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Hydrogen peroxide induces the activation of the phospholipase C-γ1 survival pathway in PC12 cells: protective role in apoptosis

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It has been reported that phospholipase C-γ1 (PLC-γ1) plays an important protective role in hydrogen peroxide (H_2O_2)-induced pheochromocytoma (PC) 12 cells death. However, most studies have used high doses of H_2O_2 and the downstream targets of PLC-γ1 activation remain to be identified. The present study was designed to examine the roles of PLC-γ1 signaling pathway in the apoptosis of PC12 cells induced by low dose of H_2O_2, as well as the downstream factors involved in this pathway. Low-dose treatment of H_2O_2 resulted in PLC-γ1 tyrosine phosphorylation in a time-dependent manner and H_2O_2 killed the PC12 cells by inducing necrosis. In contrast, pretreatment of PC12 cells with U73122, a specific inhibitor of PLC, markedly increased the percentage of dead cells. The mode of cell death was converted to apoptosis as determined by Hoechst/PI nuclear staining and fluorescence microscopy. Western blot analysis demonstrated that the expression of Bcl-2 protein and the activation of pro-caspase-3 were not significantly affected by low dose of H_2O_2 alone. However, after pretreatment with U73122, Bcl-2 protein expression was dramatically decreased and the activation of pro-caspase-3 was significantly increased. We concluded that PLC-γ1 plays an important protective role in H_2O_2-induced PC12 cells death. Bcl-2 and caspase-3 probably participate in the signaling pathway as downstream factors.

Keywords phospholipase C-γ1; hydrogen peroxide; apoptosis; Bcl-2; caspase-3

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Introduction

For organisms living in an aerobic environment, exposure to reactive oxygen species (ROS) is unavoidable. ROS encompass a variety of partially reduced metabolites of oxygen (e.g. superoxide anions, hydrogen peroxide (H_2O_2), and hydroxyl radicals) possessing higher reactivity than molecular oxygen. When the production of ROS exceeds the capacity of cellular antioxidant defense system, it will result in what is termed a state of oxidative stress. At the cellular level, oxidant injury elicits responses ranging from proliferation to growth arrest, senescence, and cell death.

Oxidative stress has been implicated in a wide variety of disease processes including atherosclerosis, diabetes, pulmonary fibrosis, neurodegenerative disorders, and arthritis, and is believed to be a major factor in aging [1–3]. Recent studies have shown that infiltrating neutrophils release ROS in the site of spinal cord injury and ROS play a crucial role in the secondary damage of spinal cord injury. ROS are also involved in the cytochrome *c* release and caspase-dependent neuronal cell apoptosis [4,5]. Thus, ROS would be a potential treatment strategy for reducing secondary injury.

Phospholipase C-γ (PLC-γ) is a member of the family of phosphoinositide-specific PLCs (PI-PLC) that converts phosphatidylinositol 4,5-bisphosphate (PI4,5P2) to 1,2-diacylglycerol and inositol 1,4,5-trisphosphate. There are two forms of PLC-γ, namely PLC-γ1 and PLC-γ2. PLC-γ1 is widely distributed but the expression of PLC-γ2 is primarily limited to cells of hematopoietic lineage, though its overexpression was reported in the central nervous system [6]. PLC-γ1 has an essential role

in mammalian growth and development. Mice deficient in PLC-γ1 develop normally to embryonic day 8.5 but die soon after due to unknown defects in growth and development [7]. PLC-γ1 plays a key role in growth factor-dependent signal transduction [8]. PC12 cells are rat neuroendocrine cells in which PLC-γ1 was overexpressed. It has been reported that elevated PLC-γ1 expression suppressed ultraviolet C-induced apoptosis in PC12 cells [9]. However, the same authors found no protective effect of PLC-γ1 overexpression in NIH3T3 cells subjected to several different oxidative stress-inducing agents [10]. Recently, we reported that oxidative stress suppressed the osteoblastic differentiation process of primary rabbit bone marrow stromal cells and calvarial osteoblasts, and stimulated PLC-γ1 activation [11]. Furthermore, PLC-γ1 plays an important protective role in H_2O_2-induced PC12 cells apoptosis [12]. Remarkably, activation of PLC-γ1 alone is sufficient to protect the cells from H_2O_2-induced apoptosis in PC12 cells. The downstream targets of PLC-γ1 activation involved in mediating the protective effects, however, remain to be identified.

