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PROTECTIVE EFFECT OF H_2O_2 AGAINST SUBSEQUENT H_2O_2-INDUCED CYTOTOXICITY INVOLVES ACTIVATION OF THE PI3K-AKT SIGNALING PATHWAY

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Abstract – Preconditioning of sublethal ischemia implies a cytoprotective mechanism against subsequent ischemia-induced cell death; however, the precise mechanism by which preconditioning protects against ischemic injury is not known. In the present study, we clarified whether pretreatment with a sublethal concentration of H_2O_2 could counter subsequent H_2O_2-induced cytotoxicity and also investigated the mechanisms of the cytoprotective effect of a sublethal concentration of H_2O_2. Using the MTT reduction assay and Calcein-AM staining assay, we showed that pretreatment with H_2O_2 (10 μM, 24 hr) of COS7 cells partially protected cells against subsequent H_2O_2 (6 mM, 1 hr) -induced cytotoxicity. The phosphorylation of Akt/PKB, a downstream target of phosphatydylinositol-3 kinase (PI3K), at Ser473 was augmented by H_2O_2 (10 μM) administration. This augmentation peaked at 10 minutes after H_2O_2 (10 μM) treatment and fell to the basal level at 24 hr. A blocker of PI3K, LY294002, significantly attenuated H_2O_2 (10 μM, 24 hr) -induced cytoprotection. In addition, pretreatment with LY294002 reduced H_2O_2 (10 μM, 10 min) -induced phosphorylation of Akt at Ser473. These findings suggest that a sublethal concentration of H_2O_2 exerts a cytoprotective effect against subsequent H_2O_2-induced cell death and that this cytoprotective effect of H_2O_2 is mediated by activation of the PI3K-Akt signaling pathway.

Key words: H_2O_2, cytoprotective effect, PI3K-Akt signaling pathway, preconditioning.

INTRODUCTION

The production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), is a normal physiological process, but an imbalance between the production of ROS and their removal may induce oxidative stress. Accumulation of ROS may cause oxidative damage to nucleic acids, lipids, and proteins and affect cell membrane properties and, as a consequence, lead to the oxidative destruction of cells (17). Indeed, in neuronal degenerative diseases such as Alzheimer's disease and Parkinson disease, an excessive amount of ROS is accumulated in the brain (10, 13, 16). ROS also play central roles in cardiac physiology and pathophysiology (1, 9).

It has been reported that sublethal transient ischemia, known as ischemic preconditioning, salvages subsequent global ischemia-induced neuronal death (15) and ischemic myocardium (11). Ischemic preconditioning also protects kidneys from ischemic acute renal failure (20); however, the precise mechanisms by which preconditioning decreases ischemia-induced cytotoxicity remain unknown.

The serine/threonine protein kinase Akt is a signaling kinase downstream of phosphatidylinositol 3-kinase (PI3K) (3). The PI3K-Akt pathway is a critical transducer of several major survival signals in CNS neurons (5). Indeed, we have previously reported that nicotinic acetylcholine receptor stimulation protects neurons against glutamate excitotoxicity

Abbreviations: H_2O_2: hydrogen peroxide; PI3K: phosphatidylinositol 3-kinase**;** ROS: reactive oxygen species.

through the PI3K-Akt system (6). Also, we have reported that prestimulation of the glutamate receptor protects neurons from excess glutamate-induced excitotoxicity (12) via activation of Akt. Combining our results and other reports, we speculate that cytoprotective effects of ischemic preconditioning might involve the generation of sublethal concentration of ROS, such as H_2O_2, and the PI3K-Akt signaling pathway.

