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# Characterization of $H_2O_2$-induced acute apoptosis in cultured neural stem/progenitor cells

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**Abstract In the present study, we characterized hydrogen peroxide ($H_2O_2$)-induced cell apoptosis and related cell signaling pathways in cultured embryonic neural stem/progenitor cells (NS/PCs). Our data indicated that $H_2O_2$ induced acute cell apoptosis in NS/PC in concentration- and time-dependent manners and selectively, it transiently increased PI3K-Akt and Mek-Erk1/2 in a dose-dependent manner. Inhibition of PI3K-Akt with wortmannin, a PI3-K inhibitor, was found to significantly increase $H_2O_2$-induced acute apoptosis and dramatically decrease basal pGSK3β levels. The level of pGSK3β remained unchanged with $H_2O_2$ exposure. We conclude that the transient activation of PI3K-Akt signaling delays the $H_2O_2$-induced acute apoptosis in cultured NS/PCs in part through maintaining the basal pGSK3β level and activating other downstream effectors.**


*Keywords:* Oxidative stress; Apoptosis; Akt; Survival; Neural stem cell; Erk1/2

## 1. Introduction

The production of intracellular reactive oxygen species (ROS) caused by exposure to external oxidants is very destructive to normal cell types and plays critical roles in diverse cellular processes from tumorigenesis to aging [1]. Bursts of intracellular ROS result in cell apoptosis, which is a process regulated by cell signaling pathways and leads to cell death accompanied by the reduction of the total cell volume, general compaction of cell organelles and DNA fragmentation. Neural stem/progenitor cells (NS/PCs) are very sensitive to increases of ROS and result in cell apoptosis. Oxidative stress-induced apoptosis in NS/PCs has often been observed during NS cell therapy and therapeutic irradiation of brain [2–4]. The characterization of oxidative stress-induced apoptosis in NS/PCs will help to improve this disadvantage.

Hydrogen peroxide ($H_2O_2$) is one of the ROS generated during cellular metabolism. Previous studies have shown that it can activate several downstream signaling pathways involved in cell survival or apoptosis in various cell types during oxidant insults [5–8]. However, there is limited information about the emergence of linking $H_2O_2$-induced signaling pathways with NS/PC apoptosis. In the present study, we used cultured NS/PCs dissociated from embryonic day 13 (E13) rat cerebral cortexes to characterize $H_2O_2$-induced NS/PC apoptosis and related cell signaling pathways. We have concluded that the PI3K-Akt signaling pathway is transiently activated by exposure to $H_2O_2$ and plays a protective role in delaying acute apoptosis in the NS/PCs.

## 2. Materials and methods

### 2.1. Primary embryonic NS/PC cultures

To prepare the adherent NS/PC cultures, the dissected cortical neuroepithelia of embryonic day (E) 13 rat brains were subjected to papain dissociation according to a previous protocol [9]. Then the dissociated cells were plated in 35 mm dishes coated with poly-D-lysine and fibronectin and maintained in serum-free NeuralBasal (NB) medium supplemented with B27 (Invitrogen, CA), L-glutamine and basic fibroblast growth factor (bFGF) [10]. All animals were treated humanely according to NIH guidelines.

### 2.2. Exposure to $H_2O_2$ and pretreatment with inhibitors

Newly opened bottles of $H_2O_2$ were used and freshly prepared diluted $H_2O_2$ stock solution (100 mM) was immediately added into cell culture medium to achieve the desired working concentration. For inhibitor studies, each inhibitor was added to the culture medium 30 min before the addition of $H_2O_2$.

### 2.3. Immunocytochemistry and propidium iodide (PI) staining

To assess the purity of NS/PCs, cell cultures (Day 6) with approximately 75% confluence on the plates were fixed with 4% formaldehyde and immunostained for nestin, a specific marker for NS/PCs. Fluorescein-conjugated secondary antibody was used and cells were stained green [11]. Cells were subjected to nuclei staining with propidium iodide (PI) (2.5 μg/ml) for 10 min to obtain the total cell count.

### 2.4. Calcein-AM/ethidium homodimer-1 (EthD-1)

To quantify $H_2O_2$-induced cell death, cells were stained with calcein-AM for live cells and ethidium homodimer-1 (EthD-1) for dead cells using a Live/Dead viability/cytotoxicity kit according to the manufacturer's protocol (Molecular Probes Inc., OR). Approximately, 1500–2000 cells were counted to obtain the ratios of dead cells to total cell count.