The present study sought to characterize the protective role of PLC-γ1 in low-dose H_2O_2-induced PC12 cells apoptosis as well as to identify the downstream signaling factors involved in the PLC-γ1 pathway.

Materials and Methods

Reagents

RPMI 1640 medium and fetal calf serum (FCS) were from Gibco BRL (Gaithersburg, USA). Antibodies specific to Bcl-2, pro-caspase-3, and the enhanced chemiluminescence (ECL®) Western-blotting detection system were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies specific to PLC-γ1 and phospho-PLC-γ1 were purchased from Cell Signaling Technology (Beverly, MA, USA). H_2O_2, Hoechst 33342, propidium iodide (PI), β-actin, and U73122 were purchased from Sigma (St. Louis, USA). All antibodies were used according to the manufacturers' instructions.

Cells and treatments

Rat neuroendocrine cell line PC12 cells were provided by Dr Cheng-gang Zou (School of life science, Yunnan University, Kunming, China). Cells were grown in RPMI 1640 medium containing 10% heat-inactivated FCS, 2-mM-glutamine, and antibiotics (100-IU/ml penicillin and 100-μg/ml streptomycin) at 37°C in 5% CO_2 in air. Exponentially growing cells were harvested by centrifugation and resuspended in fresh medium to achieve a culture density of 1×10^6 cells/ml. U73122, a specific inhibitor of PLC-γ1, was added to the culture medium 30 min before the addition of 10 μM H_2O_2. This time point was chosen to minimize the possibility of any direct interactions between U73122 and H_2O_2. Cell incubations were for 5 min to 48 h, as indicated in the text.

Quantification of cell death using Hoechst PI nuclear staining and fluorescence microscopy

Hoechst PI nuclear staining was carried out as previously described [13] with slight modifications. Briefly, cells (5×10^6 cells/ml) were incubated for 15 min at 37°C with Hoechst 33342 dye (10 μg/ml in phosphate-buffered saline, PBS), centrifuged, washed once in PBS, and then resuspended at an approximate density of 1×10^7 cells/ml. PI (50 μg/ml in PBS) was added just before microscopy. Cells were visualized using an Olympus microscope (Olympus, Olympus Optical Ltd, Tokyo, Japan) equipped with a fluorescent light source and a UV-2A filter cube with excitation wavelength of 330–380 nm and barrier filter of 420 nm. Cell morphology was scored as follows: 1, viable cells had blue-stained nuclei with smooth appearance; 2, viable apoptotic cells had blue-stained nuclei with multiple bright specks of condensed chromatin; 3, non-viable apoptotic cells had red-stained nuclei with either multiple bright specks of fragmented chromatin or one or more spheres of condensed chromatin (significantly more compact than normal nuclei); 4, non-viable necrotic cells had red-stained, smooth and homogeneous nuclei that were about the same size as normal (control) nuclei. Samples were randomized and examined after blinding. At least 200 cells were counted for each treatment. Experiments were repeated at least three times.

Western blot analysis

H_2O_2-stimulated cells (5×10^7 cells/ml) were lysed in ice-cold lysis buffer [62.5-mM Tris–HCl, pH 6.8, 25% glycerol, 2% sodium dodecyl sulphate (SDS), 0.01% bromphenol blue, and 5% β-mercaptoethanol]. Cell lysates were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto immunoblot polyvinylidenedifluoride membranes (Bio-Rad, Richmond, CA, USA). The membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 2 h at room temperature and incubated separately with various primary antibodies

(1:1000 dilution) for 1.5 h at room temperature. The membranes were then washed three times for 15 min with TBS-T and incubated with horseradish peroxidase-conjugated secondary antibody (1:2000 dilution) for 1 h at room temperature. Blots were again washed three times for 5 min each in TBS-T and developed by the ECL® detection system. Membranes were exposed to Fuji Medical X-Ray Film (Fuji Photo Film Co., Ltd, Karagawa, Japan).