In the present study, we investigated the effects of a sublethal concentration of H_2O_2 on H_2O_2-induced cell death, and demonstrated that it, 1) protects cells from subsequent H_2O_2-induced cell death, 2) enhances the phosphorylation of Akt/PKB at Ser473 and that this phosphorylation of Akt was attenuated by the inhibition of PI3K and 3) the cytoprotective effect of H_2O_2 is attenuated by the specific PI3K inhibitor. These results suggest that H_2O_2 protects cells from a subsequent excess amount of H_2O_2-induced cytotoxicity and this protective effect is mediated by activation of the PI3K-Akt signaling pathway. Against oxidative stress, a sublethal concentration of H_2O_2 may enhance cellular defense systems, including survival signals such as the PI3K-Akt signaling system.

MATERIALS AND METHODS

Materials

The sources of drugs and materials are as follows: Dulbecco's modified Eagle's medium and fetal bovine serum (Invitrogen, Carlsbad, CA, U.S.A.); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Nacalai Tesque, Kyoto, Japan); anti-phospho-Akt (Ser 473) antibody and anti-Akt antibody (Cell Signaling Technology, Inc, Danvers, MA, U.S.A.); anti-Actin antibody (Millipore, Billerica, MA, U.S.A.); LY294002 (Calbiochem, Cambridge, MA, U.S.A.); hydrogen peroxide (Wako, Osaka, Japan); Calcein-AM (Dojindo, Kumamoto, Japan).

Cell Culture

COS7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were incubated at 37°C in a 95% air/5% CO_2 humidified atmosphere.

Calcein-AM staining

Cultures were treated with 5 μM calcein-AM. Cell viability was assessed by monitoring the uptake and intracellular conversion of non-fluorescent calcein-acetoxymethyl ester (calcein-AM) to fluorescent calcein by intracellular esterases. Stained cells were scored as living cells. Cell viability is presented as a percentage of the control.

MTT assay

Cell viability was assessed using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Briefly, after each treatment, cells were incubated at 37°C for 30 min with media containing 0.5 mg/ml MTT. After incubation, the MTT solution was replaced with 2-propanol (1 ml) and the cells were submitted to 1 min of shaking. The solution was transferred to a cuvette. Absorption was measured at 570 nm using a spectrophotometer (Model U-1100; HITACHI, Tokyo, Japan).

Preparations of Cell Extracts

After each treatment, cells were lysed in buffer consisting of 20 mM Tris hydrochloride, pH 7.0, 2 mM EGTA, 25 mM 2-glycerophosphate, 1% Triton X-100, 2 mM dithiothreitol, 1 mM vanadate, 1 mM phenylmethylsulfonyl fluoride and 1% aprotinin and then centrifuged at 15,000 rpm for 30 min at 4°C. The supernatants were used as the cell extracts for immunoblot analysis.

Immunoblotting

SDS-solubilized samples were loaded onto SDS–polyacrylamide gels. After electrophoresis, proteins were electrotransferred to a polyvinylidene difluoride membrane. Membranes were incubated with primary antibodies in 5% non-fat dry milk containing 20 mM Tris hydrochloride (pH 7.6), 135 mM NaCl and 0.1% Tween 20 overnight. Subsequently, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody. Immunoreactive bands were detected by enhanced chemiluminescence.

Statistical Analysis

Statistical significance of the differences between groups was determined by one-way ANOVA followed by Dunnett's multiple comparison's tests or Student-Newman-Keuls tests.

RESULTS

Sublethal Concentration of H_2O_2 Protects Cells From Subsequent H_2O_2-induced Cell Death

We initially investigated the effect of sublethal concentration of H_2O_2 on subsequent H_2O_2-induced cytotoxicity in our culture (Fig. 1). For our assay of cell viability, we used a fluorescent dye, calcein-AM. This dye is able to cross the cell membrane while associated with its acetoxymethyl (AM) group, but only becomes fluorescent after the –AM group is cleaved by functional intracellular esterases. In dead or dying cells, esterases are not present to perform this conversion, providing an effective loss-of-function assay for cell viability (14). As shown in Fig. 1 (a) and (b), H_2O_2 (6 mM, 1 hr) alone caused significant cell death compared to the control. Prestimulation of H_2O_2 (10 μM) for 24 hr partially but significant reduced H_2O_2 (6 mM, 1 hr) -induced cytotoxicity. Alternatively, we also assessed cytotoxicity using the MTT assay. As shown in Fig. 1 (c), pretreatment of H_2O_2 (10

