Biomolecules & Therapeutics, **17(4)**, 395-402 (2009)
www.biomolther.org

ISSN: 1976-9148(print)/2005-4483(online)
DOI: 10.4062/biomolther.2009.17.4.395

The Protective Effects of Insulin on Hydrogen Peroxide-Induced Oxidative Stress in C6 Glial Cells

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(Received September 7, 2009; Revised October 7, 2009; Accepted October 8, 2009)

Abstract − Insulin appears to play a role in brain physiology, and disturbances of cerebral insulin signalling and glucose homeostasis are implicated in brain pathology. The objective of the present study was to investigate the protective effects of insulin under conditions of oxidative stress induced by hydrogen peroxide (H_2O_2) in C6 glial cells. Insulin at concentration of 10^{-7} M could prevent 12 h H_2O_2-induced cell death. The formation of reactive oxygen species (ROS), nitric oxide (NO) and 2-thiobarbituric acid-reactive substances (TBARS) were significantly scavenged by insulin pre-treatment in C6 glial cells after H_2O_2-induced oxidative stress. Insulin significantly stimulated the phosphorylation of Akt in the cells and the activation of Akt was maintained in response to insulin under H_2O_2 incubation for 12 h. In conclusion, these results provide evidence that insulin acts as a free radical scavenger and stimulating Akt activity. These data suggest that insulin may be effective in degenerative diseases with oxidative stress.

Keywords: Insulin, Oxidative stress, Akt, Glial cells

INTRODUCTION

Oxidative stress in brain is particularly vulnerable occurrence as a consequence of increased intracellular levels of reactive oxygen species, possibly due to its high consumption of oxygen during oxidative phosphorylation (Castagne *et al*., 1999) forms a common pathway leading to neuronal death (Ames *et al*., 1993) and the processes can become disturbed as reported for aging (Beckman and Ames, 1998) and several neurological disorders such as Parkinson's disease (PD) and Alzheimer's disease (AD) (Dringen, 2000).

Free radical reactions, which involves excess accumulation of reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide (H_2O_2) and hydroxyl radical, can damage cells by lipid peroxidation and alteration of protein and nucleic acid structure (Berlet and Stadtman, 1997). H_2O_2 is the major mediator of oxidative stress and a potent mutagen. Large quantities of H_2O_2 can be generated by the respiratory chain and several other metabolic pathways. Moreover activated inflammatory cells (e.g. microglia) are able to produce this molecule via the oxidative burst mechanism. Although this peroxide is a weak oxidant, it can be converted, in the presence of reduced transition metals such as ferrous and cuprous ions, to highly reactive hydroxyl radicals that are believed to mediate the genotoxicity of this compound (Halliwell and Gutteridge, 1990).

In neuropathologies, as well as the natural process of aging, the involvement of oxygen free radical overproduction is strongly suspected as a major factor related to the progressive derangement from normal function (Beckman and Ames, 1998) and could involve several mechanisms of cell signaling. Therefore, we reasoned that cellular responses to oxidative stress might be mediated by signal transducing proteins. The characterization of mechanisms underlying growth-survival decisions of the resistance of some cells to oxidative stress and may lead to find targets that would be modulated by drugs to prevent cell death in brain (Altiok *et al*., 2006). Therefore, the prevention of H_2O_2-induced oxidative stress may be a useful model to test molecules for the prevention and treatment of neurodegenerative diseases.

Insulin has functions in the brain and dysregulation of these functions may contribute to the expression of late-life neurodegenerative diseases. The effect of insulin in peripheral tissues is the stimulation of glucose uptake, oxida-

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tion, and storage. However, it's very high concentration in brain, 25 times greater than in plasma suggesting a greater involvement in brain functions (Margolis and Altszuler, 1967; Szabo and Szabo, 1972). The effect of insulin on the brain is less well defined. Elevations of circulating insulin can alter brain function, augmenting the counterregulatory response to hypoglycemia (Davis *et al*., 1993; Fruewald-Schultes *et al*., 1999), altering feeding behaviour (Debons *et al*., 1970; Rodin *et al*., 1985), and modulating auditory evoked potentials (Kern *et al*., 1994). How insulin abnormalities may contribute to the symptoms and pathogenesis have been examined in various experimental model systems and role for insulin has been established in molecular and neurophysiological features of memory processing (Zhao and Alkon, 2001). The giving of insulin while maintaining euglycaemia improves memory in both healthy adults and people with AD (Craft *et al*., 1999; Kern *et al*., 2001). Insulin also exerts neuroprotection via activation of Akt in neuronal cells (Kim and Han, 2005).