Statistical analysis

Each experiment was repeated at least three times. Statistical analyses were performed using Student's *t*-test. $P < 0.05$ was considered significant. Data were presented as mean ± SD.

Results

Low-dose oxidant stimulates phosphorylation of PLC-γ1 in PC12 cells

To examine the effect of H_2O_2 treatment on PLC-γ1 phosphorylation, PC12 cells were exposed to 50-μM H_2O_2 at different time points. Expressions of PLC-γ1 and tyrosine phosphorylation of PLC-γ1 were examined by Western blot analysis with PLC-γ1- and phosphotyrosine-specific antibody. As shown in **Fig. 1**, H_2O_2 treatment resulted in PLC-γ1 tyrosine phosphorylation in a time-dependent manner. A significant increase in PLC-γ1 phosphorylation was detected after 50-μM H_2O_2 treatment and activation was rapid, occurring within 10 min of treatment. Maximum levels of PLC-γ1 phosphorylation were detected at 20–30 min after the addition of H_2O_2.

Effects of PLC-γ1 on PC12 cells apoptosis following low-dose oxidant challenge

To investigate the possible influence of PLC-γ1 on the effects of H_2O_2-induced apoptosis, we determined the cell death by fluorescence microscopy after staining the cells with the nuclear dyes Hoechst 33342 and PI. This method enabled us to distinguish apoptotic from non-apoptotic cells and also showed whether the cells had lost their plasma membrane permeability barrier (see Materials and Methods). In this experiment, cells treated with 50-μM H_2O_2 were incubated for 24, 36, and 48 h, respectively. The mode of cell death was determined by fluorescence microscopy using Hoechst/PI staining. **Fig. 2** showed that H_2O_2 killed the PC12 cells by inducing necrosis. Dead cells in 50-μM H_2O_2-treated cells remained at almost the same level at different time points. However, pretreatment with U73122 (10 μM) for 30 min, an inhibitor of PLC-γ1, effectively enhanced the effects of subsequent treatment with H_2O_2. More strikingly, U73122 converted the mode of cell death to apoptosis. Quantitative results in **Fig. 2(B)**, with 50-μM H_2O_2 treatment for 24, 36, and 48 h showed that dead cells were significantly increased from 19 ± 3%, 21 ± 2%, and 24 ± 2%, respectively ($P < 0.05$, compared with 10-μM U73122 treatment alone). However, pretreatment of cells with 10-μM U73122 markedly increased the percentage of dead cells to 59 ± 4%, 72 ± 2%, and 81 ± 3%, respectively, and converted the mode of cell death to apoptosis. ($P < 0.05$ compared with 50-μM H_2O_2 treatment alone).

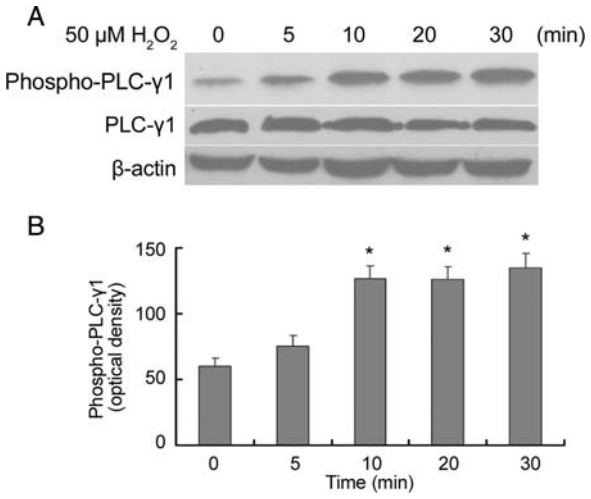

Figure 1 Fifty micromolar H_2O_2 treatment induces tyrosine phosphorylation of PLC-γ1 at different time points in PC12 cells
(A) PC12 cells treated with 50-μM H_2O_2 at different time points. PLC-γ1 expression and PLC-γ1 phosphorylation are assessed. Results are repeated at least in three independent experiments. (B) Optical densities of phospho-PLC-γ1 at various time points. Significant difference between H_2O_2 treated and control groups is indicated by $^*P < 0.05$.