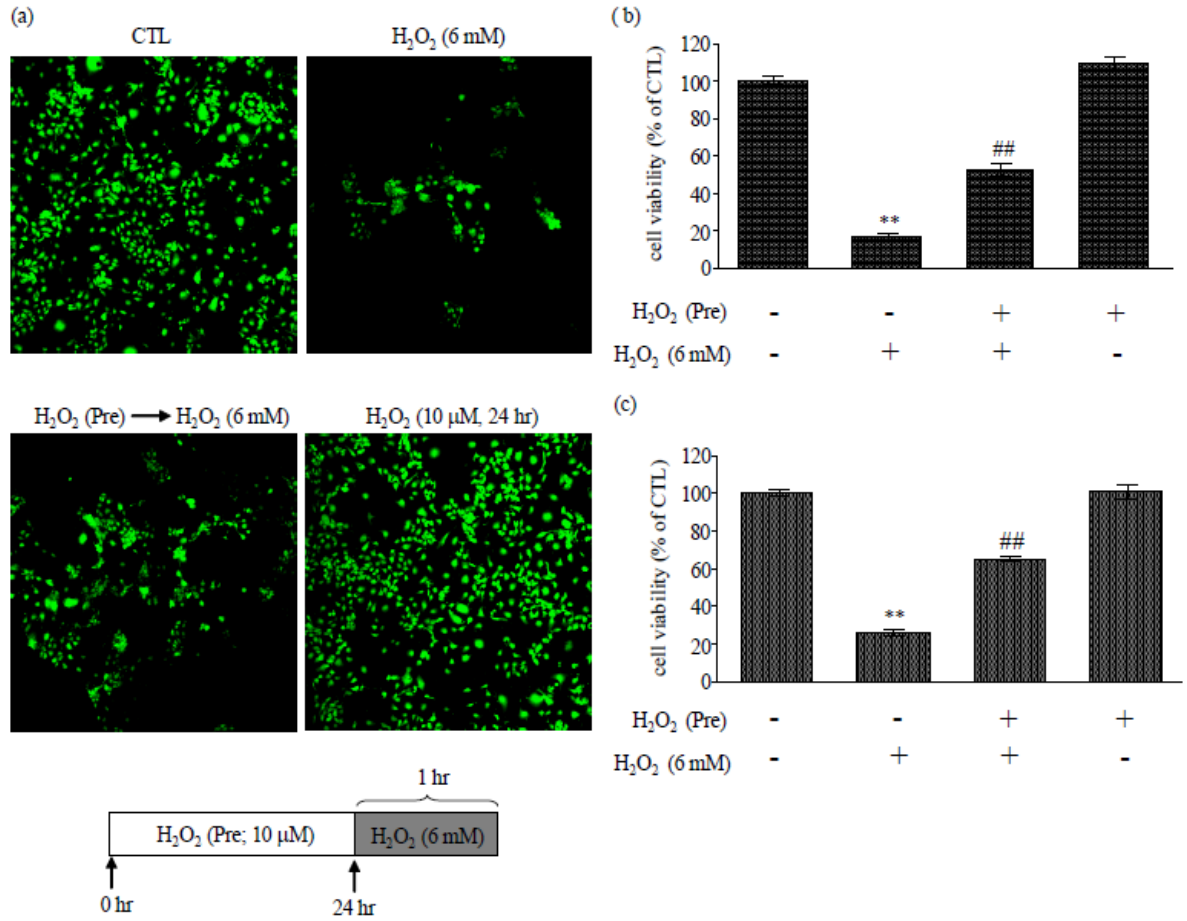

Figure 1. Sublethal concentration of H_2O_2 protects cells from subsequent H_2O_2-induced cytotoxicity.
(a) COS7 was pretreated with H_2O_2 (10 μM) for 24 hr, followed by H_2O_2 administration (6 mM, 1 hr). Cell viability was assessed by uptake of calcein and its conversion of fluorescent calcein. Calcein-AM (5 μM) was added to the cultures for 10 minutes, and fluorescence was observed (excitation 485 nm, emission 528 nm). Magnification×100. (b) Quantitative analysis of cell viability. Cells stained by calcein were scored as living cells. Cell viability was represented as a percentage of the control. (c) H_2O_2 (10 μM, 24 hr) was administered prior to H_2O_2 exposure (6 mM, 1 hr). Cell viability was analyzed by the MTT assay. Pretreatment with H_2O_2 (10 μM, 24 hr) is represented as "H_2O_2 (Pre)".
Error bars are SEM. Similar results were obtained in three experiments. ** P <0.01, H_2O_2 (6 mM) alone vs. non treatment. ## P <0.01, H_2O_2 (Pre) + H_2O_2 (6 mM) vs. H_2O_2 (6 mM) alone.

μM, 24 hr) exerts a partial but significant reduction of H_2O_2 (6 mM, 1 hr) -induced cytotoxicity. Incubation with 10 μM H_2O_2 for 24 hr had no effect on cell viability. These results indicate that sublethal concentration of H_2O_2 partially attenuates subsequent H_2O_2-induced cytotoxicity.