Since free radicals induced oxidative stress/damage play a critical role in several brain diseases. In the present study, we explored to investigate whether insulin has any effect on the Akt signaling mechanisms and oxygen stress in C6 glial cells as a model for brain glial cells after oxidative damage induced by H_2O_2.

MATERIALS AND METHODS

Chemicals

The following chemicals were obtained from the Sigma Chemical Co. (St Louis, MO, USA): 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), thiobarbituric acid (TBA), ethylene diaminetetraacetic acid disodium salt (Na_2-EDTA), 2,7-dichlorofluorescin diacetate (DCF-dAc), sodium azide, hydrogen peroxide (H_2O_2), glutathione. Cell culture materials were purchased from Gibco BRL (Gaithersburg, MD, USA). All other chemicals were of the highest analytical grade and purchased from common sources.

Cell culture

C6 glial cells were grown in DMEM media with 10% fetal bovine serum in a 5% humidified CO_2 atmosphere at 37°C. Cells were washed with phosphate buffered saline (PBS) three times and incubated with serum-free media for 24 h.

Induction of stress and drug treatment

In the C6 glial cell culture, induction of stress was made by 0.5 mM hydrogen peroxide (H_2O_2) in serum free media for 12 h. The stock solution of Insulin was diluted with serum free media and added to cultured cells, 10 min, 30 min and 60 min before oxidative stress induction, reaching final concentrations of 10^{-7} M.

Determination of cell viability

Cell viability was measured by the MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) method. The assay is based on the ability of living cells to convert dissolved MTT into insoluble formazan by mitochondrial dehydrogenases in viable cells. The amount of formazan produced is proportional to the number of living cells (Amoroso *et al*., 1999). The cells were plated in 96 well plates at a density of 1×10^5 cells/well. Serum-starved cells were preincubated with insulin (10^{-7} M) for 10, 30 and 60 min, followed by H_2O_2 (0.5 mM) treatment for 12 h. After treatments, cells were incubated with 40 μl of MTT stock solution (2 mg/ml in PBS) in 100 μl of medium. Following additional 3 h incubation at 37°C, the medium was removed and 100 μl of DMSO was added to dissolve the formazan crystals (Mosmann, 1983). The absorbance was read at 570 nm using a microplate reader (BioRad Model 550). The optical density of the formazan formed in the control cells was taken as 100% viability. Data are mean percentages of viable cells versus the respective controls.

Detection of intracellular reactive oxygen species (ROS)

The intracellular ROS was measured using a non-fluorescent dye, 2,7-dichlorofluorescin diacetate (DCF-dAc), which is a membrane permeable fluorigenic tracer that is oxidized by various species of ROS. The nonploar DCF-dAc is diffusing into the cells through the cell membrane. Then, the dye is deacetylated by intracellular esterases to the non-fluorescent 2',7'- dichlorohydrofluorescein (DCFH) (Frenkel and Gleichauf, 1991), which is oxidized to a measurable fluorescent compound 2',7'-dichlorofluorescein (DCF) by ROS (LeBel *et al*., 1992). The serum-starved cells were pre-incubated with Insulin (10^{-7} M), followed by 0.5 mM H_2O_2 for 12 h. The cells were then treated with 100 μM H_2DCFDA for 30 min at 37°C, and then washed twice with PBS. Finally, fluorescence reading was realized at the top of the plastic microplate at an excitation of 485 nm and an emission of 530 nm using a fluorescence microplate reader (TRIAD Series Multimode Detector, DYNEX Technologies, Inc. VA, USA). The values were calculated as the relative intensity of DCF fluorescence, compared with the control.