### 2.5. TUNEL (terminal deoxynucleotidyl transferase-mediated UTP end-labeling) assay

To characterize cell apoptosis, cells were fixed and permeabilized before staining with anti-fluorescein antibody according to the manufacturer's protocol (Roche Diagnostics GmbH, IL). Fragmented or

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*Abbreviations:* NS/PC, neural stem/progenitor cell; CNS, central nervous system; ROS, reactive oxygen species

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nick DNA stained with fluorescein-conjugated antibody was observed with fluorescence microscopy.

### 2.6. DNA fragmentation assay

To measure oligonucleosome-sized fragments resulting from cleavage of nuclear DNA, the DNA fragmentation assay was modified and performed as previously described [12]. In brief, $3 \times 10^6$ control/treated cells were recovered by trypsinization, and the cells were lyzed in 5 mM Tris–HCl, pH 8, 20 mM EDTA, 0.5% Triton at 4 °C for 20 min. The soluble fractions in the lysates were collected after centrifugation and contained the fragmented DNA only. Samples were then treated with RNAase A and proteinase K subsequently. DNA was extracted sequentially with phenol, phenol/chloroform/isoamyl alcohol, and chloroform and precipitated in ethanol in the presence of 0.5 M NaCl at −80 °C overnight. The precipitated DNA was electrophoresed on 1.2% agarose gels.

### 2.7. Western blotting and densitometry analysis

bFGF was withdrawn from NB/27 medium overnight before conducting $H_2O_2$ exposure. Fifteen μg of protein was used to conduct electrophoresis on 4–20% denature PAGE gels (Invitrogen) and Western blotting as previously described [13]. Most of the antibodies were purchased from New England Bio Lab. Inc. The ratio of phosphorylated protein level to total protein level was calculated using Scion Image software (Scion, MD).

## 3. Results and discussion

NS/PCs in the central nervous system (CNS) during development are self-renewing and can generate neurons and glia [14,15]. Consistent with in vivo studies, neurons are the first differentiated cells to appear in the expanded NS/PC cultures. Therefore, it is necessary to examine the purity of NS/PCs in the expanded cultures by immunocytochemistry. NS/PC cultures at Day 6 (D6) contained approximately 95% nestin$^+$ cells (Fig. 1A) and were used to conduct the subsequent experiments in the present study. Nestin is an intermediate filament mainly located in the cytoskeleton close to the cell membrane and is a specific marker for NS/PCs. Nuclei were visualized by PI staining (Fig. 1A).

$H_2O_2$ is a strong oxidant and very toxic to cells. To quantify $H_2O_2$-induced cell death, calcein-AM (live) and ethidium