Sublethal Concentration of H_2O_2 Enhances Phosphorylation of Akt at Ser473

It has been reported that the PI3K-Akt signaling pathway is a critical transducer for several major survival signals in cultured cells (5). Thus, we hypothesized that the cytoprotective effect of a sublethal concentration of H_2O_2 might be mediated by activation of the PI3K-Akt signaling pathway. We investigated the effect of a sublethal concentration of H_2O_2 on Akt Ser473 phosphorylation, the active form of Akt, by immunoblotting (Fig. 2, a, b). Administration of H_2O_2 (10 μM, 24 h) to COS7 cells resulted in an increase in the phosphorylation of Akt at Ser473, which peaked at 10 min of exposure and returned to the basal level in 24 hr. This result indicates that a sublethal concentration of H_2O_2 increases Akt activity.

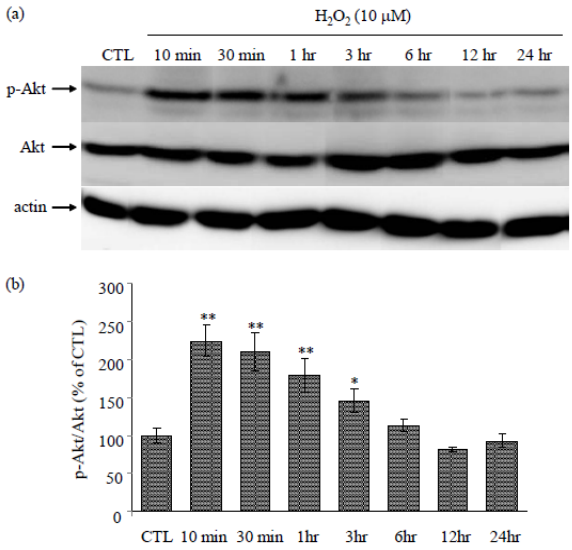

Figure 2. Sublethal concentration of H_2O_2 enhances Akt phosphorylation at Ser473.
(a) The activity of Akt was monitored by Western blotting using a phospho-specific antibody (anti-phospho-Ser473 Akt). COS7 cells were exposed to H_2O_2 (10 μM) for 10 min, 30 min, 1 hr, 3 hr, 6 hr, 12 hr, 24 hr. (b) Quantitative analysis of H_2O_2-induced Akt phosphorylation (Ser473). Error bars are SEM. Similar results were obtained in three experiments. ** P <0.01, H_2O_2 (10 μM) alone vs. control (CTL). * P <0.05, H_2O_2 (10 μM) alone vs. control (CTL).

