HO-1 may be an important intrinsic mediator involved in preconditioning-induced adaptive cytoprotection, in particular, oxidative preconditioning.

Accumulating evidence indicates that HO-1 is highly inducible by agents causing oxidative stress, such as H_2O_2 (14,22,32). HO-1 induction is often connected with increased resistance to oxidant-mediated cell injury. Multiple mechanisms are involved in the protection of HO-1 from pathophysiological conditions. One of the key mechanisms may be associated with its antioxidant effect. For example, bilirubin, one of the main byproducts of the catabolism of heme by HO-1, acts as a radical scavenger (32); nanomolar amounts of bilirubin can reduce micromolar amounts of H_2O_2 (34). The increased formation of this antioxidant could therefore explain the observed roles of HO-1 in the adaptive protection of H_2O_2 preconditioning. Besides an increased bililrubin production, both CO and ferritin (another product of HO-1 enzyme activity) have also been shown to have an antioxidant effect (32,35,36), which might also contribute to the cytoprotection of H_2O_2 preconditioning. Moreover, other antioxidant enzymes may be regulated by byproducts of HO-1 activity, thus contributing to ROS detoxification. For example, HO-1 activates the expression of mitochondrial superoxide dismutase in neonatal rat astroglia challenged with dopamine (37). Furthermore, it has been demonstrated that upregulation of HO-1 improves mitochondrial function and prevents ATP depletion after oxidative stress (38). Noteworthily, some reports have suggested a duality of effects of HO-1 overexpression in oxidative stress (39,40). The release of ferric iron from the porphyrin ring of heme may result in detrimental effects, because this form of iron is known to catalyze oxidative stress (41).

Akt is a central node in cell signaling downstream of growth factors, cytokines, and other cellular stimuli. Akt can promote cell survival and protect against apoptosis initiated by the mitochondrial pathway through phosphorylation and inhibition of the mitochondrial pro-apototic proteins Bad, Bax and caspase-9 (42). Since HO-1 is induced by H_2O_2 preconditioning, and has been identified as a new substrate of Akt (43), we explored the effect of preconditioning with H_2O_2 on the activation of Akt. The results of this study showed that preconditioning markedly enhanced the expression of p-Akt, indicating that Akt is activated by preconditioning with H_2O_2. These results are consistent with previous evidence that Akt is rapidly activated in response to strong oxidants, such as H_2O_2 (44,45) and that oxidative preconditioning increases Akt activation in L-cells (7). In agreement with findings of previous studies (9,43), we found that Ly294002, a selective inhibitor of PI3K, blocked the induction of HO-1 by H_2O_2 preconditioning, suggesting that the PI3K/Akt pathway mediates the expression of HO-1. Similarly, recent studies have shown the transcriptional regulation of HO-1 by the PI3K/Akt pathway in response to nerve growth factor and to the antioxidant polyphenol, carnosol (27,30). Importantly, our data showed that treatment with Ly294002 also blocked the protective effects of H_2O_2 preconditioning against cytotoxicity induced by H_2O_2, which is comparable with the findings reported by Han *et al* (7) and Angeloni *et al* (9) . These results suggest that the PI3K/Akt pathway is involved in the adaptive effect of H_2O_2 preconditioning.

In conclusion, we have provided new evidence to elucidate an important mechanism responsible for the adaptive cytoprotective effect of H_2O_2 preconditioning against oxidative stress-induced injuries, including cytotoxicity, apoptosis and mitochondrial dysfunction in PC12 cells. We have observed that activation of PI3K/Akt-HO-1 pathway is involved in the protective effects of oxidative preconditioning. A better understanding of the role of PI3K/Akt-HO-1 pathway in the adaptive cytoprotection against oxidative stress may provide new therapeutic approaches for oxidative stress-related diseases. The findings of this study also support the notion that the lower levels of ROS generated by physiological metabolism may continually precondition cells and defend them against oxidative stress-induced insults under both physiological and pathophysiological conditions.

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