RESEARCH ARTICLE

Insulin involved Akt/ERK and Bcl-2/Bax pathways against oxidative damages in C6 glial cells

Mahesh Ramalingam and Sung-Jin Kim

Department of Pharmacology and Toxicology, Metabolic Diseases Research Laboratory, School of Dentistry, Kyung Hee University, Seoul, Republic of Korea

Abstract

Insulin, a hypoglycemic hormone, has multiple functions in the brain. The aim of this study to identify the mechanisms of insulin in hydrogen peroxide (H2O2)-induced toxicity in the C6 glial cells. Cytotoxicity, lactate dehydrogenase, nitric oxide, reactive oxygen species and calcium ion, lipid peroxidation, protein oxidation and glutathione levels were determined. Signaling pathway molecules were assessed by western blotting and RT-PCR. The results showed that treatment with insulin reduced the cell death and cell membrane damages against H2O2-induced toxicity. Furthermore, insulin interfered H2O2-induced intracellular generation of reactive oxygen species and calcium-ion transport, apoptosis, including lipid and protein oxidation products. Cells treated with insulin reverted H2O2-induced suppression of reduced glutathione levels by blocking oxidized glutathione. Moreover, insulin treatment activates Akt, restores ERK1/2 and Bcl-2 by preventing Bax and Bax/Bcl-2 ratio. Our results suggest that treatment of insulin exerts potential role against 24 h of H2O2-induced toxicity in C6 cells.

Keywords

Apoptosis, glial cells, insulin, neuroprotection, reactive oxygen species

Introduction

Insulin is a peptide hormone secreted from beta cells of the endocrine pancreas (1) and effects on glucose metabolism in the liver, skeletal muscle and adipose tissue (2). Though, insulin crosses the blood-brain barrier (BBB) and enters the brain via a receptor-mediated active transport system (3). Brain insulin signaling is required for a normal mitochondrial function and metabolism that could be helpful to maintain the central regulation of energy balance in the brain and to avoid the development of neurodegenerative diseases (4). Oxygen is necessary for energy production in living organisms, though, by-product of the partial reduction of oxygen leads to formation of reaction oxygen species (ROS) has long been implicated in the process of aging and neurodegenerative diseases (5). Hydrogen peroxide (H2O2) is a major ROS and a by-product of normal cellular function produced from superoxide by superoxide dismutase and monoamine oxidase. Exogenously applied H2O2 induces mitochondrial swelling, plasma membrane blebs, loss of retained mitochondrial function, peroxidation of lipids, oxidation of proteins and apoptosis resulting to cell death (6,7).

Many of the intracellular signaling pathways involved in cellular apoptosis/survival (8,9). To elucidate the molecular pathways of brain cell death, the Akt (protein kinase B; PKB), extracellular regulated kinase (ERK) and Bcl-2 (B-cell lymphoma 2) have been implicated (7). Akt, a serine/threonine kinase, plays a critical role in cell growth, differentiation and survival (10). Hence, the activation of Akt influences on several down-stream targets which could result in attenuation of apoptosis among which plays a very important role is mediated by pro- and anti-apoptotic proteins of Bcl-2 family (5,9). In addition, Bcl-2 proteins possess non-apoptotic roles as different as cell-cycle regulation, neuroplasticity or calcium ion (Ca2+) homeostasis regulation among other (11). Astrocytes, the most abundant glial cell type in the brain, are believed to play a major role in the central nervous system (CNS). It has been proposed that astrocytes have dynamic role in regulating neuronal function via the modulation of synaptic transmission and plasticity, secretion of growth factors, uptake of neurotransmitters, and regulation of extracellular ion concentrations and metabolic support of neurons (5,6). Therefore, the present study was aimed to determine the potential role of insulin against continuous H2O2-induced oxidative damages in rat C6 glial cells.
**Materials and methods**