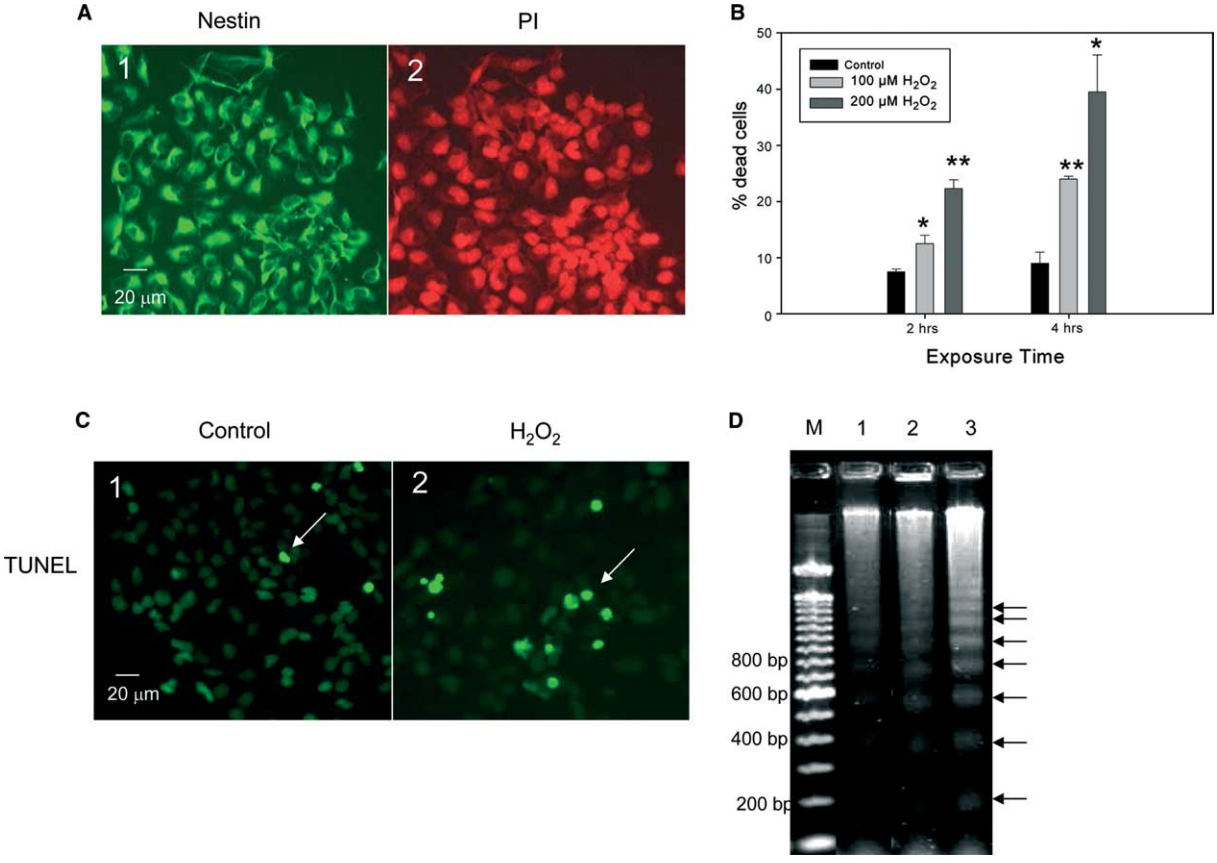


Fig. 1. $H_2O_2$-induced acute cell death is apoptotic. To characterize $H_2O_2$-induced apoptosis, TUNEL, DNA fragmentation assay, and Western blotting were performed. Dissociated NS/PCs were maintained in NB/27 medium supplemented with bFGF for 6 days. (A) Photographs of two exposures of a single field in a cell culture. NS/PCs were double stained with anti-nestin (A1) and propidium iodide (PI) for visualizing nuclei. PI staining yields the total cell count (A2). (B) $H_2O_2$-induced cell death was quantified with live/dead (calcein-AM/EthD-1) dye staining. The cell cultures exposed to 100 μM $H_2O_2$ for 2 and 4 h contain 12.5% and 25% dead cells, respectively. The cultures exposed to 200 μM $H_2O_2$ for 2 and 4 h have 23% and 40% dead cells, respectively. "*" and "**" represent $P < 0.05$ and $P < 0.005$ by $t$ test, respectively. Bars represent means ± S.E. obtained from three individual experiments with duplicate samples. (C) DNA fragmentation in apoptotic cells was visualized by TUNEL staining with fluorescein-conjugated antibody (arrows). Few apoptotic cells appear in the control cell culture (C1). In contrast, the cell culture exposed to 200 μM $H_2O_2$ for 2 h has significantly more apoptotic cells (C2). Scale bars in (A,C): 20 μm. (D) DNA ladders with an increment of 200 bp in size resulting from $H_2O_2$-induced apoptosis were measured by DNA fragmentation assay as described in Section 2. Control cells (lane 1) show some intrinsic apoptosis, while cells treated with 200 μM $H_2O_2$ for 2 and 4 h (lanes 2 and 3, respectively) show the distinctive pattern of oligonucleosomes (the arrows) resulting from DNA cleavage in apoptotic cells. Lane M is a marker for DNA length.

homodimer-1 (dead) staining was conducted. Our results indicated that the increase (twofold) in $H_2O_2$-induced cell death became statistically ($P < 0.05$) significant in the cell cultures exposed to 200 μM for 2 and 4 h (Fig. 1B). Twofold increase in cell death was also observed at 100 and 200 μM $H_2O_2$ for 4 h (Fig. 1B). $H_2O_2$ significantly increases the acute cell death in cultured NS/PCs in time- and dose-dependent manners.

To measure $H_2O_2$-induced apoptosis, terminal deoxynucleotidyl transferase-mediated UTP end-labeling (TUNEL) staining, a DNA fragmentation assay and Western blotting were performed. TUNEL preferentially labels apoptosis in comparison to necrosis and the staining data indicated that there was a significant increase in NS/PC cell death via apoptosis in cells exposed to $H_2O_2$ (Fig. 1C), while control cells showed only some intrinsic apoptosis (Fig. 1C). To further prove $H_2O_2$-induced cell death is apoptotic, a DNA fragmentation assay was used to detect the presence of a DNA ladder. We found that a distinctive pattern of oligonucleosome sized DNA fragments (arrows in Fig. 1D) resulting from apoptosis was present and significantly stronger in cells exposed to $H_2O_2$ relative to control cells which had some background DNA fragmentation due to the intrinsic apoptosis (Fig. 1D). The intrinsic apoptosis is often observed in cultured NS cells [16] and the resulting mechanism is not completely known yet. Our result consistently suggests that $H_2O_2$ induced acute apoptosis in cultured NS/PCs.