Nitrite assay

The cells were treated with Insulin and for H_2O_2 in the 96-well multiple plates. Then the cells were centrifuged at

1,000× g for 10 min and the supernatants were collected. The NO production was measured as the nitrite (NO_2^-) concentration according to the method of Green *et al*. (1982). Supernatants (50 μl) were mixed with 100 μl of 0.1% sulfanilamide and 100 μl 0.1% N-1-naphthylethylenediamine dihydrochloride in 2.5% polyphosphoric acid. The absorbance was measured at 540 nm with a Micro-Reader (BioRad, Model 550). Nitrite was quantified using sodium nitrate as a standard.

Determination of 2-thiobarbituric acid-reactive substances (TBARS)

The extent of lipid peroxidation was evaluated as 2-thiobarbituric acid-reactive substances (TBARS) (Fraga *et al*., 1988). The measurement of TBARS formation was performed as described in Ohyashiki *et al*. (1991) with some modification. The reaction was terminated by the addition of 50 μl of butylated hydroxytoluene (BHT, 4% w/v in ethanol) and then the cell suspension was heated at 90°C for 30 min with 200 μl sodium dodecyl sulphate (SDS, 0.3% w/v) and 1 ml 0.67% 2-thiobarbituric acid (50% acetic acid). After cooling to room temperature, the complex formed with 2-thiobarbituric acid was extracted with 3 ml butanol. After centrifuged at 5,200× g for 10 min, the absorbance of the supernatant was measured at 532 nm. A standard was prepared from 1,1,3,3-tetra-ethoxy propane.

Western blotting

Treated cells (5×10^6 cells/10 ml in 100-mm dish) were collected and washed with PBS. After centrifugation, cells were lysed in 200 μl of lysis buffer (20 mM Hepes, 50 mM $MgCl_2$, 25 mM KCl, 2 mM PMSF, 0.1 mg/ml aprotinin, 1 mM sodium vanadate, 1 mM sodium molybdate, 10 mM beta-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 0.25 M sucrose and 1% Triton-X 100). The lysate was incubated on ice for 2 h and centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was collected and followed by protein concentration determination using Bradford assay. After addition of sample loading buffer, equivalent amounts of protein samples were electrophoresed on a 10% sodium dodecyl sulfate denaturing polyacrylamide slab gels (SDS-PAGE) and subsequently transferred to nitrocellulose transfer membrane (Whatman PROTRAN). The membrane was incubated in fresh blocking buffer (0.1% Tween 20 in Tris buffered saline (TTBS), pH 7.4, containing 5% non fat dried milk) at room temperature for 60 min and then probed with the p-Akt and Akt (Cell Signaling Technology Inc., Beverly, MA, USA) antibody in blocking buffer at 4°C overnight. The membrane was washed three times for 10 min each using TTBS. After that it was incubated in the appropriate HRP-conjugated secondary antibody at room temperature for another 2 h and washed again three times in TTBS buffer. The membrane was developed using the enhanced chemiluminescence reagents (ECL) for 5 min according to the manufacturer's instructions and was exposed to Amersham Hyperfilm ECL (GE Healthcare Limited, UK). The results obtained from the immunoblot assay were calculated using IMAGEJ imaging software (NIH, USA) as the integrated density.

Statistical analysis

All data analysis was completed using the Graphpad PRISM 5.0 software. Data are expressed as mean ± standard deviation (S.D.). The significance level of treatment effects was determined using one way analysis of variance (ANOVA) followed by Tukey's post-hoc analysis and *p*-values lower than 0.05 was considered statistically significant. All experiments were performed a minimum of three times.