Downstream factors in PLC-γ1 signaling pathway involved in low-dose H_2O_2-induced oxidant injury

To examine the possible involvement of PLC-γ1 in the regulation of Bcl-2 expression and caspase-3 activation, we investigated whether PLC-γ1 could regulate Bcl-2 expression and pro-caspase-3 activation by Western blot analysis in PC12 cells. As shown in **Fig. 3**, the level of Bcl-2 protein and activation of pro-caspase-3 were not significantly affected by 50-μM H_2O_2 alone. However, after pretreatment with 10-μM U73122 for 30 min, Bcl-2 protein expression was dramatically decreased and activation of pro-caspase-3 was significantly increased.

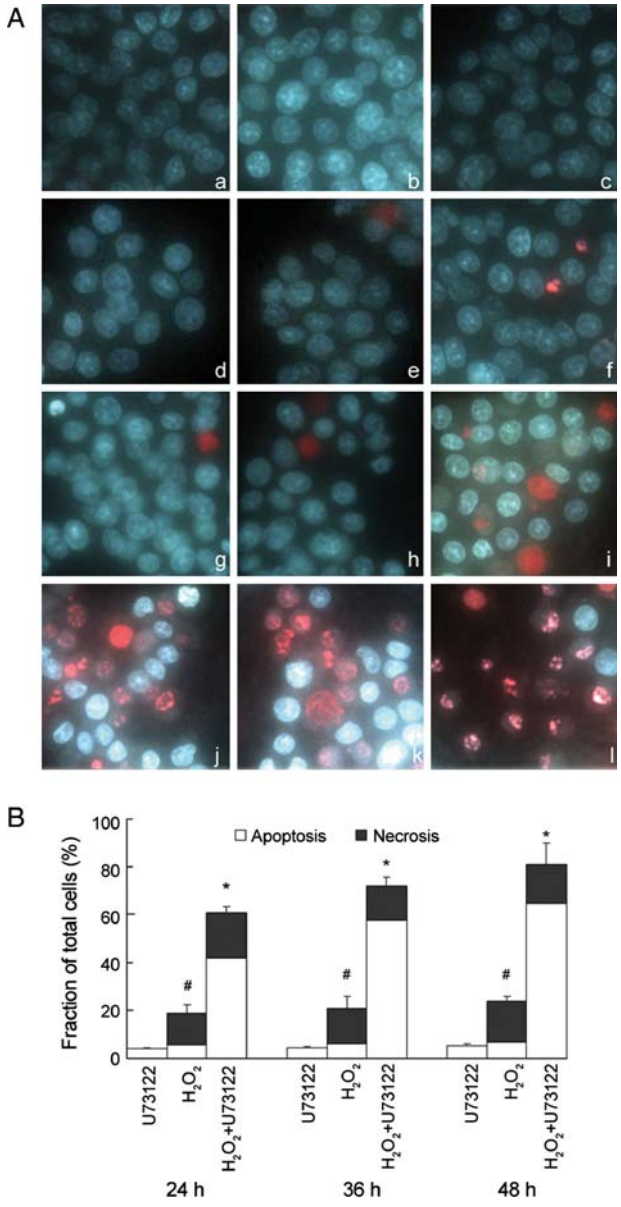

Figure 2 PLC-γ1 provides protection against H_2O_2-induced PC12 cell death (A) Morphology of cells as examined by fluorescence microscopy as described in Materials and Methods (original magnification, 400×). a, b and c: Cells with no treatment for 24, 36 and 48 h, respectively; d, e and f: cells treated with 10-μM U73122 for 24, 36 and 48 h, respectively; g, h and i: cells treated with 50-μM H_2O_2 for 24, 36 and 48 h, respectively; j, k and l: cells treated with 10-μM U73122 and 50-μM H_2O_2 for 24, 36 and 48 h, respectively. (B) Quantitative analysis of necrotic and apoptotic cells by fluorescence microscopy in various treatments. For ease of interpretation, the numbers of blue (membrane-intact) and red (PI-permeable) apoptotic cells are expressed as open bars and the numbers of necrotic cells are expressed as filled bars. Error bars show the SD for the total number of dead cells. Each experiment was repeated at least three times. $^{\#}P < 0.05$ compared with 10-μM U73122 treatment alone; $^{*}P < 0.05$ compared with 50-μM H_2O_2 treatment alone.