We have examined the levels of p38MAP, JNK, pERk1/2 and pAkt in the NS/PCs exposed to $H_2O_2$ by Western blotting. There was no significant increase in p38MAPK or JNK levels (data not shown). Only pAkt and pErk1/2 levels increased in response to $H_2O_2$ exposure. To further characterize these two signaling pathways, cells were exposed to different concentrations of $H_2O_2$ for 15 min. The pAkt level appeared to increase in a concentration-dependent manner in the presence of 20, 50, 100, and 200 μM $H_2O_2$, while the total Akt levels did not change among all tested concentrations (Fig. 2A). For time course measurements, cells were exposed to 200 μM $H_2O_2$. The pAkt levels increased shortly after the exposure and returned to basal level within 4 h, however, Akt levels remained unchanged throughout the experiment (Fig. 2B). Our data demonstrate that $H_2O_2$ transiently activates PI3K-Akt in cultured NS/PCs in a concentration-dependent manner.

The pErk1/2 levels were assessed at different concentrations of $H_2O_2$ for 15 min. The exposure of $H_2O_2$ induced increase in pErk1/2 level in a concentration-dependent manner at 10, 20, 50, and 100 μM, while the total Erk1/2 levels were constant for all tested concentrations (Fig. 3A). Time course measurements were conducted with 100 μM $H_2O_2$. The pErk1/2 level reached the maximum in 15 min and declined slowly (Fig. 3B). The levels remained stable for 4 h, while the total Erk1/2 level remained the same (Fig. 3B). Our data suggest that $H_2O_2$ exposure transiently increases Mek-Erk1/2 signaling pathway in cultured NS/PCs in a concentration-dependent manner. Both $H_2O_2$-induced pAkt and pErk1/2 levels reached the maximum at 200 μM and decreased slightly at the concentrations over 200 μM, possibly due to the extensive cell death caused by those concentrations.

The physiological consequence of $H_2O_2$-induced PI3K-Akt and Mek-Erk1/2 signaling was examined with the inhibitors, wortmannin and U0126, respectively. U0126, a new Mek inhibitor, blocked the activation of Erk1/2 efficiently (Fig. 3B), but there was no significant increase in $H_2O_2$-induced cell apoptosis with the pretreatment of U0126 (U + H in Fig. 4A). In contrast, pretreatment with wortmannin, a PI3K inhibitor,

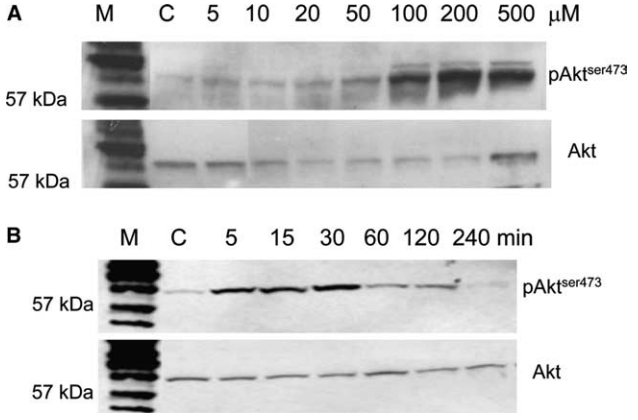


Fig. 2. $H_2O_2$ transiently increases Akt phosphorylation in a dose-dependent manner. Western blotting was used to analyze the cell extracts with pAkt ($Ser^{473}$) and Akt antibodies. (A) NS/PC cultures at day 6 were exposed to 0, 5,10, 20, 50,100, 200, and 500 μM $H_2O_2$ for 15 min. $H_2O_2$ induces significant increase in pAkt levels in the presence of 100, 200, and 500 μM and it induces the maximal increase (twofold) at 200 μM (upper panel). The total Akt levels remain constant for all tested samples (lower panel). (B) Cells were exposed to 200 μM $H_2O_2$ for 4 h for time course measurements. The pAkt level transiently increases within 5 min and returns to the baseline within 4 h (upper panel). The total Akt levels remain unchanged (lower panel).