RESULTS

Insulin pre-treatment increases the cell viability

To determine the cytotoxic effects of H_2O_2 on C6 cells and to test the potential protection by insulin against H_2O_2

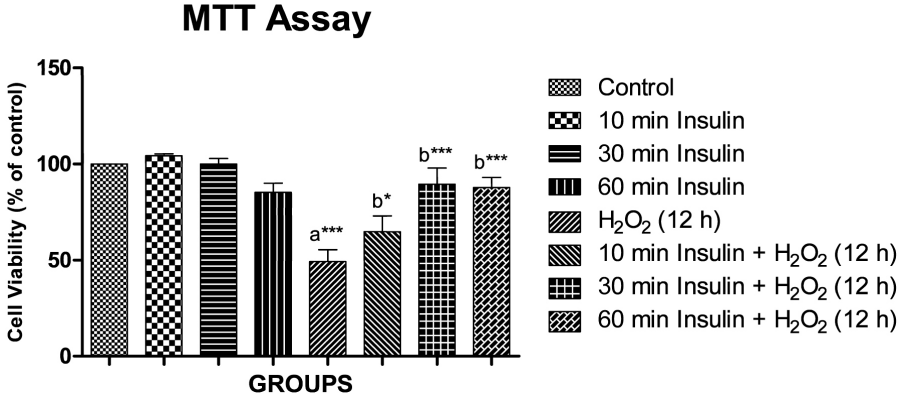

Fig. 1. Protective effect of insulin on MTT assay in C6 glial cells. Cells were incubated for 10, 30 and 60 min with insulin before the addition of H_2O_2. Oxidative damage was induced with 0.5 mM H_2O_2 for 12 h. The data shown are the mean values ± SD of three experiments. [a]with respect to control group, [b]with respect to H_2O_2-induced group. $^*p<0.05$, $^{***}p<0.001$.

induced cytotoxicity, C6 cells were pretreated with insulin for 10, 30 and 60 min and were exposed with the 0.5 mM concentration of H_2O_2 for 12 h and measured cell death and survival rates with the MTT assay (Fig. 1). This assay is used to measure cell viability and is based on the reduction of MTT to a blue formazan product by dehydrogenase enzymes of intact mitochondria. Thus, the MTT assay measures mitochondrial function or integrity. The results showed that when the cells were exposed with H_2O_2, the cell viability significantly decreased (49.31%) and cells lost processes. Pretreatment of Insulin time ranging 10, 30 and 60 min prior to H_2O_2 treatment showed the protective effect at 64.87, 89.59 and 87.87% respectively. In the case of insulin treatment only to the control cells, cell viability measured by the MTT assay was not significantly changed at the times of 10, 30 and 60 min treatment with the concentration studied.

Insulin inhibit H_2O_2 induced elevation in intracellular ROS level

We examined the intracellular ROS level with DCFH-DA. As shown in Fig. 2, exposure of C6 cells to H_2O_2 for 12 h led to a significant increase in DCF signal compared with the control group (139.6%). Insulin pretreatment inhibited the increase in DCF fluorescence in a time-dependent manner in the 10-60 min range (104.6, 93.35 and 86.66% for 10, 30 and 60 min respectively). From this result, the 60 min insulin treatment at the concentration of 0.5 mM respect to maximum inhibition in ROS formation within the H_2O_2-induced toxicity for 12 h. In the pre-treatment with insulin only groups, the 60 min pre-treatment significantly decreased (83.45%) the ROS formation in C6 glial cells than 10 and 30 min treatment.

Insulin pre-treatment decreases the nitrite in cells

The free radical, NO, is synthesized by the action of nitric oxide synthase (NOS) in mammalian cells and is utilized for normal cellular functions. The NO produced is further metabolized to nitrite and nitrate, the levels of which are used as an indicator of NOS activity in cells. We determined the concentration of nitrite in the culture medium. The medium of the cells treated with H_2O_2 showed a 158.5% increase in nitrite levels as compared with the medium of the controls (Fig. 3). The insulin pre-treatment inhibits the H_2O_2-induced generation of nitrite at 10-60 min range (71.53, 72.62 and 78.50% at 10, 30 and 60 min respectively) indicating that insulin has a strong protective against RNS.