**Cell culture and treatments**

The rat glioblastoma cell line C6 (CCL-107) was maintained in Dulbecco’s modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin streptomycin (Pen Strep) and 2 mM L-glutamine, at 37 °C in a humidified atmosphere containing 5% CO₂/95% air. Confluent cultures from passages 19 to 28 were washed with phosphate-buffered saline (PBS), detached with 0.25% trypsin-EDTA solution, re-seeded as 5 x 10⁵ cells/ml of DMEM containing 1% bovine serum albumin (BSA) and used for experiments after 16 h. C6 cells were incubated with H₂O₂ in the absence (24 h) or presence of insulin (1 h pre-treatment and 24 h co-incubation) and insulin alone (25 h).

**Cell viability, lactate dehydrogenase (LDH) and nitric oxide (NO) assays**

Cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the absorbance at 570 nm measured using a TRIAD Series Multimode Detector (Dynex Technologies Inc., Chantilly, VA). The optical density of the formazan formed in the normal (untreated control) cells was taken as 100% viability. The LDH in vitro toxicity assay kit (TOX-7, Sigma-Aldrich, St. Louis, MO) and Griess reagent (G-4410, Sigma, St. Louis, MO) were used to measure membrane viability as a function of the amount of cytoplasmic LDH and NO released into the medium. The absorbance was measured at 490 nm (for LDH) and 540 nm (for NO).

**Detection of intracellular ROS and calcium (Ca²⁺)**

The intracellular ROS and Ca²⁺ were measured using 2,7-dichlorofluorescin diacetate (DCF-dAc) and fura-2AM, respectively. The fluorescence intensity was read from the top (1.0 Second; Top Read Mode) of the wells of a black plastic microplate (excitation of 485 nm and an emission of 530 nm for DCF-dAc; 340 nm and 380 nm excitation and 510 nm emission for fura-2AM). The values were calculated as the relative intensity of fluorescence compared with the normal (untreated control) cells.

**Measurement of MDA, PCO, GSH and GSSG**

C6 cells were homogenized in 50 mM phosphate buffer (pH 7.4) for oxidants and antioxidant assays. The extent of lipid peroxidation (LPO) in cell homogenates was determined by measuring the release of thiobarbituric acid reactive substance (TBARS) as malondialdehyde (MDA) equivalents. The results are expressed as nmoles MDA formed per milligram of protein. The protein carbonyl (PCO) content was evaluated and the results are presented as nmoles of DNPH incorporated per milligram of protein. Reduced glutathione (GSH) was determined by using Ellman’s reagent. Oxidized glutathione (GSSG) was measured by masking GSH with 2-vinylpyridine by the same. Protein concentration was determined using Bio-Rad (Hercules, CA) protein assay reagent.

**Western blot analysis**

C6 cells were lysed with lysis buffer containing 20 mM tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% NP-40, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 30 mM NaF, 2.5 mM Na₃P, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mM PMSF. Lysates were then centrifuged at 12,000 rpm for 10 min at 4 °C and the supernatants were used as the total cell lysates. The protein concentration of each sample was determined by the Bradford assay. Exactly, 50 μg of protein were separated on 7–12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to PVDF membranes (Millipore, Bellerica, MA). Each membrane was blocked and then incubated with primary antibodies of the phospho- or total forms of Akt and ERK (Cell Signaling, Danvers, MA) or that recognized for Bcl-2, Bax (Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (Sigma). Each protein was detected by the enhanced chemiluminescence (ECL) system (Amersham, London, UK) with a ChemiDoc XRS imaging system (Bio-Rad, Hercules, CA). The densitometric analysis was performed using ImageJ (National Institute of Health, Bethesda, MD) software.