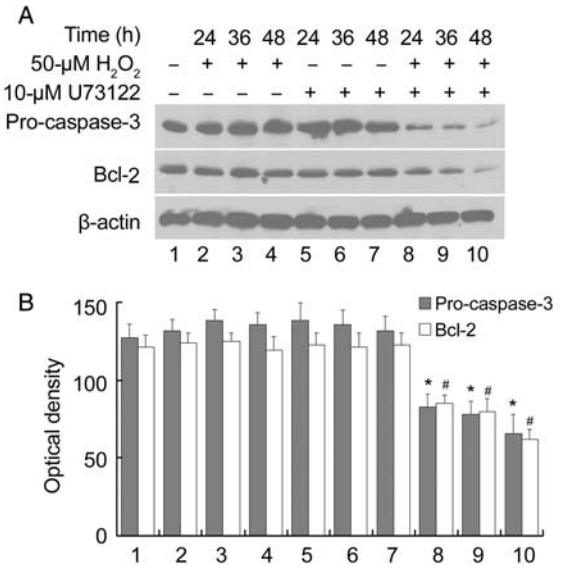

Figure 3 U73122 downregulates Bcl-2 expression and upregulates pro-caspase-3 activation in 50 μM H_2O_2-induced PC12 cell death in a time-dependent manner (A) PC12 cells treated with 10-μM U73122, or left untreated, for 30 min. Then, the cultures were exposed to 50-μM H_2O_2 for 24, 36 and 48 h, respectively. Experiments were repeated at least three times independently. β-actin was used as an internal control. (B) Optical densities of pro-caspase-3 and Bcl-2 in various treatment groups. $^{*,\#}P < 0.05$ compared with 50-μM H_2O_2 treatment alone or 10-μM U73122 treatment alone.

Discussion

It is well known that ROS elicit a wide spectrum of responses. These responses depend upon the severity of the damage, which is further influenced by the cell type, the magnitude of the dose, and the duration of the exposure. Typically, low doses of ROS, particularly H_2O_2, are mitogenic and promote cell proliferation, whereas medium doses result in either temporary or permanent growth arrest, such as replicative senescence. Very severe oxidative stress ultimately causes cell death via either apoptotic or necrotic mechanisms [2].

There are ample evidence, derived from some studies, suggesting that the use of pharmacological inhibitors of PLC-γ1 led to cell apoptosis or loss of anti-apoptotic effects of oxidative stress [9,10]. Mouse embryonal fibroblasts derived from mice rendered deficient for PLC-γ1 by targeted disruption of both PLC-γ1 alleles are more sensitive to H_2O_2 treatment relative to normal fibroblasts [14].