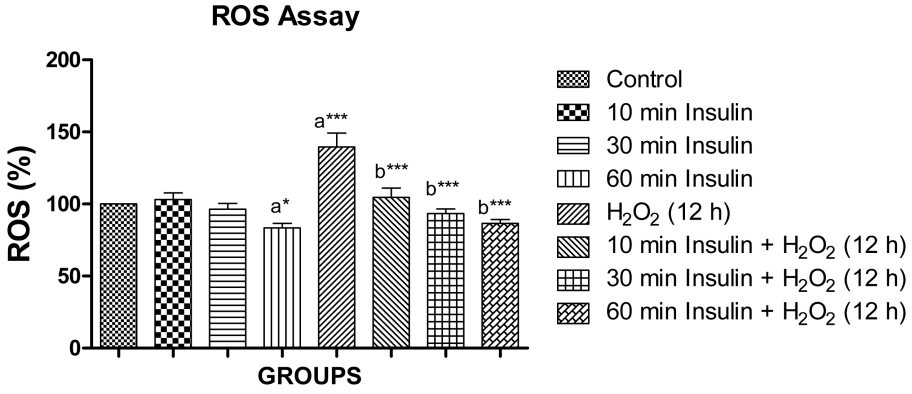

Fig. 2. Protective effect of insulin on ROS formation assay in C6 glial cells. Cells were incubated for 10, 30 and 60 min with insulin before the addition of H_2O_2. Oxidative damage was induced with 0.5 mM H_2O_2 for 12 h. The data shown are the mean values ± SD of three experiments. [a]with respect to control group, [b]with respect to H_2O_2-induced group. $^*p<0.05$, $^{***}p<0.001$.

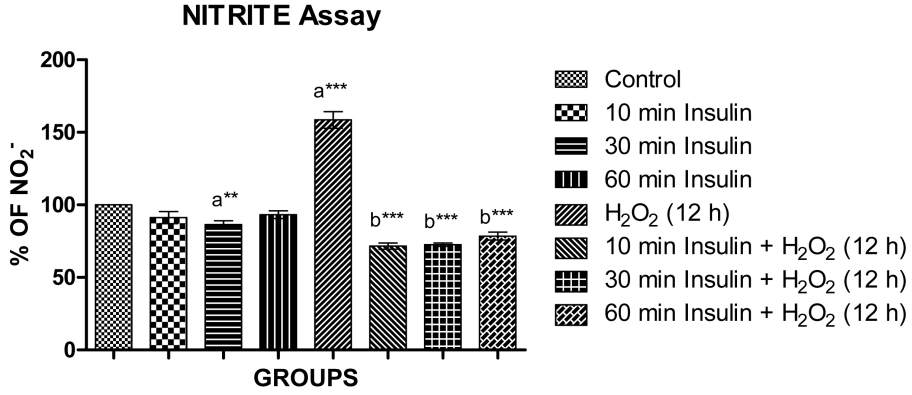

Fig. 3. Protective effect of insulin on NO assay in C6 glial cells. Cells were incubated for 10, 30 and 60 min with insulin before the addition of H_2O_2. Oxidative damage was induced with 0.5 mM H_2O_2 for 12 h. The data shown are the mean values ± SD of three experiments. [a]with respect to control group, [b]with respect to H_2O_2-induced group. $^{**}p<0.01$, $^{***}p<0.001$.

Insulin inhibit the TBARS formation

The involvement of oxidative stress is further confirmed by formation of TBARS in insulin treated cells since TBARS represents an index of oxidative damage to membrane lipids. As show in Fig. 4, after incubation with 0.5 mM H_2O_2 for 12 h, the level of TBARS in C6 glial cells was significantly increased. Pre-treatment with insulin significantly decreased the TBARS levels at 10, 30 and 60 min. The unstimulated C6 glial cells with insulin treatment also express the inhibition of TBARS formation at 30 min pre-treatment level.