**Reverse transcriptase-polymerase chain reaction (RT-PCR)**

Total RNA was extracted using TRIzol reagent (Life Technologies, Grand Island, NY) and estimated. Total RNA (2 μg) was reverse transcribed for 60 min at 42 °C, followed at 94 °C for 15 min. Resultant cDNA was amplified in the presence of 1 nM sense and antisense primers. The PCR consisting of denaturation at 94 °C for 30 s, primer annealing at 49.1 °C (β-actin) or 43.3 °C (Akt1 and Bcl2l2) for 30 s, and extension at 72 °C for 30 s for 30 cycles was carried out. The amplified samples were then loaded at equal volumes onto 1.5% agarose gels in Tris-Acetate-EDTA (TAE) buffer. The PCR products were visualized with ethidium bromide by a Bio-Rad ChemiDoc. The band intensity was quantified by NIH ImageJ densitometric analysis. The primer pairs used for amplification are given below: Akt1: Sense 5'-GCCACGGATACCATGAAAGC-3' Bcl2l2: Antisense 5'-ATGCCGGTTCAGGTACTCAG-3' Akt1: Antisense 5'-TGATGAAGGTGTTGGGCCTC-3' Bcl2l2: Sense 5'-GGCTGTGTTGTCCCTGTAT-3' β-actin: Sense 5'-GCCGTGTTGTCCTCCGTAT-3' β-actin: Antisense 5'-CCGCTCATTGCCCCATAGTG-3'

**Statistical analysis**

Data were analyzed using GraphPad Prism 5.0 software (La Jolla, CA) and the data were expressed as mean ± SD. The significance level of treatment effects was determined using a repeated measures analysis of variance (ANOVA) followed by Tukey’s multiple comparison test between groups. A probability of p < 0.05 was considered to be statistically significant.

**Results**

**Insulin on cell viability, toxicity, ROS and Ca²⁺ levels**

Cells exposed to different concentrations of H₂O₂ for 24 h results a concentration-dependent decrease in cell viability
Cell viability decreases by 68.57% at 200 μM H2O2 was chosen to assess the effect of insulin in glial cells. Pre-treatment of cells with insulin for 1 h prior to H2O2-induction prevented H2O2-induced decrease in cell viability at 24 h (Figure 1B). Significant increases in the levels of LDH (185.7%; Figure 1C), NO (138.0%; Figure 1D), ROS (153.9%; Figure 1E) and Ca2+ influx (171.9%; Figure 1F) were observed following H2O2-induction. In contrast, insulin treatment inhibited the LDH and NO releases, formation of ROS and Ca2+ influx. Meanwhile, insulin treatment to normal cells (no H2O2-induction) significantly decreased NO release, however, does not influence the LDH, ROS or Ca2+ level.

The DCFH-dAc and fura-2AM output fluorescence (Supplemental Figure 1) were increased in H2O2-induction, in contrast, insulin treatment inhibited the formation of intracellular ROS and Ca2+ influx (Supplemental Figure 1). In dual-staining of Hoechst 33342/PI, the H2O2-induced cells reacted were positive for PI (a marker of necrosis), but, no PI stained cells were observed following insulin treatment at 100 nM in conjunction with H2O2-induction. The untreated cells were not reacting to PI, but did react with nuclei staining with Hoechst 33342 (Supplemental Figure 2).

**Insulin on MDA, PCO, GSH and GSSG levels**

To further confirm the role of insulin in H2O2-induced apoptosis, we examined cellular oxidants and antioxidants in glial cells (Table 1). H2O2-induction significantly increased the oxidant products such as MDA, PCO and GSSG with decreased GSH and GSH/GSSG ratio. However, treatment with insulin significantly inhibited the H2O2-induced increase in oxidation products and increased the GSH along with GSH/GSSG ratio (p < 0.001). Meanwhile, insulin treatment to *per se* did not change the levels of MDA, PCO, and GSH levels.
whilst decreased the GSSG level and increased the GSH/GSSG ratio compared with the normal group.