Inhibition of PI3K activity attenuates H_2O_2-induced Akt activity

Next, to elucidate that the phosphorylation of Akt at Ser473 induced by sublethal concentration of H_2O_2 is PI3K dependent, we investigated the effect of PI3K inhibition on the H_2O_2-induced increase of Akt phosphorylation at Ser473 by immunoblotting. As we expected, pretreatment of LY294002 (10 μM) for 24 hr attenuated the phosphorylation of Akt at Ser473 induced by H_2O_2 (10 μM, 10 min). This result suggests that H_2O_2-induced phosphorylation of Ser473 of Akt is PI3K dependent.

Inhibition of PI3K activity attenuates H_2O_2-induced cytoprotection

Finally, we investigated the effect of PI3K, which is the upstream signal molecule of Akt, on the H_2O_2-induced cytoprotective effect. Simultaneous administration of a specific PI3K inhibitor, LY294002 (10 μM), and H_2O_2 (10 μM) attenuated the protective effect of H_2O_2 (10 μM, 24 hr) against H_2O_2 (6 mM, 1 hr) -induced cytotoxicity. This result suggests that the protective effect of H_2O_2 is mediated by activation of PI3K.

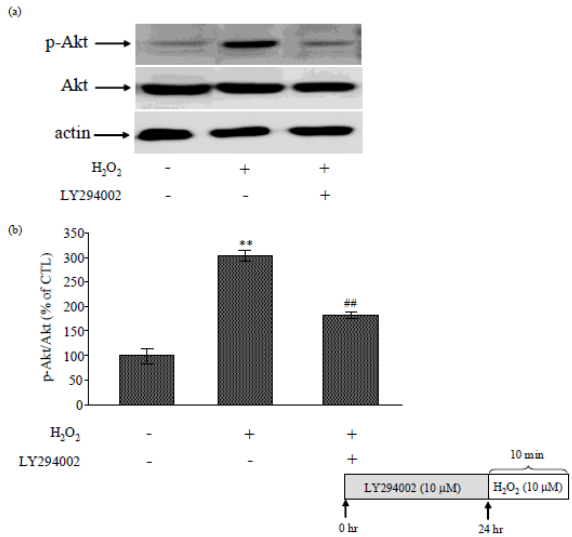

Figure 3. Inhibition of PI3K attenuates H_2O_2-induced phosphorylation of Akt at Ser473.
(a) H_2O_2 (10 μM) was added to the culture for 10 minutes. LY294002 (10 μM, 24 hr) was administered to the culture prior to H_2O_2 exposure (10 μM, 10 min). The activity of Akt was monitored by Western blotting using a phospho-specific antibody (anti-phospho-Ser473 Akt). (b) Quantitative analysis of Akt phosphorylation (Ser473). Error bars are SEM. Similar results were obtained in three experiments. ** P <0.01, H_2O_2 (10 μM) alone vs. non treatment. ## P<0.01, LY + H_2O_2 (10 μM) vs. H_2O_2 (10 μM, 10 min) alone.