Effects of insulin on AKT phosphorylation in H_2O_2 induction

To investigate the cellular activation of AKT in the protective effect of insulin, C6 glial cells were exposed by H_2O_2 in the presence of insulin and Western blot analysis was performed. When cells were exposed to insulin, pAKT/AKT ratio was significantly increased in 10 min to 60 min. When cells were pretreated with H_2O_2 alone for 12 h, pAKT/AKT ratio was also increased as compared to control; however, insulin treatment in the presence of H_2O_2 resulted in the further enhancement of the pAKT/AKT ratio in 10 min to 30 min (Fig. 5).

DISCUSSION

Insulin receptors are present in glial cells (Clarke *et al*., 1984); however, insulin's actions in the glial cells are largely unknown. The present study is the first to focus on the role of insulin in H_2O_2-induced C6 glial cell apoptosis. When C6 glial cells were incubated with 0.5 mM H_2O_2 for 12 h, the total cell death reached over 49.31% (Fig. 1). Although H_2O_2 itself is a relatively unreactive species and

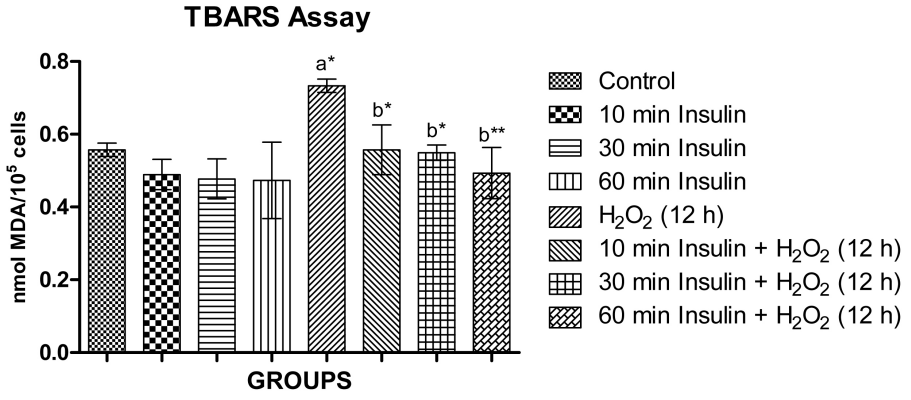

Fig. 4. Protective effect of insulin on TBARS assay in C6 glial cells. Cells were incubated for 10, 30 and 60 min with insulin before the addition of H_2O_2. Oxidative damage was induced with 0.5 mM H_2O_2 for 12 h. The data shown are the mean values ± SD of three experiments. [a]with respect to control group, [b]with respect to H_2O_2-induced group. $^*p<0.05$, $^{**}p<0.01$.