**Insulin on Akt, ERK1/2 and Bax/Bcl-2 signaling pathways**

To investigate the mechanism of insulin on H$_2$O$_2$-induced oxidative stress on survival factors, the effects of insulin on Akt, ERK1/2 (Figure 2), Bcl-2 and Bax (Figure 3) were assayed. The level of phospho-Akt(Ser473) showed non-significant activation by 24 h of H$_2$O$_2$-induction. Pre-treatment with 100 nM insulin prior to H$_2$O$_2$-induction increased the levels of p-Akt and the p-Akt(Ser473)/Akt ratio. The PI3K inhibitor, LY294002 (at 20 μM), blocked the insulin-induced phosphorylation of Akt. In addition, as shown in Figure 2(C), H$_2$O$_2$ (200 μM)-induction induced the expression of phosphorylated ERK proteins and treatment with insulin blocking ERK protein phosphorylation in C6 cells. Furthermore, we also investigated the mRNA expressions of Akt1 in C6 cells by RT-PCR (Figure 2B). Akt1 mRNA expression was changed under H$_2$O$_2$-induction alone. Concurrently, insulin treatment alone or during H$_2$O$_2$-induction also significantly increased the Akt1 mRNA expressions. H$_2$O$_2$ (200 μM)-induction significantly decreased the level of Bcl-2 protein (Figure 3A) and increased the Bax protein expression (Figure 3C). The mRNA expression of Bcl-2 in C6 cells was up-regulated under H$_2$O$_2$-induction (Figure 3B). The treatment of insulin also significantly influences the levels of Bcl-2 protein and mRNA expressions. We additionally demonstrated that the ratio of Bax/Bcl-2 was significantly

**Table 1. Effect of insulin on H$_2$O$_2$-induced oxidant products, reduced and oxidized glutathione levels in C6 cells.**

| Groups              | MDA  | PCO  | GSH   | GSSG | GSH/GSSG  |
|---------------------|------|------|-------|------|-----------|
| Normal              | 0.301±0.007 | 69.755±0.261 | 14.136±0.707 | 1.419±0.006 | 9.964±0.458 |
| Normal + Insulin (100 nM) | 0.305±0.014*NS | 70.565±0.260*NS | 14.369±0.513*NS | 1.345±0.024*** | 10.680±0.196** |
| H$_2$O$_2$ (200 μM) | 0.541±0.011**** | 105.879±1.034**** | 11.425±0.175**** | 1.824±0.005**** | 6.262±0.079**** |
| Insulin (100 nM) + H$_2$O$_2$ (200 μM) | 0.377±0.034**** | 90.368±0.148**** | 13.977±0.174**** | 1.286±0.009**** | 10.870±0.207**** |

Data are mean ± S.D. of three independent experiments and analyzed by repeated measures analysis of variance (ANOVA) followed by Tukey’s post hoc test. Statistical significance: a – compared to normal; b – compared to H$_2$O$_2$; *p<0.05, ***p<0.001 and NS – Non-significant. MDA, nmol of MDA formed/mg protein; PCO, nmol DNPH conjugated/mg protein; GSH and GSSG, nmol/mg protein.

**Figure 2.** C6 glial cells were treated with insulin for 1 h pre-treatment and another 24 h with or without H$_2$O$_2$ and assessed for p-Akt/t-Akt (A; F(4,8) = 88.90; p<0.0001) and p-ERK/t-ERK (C; F(3,6) = 3726; p<0.0001) by western blotting and Akt-1/β-actin (B; F(4,8) = 72.32; p<0.0001) by RT-PCR analyses. Data are mean ± SD of three independent experiments and analyzed by repeated measures analysis of variance (ANOVA) followed by Tukey’s post hoc test. Statistical significance: a – compared to normal; b – compared to H$_2$O$_2$; ***p<0.001 and NS – non-significant.
elevated after 24 h of H$_2$O$_2$; meanwhile, insulin reversed to normal levels (Figure 3D).