The rat PC12 cell line is a dopaminergic, neoplastic cell line in which PLC-γ1 was overexpressed. PC12 cells exhibit unique sensitivity to changes in oxygen availability and are frequently used as a cell model to

study neuronal vulnerability to hypoxia [15]. PLC-γ1 is activated by phosphorylation of specific tyrosine residues by growth factor receptors with intrinsic tyrosine kinase activities such as platelet-derived growth factor receptor, epidermal growth factor receptor, and fibroblast growth factor receptor [7]. It is well established that PLC-γ1 undergoes phosphorylation on tyrosine residues in response to H_2O_2 treatment [14]. PLC-γ1 plays an important role in regulating cell proliferation through its interactions with both receptor and non-receptor tyrosine. However, most of the previous studies have employed high doses of H_2O_2 and it is not clear whether activation of PLC-γ1 alone is sufficient to protect the cells from apoptosis and whether PLC-γ1 activation suppresses apoptosis-associated proteins activation in PC12 cells.

The present study has employed much lower concentrations of H_2O_2. We have demonstrated that, at concentrations as low as 50-μM H_2O_2, treatment with H_2O_2 results in PLC-γ1 phosphorylation, with time-dependent increases occurring over a range of 5–30 min in PC12 cells (**Fig. 1**). This suggests that H_2O_2 is not a weak activator of PLC-γ1. We further investigated the protective role of PLC-γ1 in H_2O_2-induced PC12 cells death. Our results showed that H_2O_2 killed the PC12 cells by inducing necrosis. In contrast, after pretreatment with U73122, the specific inhibitor of PLC, the percentage of dead cells was markedly increased and the mode of cell death was converted from necrosis to apoptosis. As shown in **Fig. 2**, the apoptotic bodies were enhanced in PC12 cells exposed to H_2O_2 with U73122 pretreatment, which suggest that PLC-γ1 may play a pivotal protective role in H_2O_2-induced PC12 cell death.

The Bcl-2 family plays an important role in H_2O_2-induced apoptosis in various cells [16,17]. Bcl-2 belongs to a growing family of apoptosis-regulatory proteins, which may be either death antagonists (e.g. Bcl-2 and Bcl-x/l) or death agonists (e.g. Bax, Bad, and Bak) [18]. Caspase-3 protease is synthesized as a 32-kDa inactive precursor (pro-caspase-3), which is proteolytically cleaved to produce a mature enzyme composed of 17- and 12-kDa subunits [19]. Some studies suggested that the Bcl-2 family and the protease caspase family play pivotal roles in apoptosis induced by H_2O_2 in PC12 cells [20,21]. However, it is not clear whether activation of PLC-γ1 alone is sufficient to protect the cells from apoptosis and whether PLC-γ1 activation regulates Bcl-2 expression and suppresses pro-caspase-3 activation. To investigate the molecular mechanism by which H_2O_2 induces death and which of the downstream targets of PLC-γ1 activation are involved in mediating the protective effects in this process in PC12 cells, we examined the expression of Bcl-2 proteins, the anti-apoptotic members of the Bcl-2 family, and pro-caspase-3 protein by Western blot analysis. Our data showed that the levels of Bcl-2 protein and caspase-3 activation were not modulated by low-dose H_2O_2 in PC12 cells. The results appear to be inconsistent with others [20,21]. These reports have suggested that H_2O_2-induced PC12 cells apoptosis via increasing Bcl-2 expression and elevating caspase-3 activity occurs at higher concentrations (in excess of 100 μM). Our results suggested that low concentration of H_2O_2 (50 μM) was relatively inefficient in modulating the levels of Bcl-2 protein and caspase-3 activity in PC12 cells. However, after pretreatment with U73122, the Bcl-2 protein expression was markedly decreased and activation of pro-caspase-3 was significantly increased compared with that of the cells treated with H_2O_2 alone (**Fig. 3**). Taken together, we speculate that PLC-γ1 plays a survival role by regulating the apoptosis-blocking proteins Bcl-2 and apoptosis-inducing protein caspase-3.

In conclusion, this study has provided evidence that PLC-γ1 may play an important protective role in H_2O_2-induced PC12 cell death. Bcl-2 and caspase-3 may participate in the signaling pathway as the involved downstream factors. Searching for other PLC-γ1 downstream effectors as well as the significance of them remains a challenge. Further study is required to characterize other potential downstream effectors in PLC-γ1 signaling pathway for its anti-apoptosis role.

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