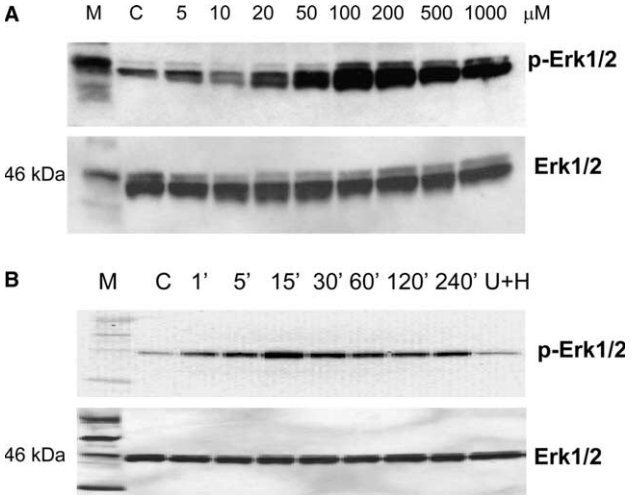


Fig. 3. $H_2O_2$ transiently increases Erk1/2 phosphorylation in a dose-dependent manner. Western-blot analysis of the pErk1/2 levels in the NS/PCs exposed to $H_2O_2$. (A) Cells were exposed to 0, 5, 10, 20, 50, 100, 200, 500, and 1000 μM $H_2O_2$ for 15 min. $H_2O_2$ significantly increases the pErk1/2 levels with the concentrations in the presence of 20, 50, 100, and 200 μM. It induces maximal increase (2.5-fold) in the pErk1/2 level at 100 μM and declines slightly at 500 and 1000 μM (upper panel). The total Erk1/2 levels are unchanged for all samples (lower panel). (B) Cells were exposed to 100 μM $H_2O_2$ for time course measurements. The pErk1/2 level increases within 1 min and reaches the maximum in 15 min. It declines slowly within 30 min and remains stable for 4 h (upper panel). The increase in pErk1/2 level in 15 min is completely eliminated by the pretreatment of a Mek inhibitor, U0126 (25 μM) (U + H in upper panel). The total Erk1/2 levels remain the same throughout the experiment (lower panel).