**Discussion**

The present in vitro model was established to determine the potential role of insulin against continuous oxidative damage of H$_2$O$_2$ in C6 cells. The result reveals H$_2$O$_2$-induced oxidative damages for 24 h in C6 glial cells decreased the cell viability in a dose-dependent manner. Pretreatment with insulin significantly decreased the cell death. Elevated levels of LDH may indicate cell injury during external stimulus. Excessive levels of NO, an important mediator of cellular communication at basal levels, have been pathologically implicated in the pathogenesis of neurodegenerative diseases (12). However, in the presence of insulin, both LDH and NO levels were significantly decreased; suggesting that insulin protect C6 glial cells against oxidative stress induced apoptosis and contributes neuroprotective effects. Our previous results with 3 and 12 h of H$_2$O$_2$-induction suggest that exogenous insulin treatment has protective effects on glial cells from H$_2$O$_2$-induced toxicity (6,7).

As ROS are produced during normal metabolism, exposure of cells to H$_2$O$_2$ causes a rapid production of ROS. In the present study, exposure of C6 glial cells to H$_2$O$_2$ resulted in significant increases in intracellular ROS and Ca$^{2+}$ levels. Large increases in intracellular Ca$^{2+}$ represent a detrimental consequence of ROS-mediated oxidative stress (4). Sustained elevation of Ca$^{2+}$ levels may impair mitochondrial function and may activate phospholipases, proteases, and endonucleases, leading to irreversible membrane, organelle, and chromatin damage, and eventually to cell death (13). Pretreatment of insulin to C6 cells prior to H$_2$O$_2$-exposure resulted in a significant decrease in ROS and Ca$^{2+}$ levels. From the above results, H$_2$O$_2$ can disrupt cellular integrity and causes apoptotic and necrotic cell death through oxidative stress. Interestingly, insulin significantly attenuates these features, indicates that insulin may possess an inhibitory effect on H$_2$O$_2$-induced apoptosis. These results were concordant with our previous results with 3 and 12 h of H$_2$O$_2$-induction and/or insulin treatment of glial cells (6,7).

Furthermore, H$_2$O$_2$ can attack proteins and lipid membranes, thereby disrupting cellular function and the reduction of the endogenous antioxidant defense system, including GSH (14). In the present study, we observed that H$_2$O$_2$-induced neurotoxicity increased the MDA, PCO, and GSSG levels and decreased the intracellular GSH content, resembling the pathophysiological state of oxidative stress in neurodegenerative diseases. In the biological system, H$_2$O$_2$ is soluble in lipid and aqueous environments (15) and ubiquitously present
with a relatively long half-life and, thus, capable of reaching its cellular targets to cause lipid and protein oxidations. Therefore, elevated LPO and PCO contents and depletion of GSH in glial cells is the main event in neuroinflammation, neurotoxicity and impairment in transporters, and has been described as the basis of neurodegenerative disorders (16). On the other hand, we found that insulin treatment increased the GSH content and completely declined the MDA, PCO and GSSG levels in the presence of H$_2$O$_2$. These results reveal an important role of insulin in preventing H$_2$O$_2$-neurotoxicity with subsequent neuroprotection.

H$_2$O$_2$-mediated signaling alters the function of various proteins, including protein phosphatases, protein kinases, phospholipases, transcription factors, and ion channel proteins (17). Studies evidence that activation of the serine/threonine protein kinase Akt (protein kinase B), is involved in neurodegenerative diseases (18). Stimulation of the protein kinase Akt was shown to be dependent on phosphoinositide 3-OH kinase (PI3K) (19). In the present study, exogenous H$_2$O$_2$-mediated oxidative stress for 24 h did not activate Akt phosphorylation supports similar report in 3 h of H$_2$O$_2$ in C6 glial (7) and SH-SY5Y cells (4) but activated by 24 h in SH-SY5Y cells (20). However, treatment of insulin at 100 nM, phosphorylation of Akt was increased significantly when compared with H$_2$O$_2$ alone. Blockade of PI3K with its selective inhibitor, LY294002, markedly prevented the protective effect of insulin on H$_2$O$_2$-induced oxidative stress. From this, we hypothesize that insulin protects C6 glial cells against oxidative stress-induced damage via Akt pathway and play an important role in the prevention of apoptotic cascades. Moreover, insulin treatment to normal cells has been shown to stimulate the Akt, in this study. Previous studies demonstrated that stimulation of the Akt pathway with insulin could improve protein synthesis and delay the progress of degeneration (6,21).