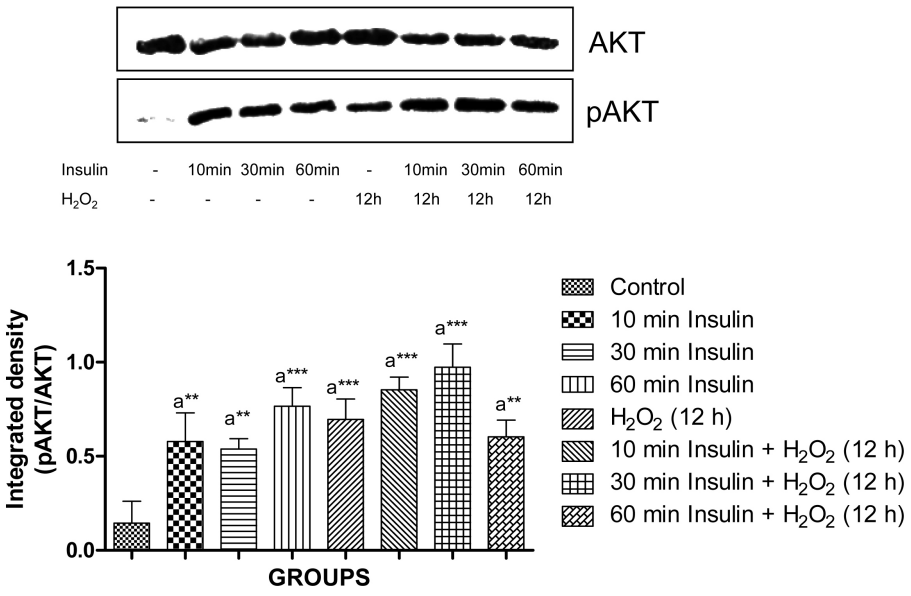

Fig. 5. Immunoblot analysis of pAKT/AKT in C6 glial cells treated with insulin. Immunoblot analysis was performed with pAKT/AKT antibody as described in the Materials and Methods. A representative blot is presented from three separate experiments and relative band intensities were determined by scanning densitometry. The data shown are the mean values ± SD of three experiments. [a]with respect to control group. $^{**}p<0.01$, $^{***}p<0.001$.

easily scavenged by cellular catalase (Gille and Joenje, 1992), it can cause membrane damage by increasing the release of arachidonic acid from the cell membrane, which may account for the prolonged damage caused by H_2O_2 even after being scavenged (Cantoni *et al*., 1989). Thus, it has been reported that H_2O_2 is excessively produced in the pathological process of acute and chronic neuronal toxicity. The neuronal cells of the brain are especially vulnerable to ROS damage as a result of their high oxygen consumption rate, abundant lipid content, high level of membrane unsaturated fatty acids that are easily oxidized by free radicals and the relative paucity of antioxidant enzymes in brain compared with other organs (Coyle and Puttfarcken, 1993).

The excess of ROS may lead to peroxidative impairment of membrane lipids and consequently disrupt neuronal functions and cause cell death (Manuchair *et al*., 1996). Furthermore, H_2O_2 can traverse cell membranes to exert its biological effects intracellularly (Halliwell and Gutteridge, 2000). This study demonstrated that hydrogen peroxide causes increased levels of intracellular ROS in C6 glial cells. DCF-dAc is commonly used to detect the generation of ROS in living cells (LeBel *et al*., 1992). The oxidation of DCFH occurs by way of strong oxidants, such as hydrogen peroxide and other hydroperoxides (Huang *et al*., 1993). Our data suggested that hydrogen peroxide increased the level of such peroxides in C6 glial cells. In this study, insulin sufficiently scavenged ROS which is supported by the decreased DCF florescence. As the data show, DCF fluorescence intensity decreased by insulin was time-dependent. A reduction of intracellular ROS by insulin was observed in the present study suggesting that hydrogen peroxide may be partly associated with oxidative toxicity in C6 glial cells.

Nitric oxide, a unique diffusible molecular messenger in the vascular and nervous systems has been shown to be involved in modulating neuronal functions with its deleterious effects attributable to more toxic peroxynitrite radical. Peroxynitrite is formed in biological systems when superoxide and NO are produced at near equimolar ratio (Beckman and Koppenol, 1996). Although it is not a free radical by chemical nature, peroxynitrite is a powerful oxidant exhibiting a wide array of tissue damaging effects including lipid peroxidation (Radi *et al*., 1991). It is therefore logical to assume that a potential way to protect neuronal cells is by nitric oxide inhibition (Contestabile, 2001). In the present study, H_2O_2 appeared to induce a marked increase in the nitric oxide levels. Supplementation of cells with insulin significantly inhibited the NO levels in the H_2O_2 induced C6 glial cells, suggesting that peroxynitrite acts as a potential mediator in the damage of C6 glial cells.

Oxidative damage can be assessed in cells by determination of the aldehyde products derived from LPO processes (Gutteridge and Halliwell, 1990). In the present study, the increased TBARS levels in H_2O_2-induced C6 glial cells reported that it might be increased LPO products. LPO constitutes a complex chain reaction of free radicals, which may contribute to tissue injury (Shi *et al*., 1998). Hydroxyl and peroxyl radicals are among the most important free radicals which can be generated as harmful byproducts and have been implicated in lipid peroxidation and some diseases. The peroxyl radical is generated in normal metabolic reactions by all aerobic organisms and also the source of the highly biologically reactive, hydroxyl radical. Therefore, scavenging of these free radicals can be an effective prevention for a living organism against oxidative stress (Bermejo-Bescós *et al*., 2008). The insulin pre-treatment decreases the TBARS as highly significant levels in H_2O_2-induced C6 glial cells. In the unstimulated cells with insulin at 30 min treatment significantly decreased the TBARS. These results indicate that the insulin scavenging the free radicals by inhibit the LPO processes.