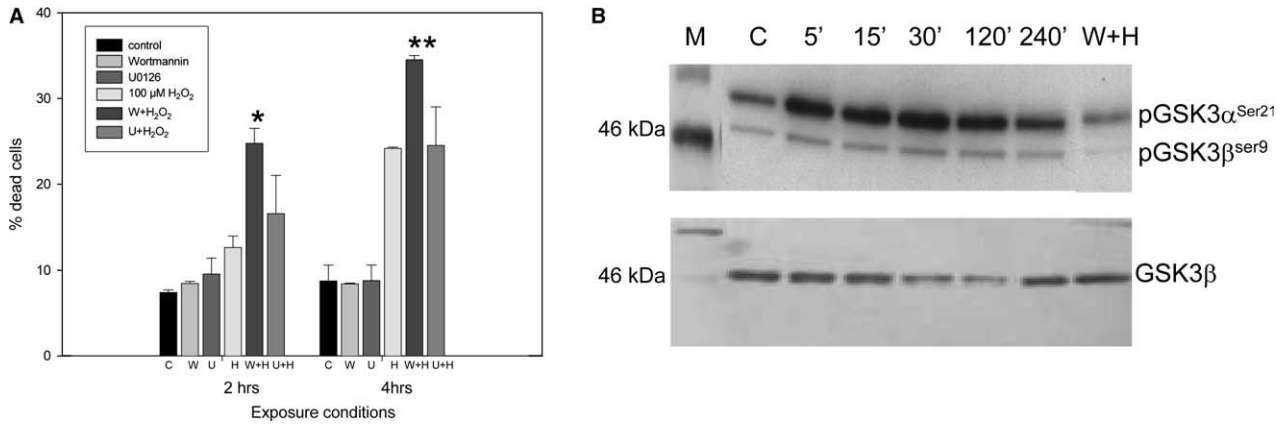


Fig. 4. Effects of PI3K and Mek inhibitors on $H_2O_2$-induced acute cell death. The quantitative analysis of cell death was assessed using calcein-AM/EthD-1 staining. **A**. Cell cultures were exposed to 100 μM $H_2O_2$ for different time durations in the absence and the presence of inhibitors. The cell cultures exposed to wortmannin in conjunction with $H_2O_2$ contain 25% dead cells for 2 h and 35% dead cells for 4 h (W + H). There are 12.5% dead cells for 2 h and 23% dead cells for 4 h appearing in the cell cultures exposed to $H_2O_2$ alone. The cell cultures treated with U0126 (U + H) in conjunction with $H_2O_2$ have 15% dead cells for 2 h and 25% dead cells for 4 h . The cell cultures treated with wortmanin (W) or U0126 (U) alone contain approximately 10% dead cells, the same as the control for 2 and 4 h . Bars represent means ± S.E. obtained from three individual experiments with duplicates. "*" and "**" represent $P < 0.05$ and $P < 0.005$ by $t$ test (H vs. W + H), respectively. (B) Western-blot analysis of the cell extracts using pGSK3α/β and GSK3β antibodies. Cells were exposed to 200 μM $H_2O_2$ for time course measurements. The level of pGSK3α significantly increases in 5 min and returns to near baseline in 4 h (upper panel), while the levels of pGSK3β do not alter throughout the exposure of $H_2O_2$. The increase of pGSK3α in 15 min is completely eliminated by the pretreatment of wortmannin (W + H). There is no alteration in the total GSK3β levels (lower panel).

significantly increased $H_2O_2$-induced apoptosis in cultured NS/PCs (W + H in Fig. 4A).

Our data suggest that the transient increase in Mek-Erk1/2 signaling in cultured NS/PCs does not play a significant role in $H_2O_2$-induced acute apoptosis. Further characterization is required to reveal the significance of $H_2O_2$-induced Mek-Erk1/2 signaling. On the other hand, the inhibition of PI3k-Akt significantly increases $H_2O_2$-induced acute cell apoptosis. This is consistent with the previous findings that the activation of PI3K-Akt signal exhibits anti-apoptotic effects against oxidative stress-induced damage in various cell types, including neural progenitor cells [17,18]. Our results suggest that the transient activation of PI3K-Akt signaling pathway delays $H_2O_2$-induced acute cell apoptosis in cultured NS/PCs.

To find the major pAkt downstream effectors for delaying $H_2O_2$-induced acute cell apoptosis, glycogen synthase kinase 3β (GSK3β) was examined using Western blotting, since it plays a critical role in regulating neuronal cell apoptosis [19,20]. Cells were exposed to 200 μM $H_2O_2$ for assessing time course alteration. Glycogen synthase kinase 3 (GSK3) contains two isoforms, GSK3α/β, which are the substrates for pAkt. Interestingly, $H_2O_2$ transiently increased pGSK3α level (Fig. 4B), but did not significantly alter the pGSK3β level (Fig. 4B). Our data suggest that pGSK3β may not be the major downstream effectors for delaying $H_2O_2$-induced acute cell apoptosis.

However, pretreatment with wortmannin completely eliminated the basal level of pGSK3β (W + H in Fig. 4B) and increased the GSK3β activity that can trigger cell apoptosis. As a result, there was significantly more $H_2O_2$-induced cell apoptosis with the pretreatment of wortmannin (W + H in Fig. 4A). Our data are consistent with the previous findings that the inhibition of GSK3β activity decreased the intrinsic apoptosis in mouse neural progenitor cells [16].

We have demonstrated that $H_2O_2$ induced acute cell apoptosis in concentration- and time-dependent manners. $H_2O_2$ exposure selectively and transiently activated PI3K-Akt and Mek-Erk signaling pathways in a concentration-dependent manner. There was no significant effect on $H_2O_2$-induced cell apoptosis for inhibiting Mek-Erk1/2 signaling. On the other hand, the inhibition of PI3K-Akt with wortmannin significantly increased $H_2O_2$-induced acute apoptosis while dramatically reducing the basal pGSK3β level, one of the downstream effectors. However, $H_2O_2$ exposure did not alter the level of pGSK3β. We conclude that the transient activation of PI3K-Akt cell signaling delays $H_2O_2$-induced acute cell apoptosis in part by maintaining basal level of pGSK3β and by activating other downstream effectors.

Finding the major Akt downstream effectors for delaying $H_2O_2$-induced acute apoptosis as well as the significance of the induced MEk-Erk1/2 cell signaling remains a challenge. Further study is required for characterizing the pAkt downstream effectors for delaying $H_2O_2$-induced acute apoptosis and for analyzing the significance of the induced MEk-Erk1/2 cell signaling.

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