Moreover, ROS can activate the ERK signaling pathway in cells. In the present study, activation of ERK was detected in H$_2$O$_2$-induced C6 cells and blocking ERK activation by insulin significantly attenuated H$_2$O$_2$-induced cell death. Previous reports suggest that the phosphorylation of ERK may be due to increased hydroxyl radicals (22) or nitric oxide radicals (18). The present study indicates that insulin prevent H$_2$O$_2$-induced cell apoptosis through down-regulation of the ERK1/2 and up-regulation of the Akt pathways.

Nevertheless, it has been shown that phosphorylation and activation of Akt promote cell survival by enhancing the expression of anti-apoptotic proteins (Bcl-2, residing in the outer mitochondrial membrane) and inhibiting the activity of pro-apoptotic proteins (Bax, reside in the cytosol) (14). The translocation of Bax to the mitochondrial membrane might lead to loss of membrane potential results in the release of cytochrome c from the mitochondria which in turn activate caspase-3 that induces cell death (18). In the present study, H$_2$O$_2$-induction decreased the total Bcl-2 and increased the total Bax protein levels; however insulin treatment reverted the total Bcl-2 and Bax protein levels. Furthermore, H$_2$O$_2$-induction resulted in an increase in Bcl-2 mRNA expression. Thus, H$_2$O$_2$-induced ROS-mediated transcriptional induction may result in detected expression of Bcl-2 mRNA gene activation (23). Our previous study also showed enhanced gene expression of Bcl-2 mRNA in 24 h H$_2$O$_2$-induction in neuronal cells (20). The observation of Akt and Bcl-2 expressions were increased and Bax expression was decreased after application of insulin in the normal cells hypothesis that exogenous insulin in C6 cells may activate intracellular messenger cascades.

In conclusion, the present study shows that insulin prevents H$_2$O$_2$-induced cell death in C6 cells. Pre-treatment of cells with insulin suppresses cellular apoptosis, Ca$^{2+}$ influx and oxidation products. Further results strongly support the observation that Akt/ERK and Bcl-2/Bax pathways play a major regulatory role in response in controlling cell death/ survival in response to insulin. Further research on insulin into insulin receptors, insulin receptor substrates, and their downstream pathways could provide new therapeutic strategy in treating neurodegenerative diseases.

Declaration of interest

The authors have no conflicts of interest to declare. This study was supported by Kyung Hee University (KHU-20131115) and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2013R1A1A206613).