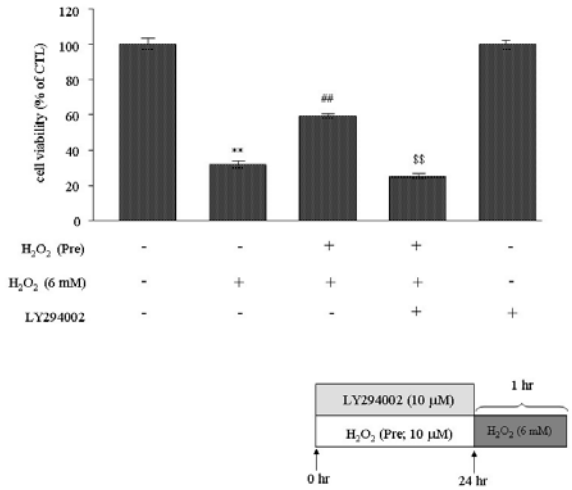

Figure 4. Inhibition of PI3K attenuates sublethal concentration of H_2O_2-induced cytoprotective effect.
H_2O_2 (10 μM) was administered 24 hr prior to H_2O_2 exposure (6 mM, 1 hr). A specific PI3K inhibitor, LY294002 (10 μM), was simultaneously administered with H_2O_2 (10 μM) for 24 hr. Cytotoxicity was assessed by the MTT assay. Pretreatment with H_2O_2 (10 μM, 24 hr) is represented as "H_2O_2 (Pre)". Error bars are SEM. Similar results were obtained in three experiments. ** P <0.01, H_2O_2 (6 mM) alone vs. non treatment. ## P <0.01, H_2O_2 (Pre) + H_2O_2 (6 mM) vs. H_2O_2 (6 mM) alone. $$ P<0.01, H_2O_2 (Pre) + LY294002 + H_2O_2 (6 mM) vs. H_2O_2 (Pre) + H_2O_2 (6 mM).

DISCUSSION

In the present study, we demonstrated for the first time that: (1) the prestimulation of cells with a sublethal concentration of exogenous H_2O_2 exerts a cytoprotective effect against subsequent H_2O_2-induced cytotoxicity; (2) a sublethal concentration of H_2O_2 enhances the phosphorylation of Akt at Ser473, which was attenuated by the selective PI3K inhibitor; (3) inhibition of PI3K reduced the cytoprotective effect of H_2O_2 against an excess amount of exogenous H_2O_2-induced cytotoxicity.

Although prior studies have shown that exogenous H_2O_2 induced the activation of Akt (7, 18, 19), consistent with our report, the relation between Akt activation and the cytoprotective effect of sublethal concentration of H_2O_2 has not been clarified. The present study revealed that H_2O_2 enhances the phosphorylation of Akt at Ser473. As phosphorylated Akt at Ser473 is considered to be an active form of Akt, the cytoprotective effect of H_2O_2 is, at least in part, mediated through the Akt pathway. Although comparatively long exposure to H_2O_2 (10 μM) for 24 hr was necessary for H_2O_2-induced cytoprotection, the phosphorylation of Akt by H_2O_2 peaked in ten minutes and returned to the basal level in 24 hr. Also, pretreatment with H_2O_2 for a relatively short time, i.e. 1 hr, did not exert a cytoprotective effect (data not shown). From these results, certain protein synthesis might be necessary downstream of Akt activation to exert the cytoprotective effect of H_2O_2 against an excess amount of H_2O_2-induced cytotoxicity. Previously, we have reported that amyloid-β-induced cytotoxicity is attenuated via activation of the PI3K-Akt pathway and up-regulation of Bcl-2, an anti-apoptotic protein downstream of the PI3K-Akt signaling pathway (6). Once Akt is activated by the phosphorylation of Ser473, Bcl-2 protein levels increase (6). It is believed that the anti-apoptotic protein Bcl-2 is responsible for suppressing cell death and acts as a cytoprotective factor. Thus, in the present study, activation of Akt induced by H_2O_2 might also lead to increased Bcl-2 at protein levels and, as a consequence, might exert H_2O_2-induced cytoprotection.

PTEN (phosphatase and tensin homolog deleted from chromosome 10) inhibits phosphoinositide 3-kinase (PI3K) -dependent activation of Akt (2). It was shown that exposure of cells to H_2O_2 resulted in the inhibition of PTEN (4, 8). From these reports and our present results, H_2O_2-induced enhancement of the phosphorylation of Akt at Ser473 might also occur through the inactivation of PTEN by H_2O_2.

Although the cytoprotective effect of preconditioning has recently attracted attention, the mechanism has not been completely clarified. In the present study, we showed that activation of PI3K-Akt signaling pathway is involved in the cytoprotective effect of H_2O_2. Further investigation is needed to elucidate how mild oxidant stresses, such as preconditioning, acquire resistance to oxidation stress.

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