Akt (protein kinase B or PKB) has been identified as a downstream target of growth factor receptor activation. In the present study, the phosphorylation of Akt was increased in insulin and H_2O_2 with insulin treatment groups. Akt phosphorylation may mediates cell protection by insulin or it may be simply a cellular response to minimize cellular damage in response to H_2O_2. However, insulin still has a tendency to transiently increase the phosphorylation of Akt in the present of H_2O_2, suggesting the potential role of Akt in the insulin's protective effect. Akt can promote cell survival by inhibiting proteins that mediate apoptosis. Akt is also involved in eliciting anti-apoptotic effects of growth factors and metabolic effects of insulin (Cross *et al*., 1995; Bevan, 2001). Exciting data from Birnbaum and colleagues establishes Akt as an essential gene for the maintenance of normal glucose homeostasis (Cho *et al*., 2001). Mice deficient in Akt display many of the typical features of type II diabetes mellitus in humans, namely hyperglycemia, elevated blood insulin levels, and insulin resistance in the liver and to a minor extent muscle. Consistent with the elevated insulin levels, the size and number of pancreatic islets is significantly increased in the Akt knockouts.

Akt also exerts a strong influence on survival of neurons. Akt mediation of IGF-1 induced survival of cerebellar neurons has been documented (Blair *et al*., 1999). Insulin acts as a survival factor for retinal neurons through activation of the PI3K/Akt pathway (Barber *et al*., 2001). Huntington's disease, a neurodegenerative disorder that

manifests itself in involuntary movements, personality changes, and dementia in patients is associated with death of neurons in the striatum and alteration in the huntingtin protein. Neuronal death induced by mutations in the huntingtin protein can be reversed by IGF-1 through Akt activation (Humbert *et al*., 2002; Sen *et al*., 2003). Treatment of cerebellar neurons with IGF-1 causes Akt phosphorylation and activation (Dudek *et al*., 1997; Kumari *et al*., 2001). This result was concurrent with the present study in the C6 glial cells.

Overexpression of Akt in several insulin responsive cell lines also stimulates the uptake of nutrients such as glucose and amino acids, and induces gene expression normally mediated by insulin (Hajduch *et al*., 2001). The mechanisms by which Akt mediates these effects remain unknown. Other data suggest that Akt phosphorylation and inactivation of GSK3 is likely to stimulate the conversion of nutrients such as glucose and amino acids to storage macromolecules (glycogen and protein) in skeletal muscle, adipose tissue and liver (Alessi, 2001).

In conclusion, the present study shows that after 12 h, H_2O_2 is able to induce oxidative toxicity in C6 glial cells. The data suggest that reactive oxygen intermediates are responsible for H_2O_2 induced lipid peroxidation in experimental cells. The insulin pre-treatment reversed the biochemical changes known to be aggravated by reactive oxygen species and underscore the beneficial effects in degenerative diseases, where oxidative stress have been implicated in their etiology. Considering insulin can bind to IGF-1 receptor, there is a possibility that insulin's protective effects observed in the present study may be mediated by binding to the IGF-1 receptor. The present report strongly support that Akt plays a vital regulatory role in multiple pathways controlling cell death/survival in response to insulin. There is also an interest in generating drugs that can activate Akt, which could potentially be used to trigger insulin dependent processes for the treatment of neurodegenerative diseases in the future.

ACKNOWLEDGMENTS

This study was supported by a grant of the Korea Health 21 R&D project, Ministry of Health & Welfare, Republic of Korea (02-PJ1-PG3-20905-0001).

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