References

1. Steiner DF, Oyer PE. The biosynthesis of insulin and a probable precursor of insulin by a human islet cell adenoma. Proc Natl Acad Sci USA 1967;57:473–80.
2. Konrad D, Rudich A, Klip A. Insulin-mediated regulation of glucose metabolism. Insulin resistance. England: John Wiley & Sons, Ltd; 2005:63–85.
3. Baur GD, Foster DM, Porte Jr D, et al. Saturable transport of insulin from plasma into the central nervous system of dogs in vivo. A mechanism for regulated insulin delivery to the brain. J Clin Invest 1993;92:1824–30.
4. Ramalingam M, Kim S-J. Insulin exerts neuroprotective effects via Akt/Bcl-2 signaling pathways in differentiated SH-SY5Y cells. J Recept Signal Transduct 2014. (Epub ahead of print).
5. Ramalingam M, Kim S-J. Reactive oxygen/nitrogen species and their functional correlations in neurodegenerative diseases. J Neural Transm 2012;119:891–910.
6. Mahesh R, Kim S-J. The protective effects of insulin on hydrogen peroxide-induced oxidative stress in C6 glial cells. Biomol Ther (Seoul) 2009;17:395–402.
7. Ramalingam M, Kim S-J. Insulin on hydrogen peroxide-induced oxidative stress involves ROS/Ca(2+) and Akt/Bcl-2 signaling pathways. Free Radic Res 2014;48:347–56.
8. Kim HJ, Kim J, Kang KS, et al. Neuroprotective effect of chebulagic acid via autophagy induction in SH-SY5Y cells. Biomol Ther (Seoul) 2014;22:275–81.
9. Yu HY, Kim SO, Jin CY, et al. beta-lapachone-induced apoptosis of human gastric carcinoma AGS cells is caspase-dependent and regulated by the PI3K/Akt pathway. Biomol Ther (Seoul) 2014;22:184–92.
10. Bozulic L, Hemmings BA. PI3K/Akt signaling in PEBK: regulation of PKB activity by phosphorylation. Curr Opin Cell Biol 2009;21:256–61.
11. Bonneau B, Prudent J, Poppegeiev N, Gillet G. Non-apoptotic roles of Bcl-2 family: the calcium connection. Biochim Biophys Acta 2013;1833:1755–65.
12. Liu B, Gao HM, Wang JY, et al. Role of nitric oxide in inflammation-mediated neurodegeneration. Ann N Y Acad Sci 2002;962:318–31.
13. Wang W, Sun F, An Y, et al. Morroniside protects human neuroblastoma SH-SY5Y cells against hydrogen peroxide-induced cytotoxicity. Eur J Pharmacol 2009;613:19–23.
14. Ramalingam M, Kim S-J. Mechanisms of action of brain insulin against neurodegenerative diseases. J Neural Transm 2014;121:611–26.
15. Ye J, Han Y, Wang C, Yu W. Cytoprotective effect of polypeptide from *Chlamys farreri* on neuroblastoma (SH-SY5Y) cells following H2O2 exposure involves scavenging ROS and inhibition JNK phosphorylation. J Neurochem 2009;111:441–51.

16. Bobermin LD, Souza DO, Gonçalves C-A, Quincozes-Santos A. Lipoic acid protects C6 cells against ammonia exposure through Na+–K+–Cl– co-transporter and PKC pathway. Toxicol In Vitro 2013;27:2041–8.

17. Fruehauf JP, Meyskens FL. Reactive oxygen species: a breath of life or death? Clin Cancer Res 2007;13:789–94.

18. Mahesh R, Jung HW, Kim GW, et al. Cryptotanshinone from *Salviae miltiorrhizae* radix inhibits sodium-nitroprusside-induced apoptosis in Neuro-2a cells. Phytother Res 2012;26:1211–19.

19. Stephens L, Anderson K, Stokoe D, et al. Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. Science 1998;279:710–14.

20. Ramalingam M, Kim S-J. The role of insulin against hydrogen peroxide-induced oxidative damages in differentiated SH-SY5Y cells. J Recept Signal Transduct 2014;34:212–20.

21. Rommel C, Bodine SC, Clarke BA, et al. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. Nat Cell Biol 2001;3:1009–13.

22. Nair VD, Yuen T, Olanow CW, Sealfon SC. Early single cell bifurcation of pro- and antiapoptotic states during oxidative stress. J Biol Chem 2004;279:27494–501.

23. Tu Y, Xu FH, Liu J, et al. Upregulated expression of BCL-2 in multiple myeloma cells induced by exposure to doxorubicin, etoposide, and hydrogen peroxide. Blood 1996;88:1805–12.

**Supplementary material available online**

Supplementary Figure 1 and 2.