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Original Article

Insulin attenuates 6-hydroxydopamine induced cell death in human neuroblastoma cells and restores p-Akt/t-Akt level

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Abstract

Introduction: Human neuroblastoma cell line is used in studying Parkinson's disease (PD) due to its similarities to dopaminergic neurons. 6-hydroxydopamine (6-OHDA), a catecholaminergic neurotoxin, has been widely used to induce cell death in cellular models of PD. Although the brain glucose entry is not dependent on insulin, this peptide has been reported to have a role in PD, in which insulin signaling disruption is reported. This study aimed to evaluate, if insulin is efficient in preventing 6-OHDA induced cell death in human neuroblastoma cells as well as its effect on phoshorylated Akt (p-Akt)/total Akt (t-Akt) ratio.

Methods: The cells -grown in DMEM/F12 media supplemented with 10% fetal bovine serum- were exposed to 6-OHDA with/without insulin for 24h, and then MTT assay was done to examine their viability. A pilot study was performed to assess the protective doses of insulin and accordingly the doses 0.9 and 1mM were selected. Western blot assay was done to evaluate the effect of 6-OHDA or insulin on p-Akt and t-Akt level.

Results: The results indicated that insulin has potency to prevent SH-SY5Y cell death, and p-Akt/t-Akt decline induced by 6-OHDA.

Conclusion: The results suggested insulin as a protective agent in dopaminergic cells.

Keywords:
Insulin;
Parkinson's disease;
6-OHDA;
SH-SY5Y;
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Introduction

Parkinson's disease (PD) is presumed as the second most common neurodegenerative disease affecting 1% of adults over the age of 60 (de Lau and Breteler, 2006). This disease is characterized by the progressive loss of dopaminergic cells of substantia nigra (Przedborski, 2005). The 6-hydroxydopamine (6-OHDA), as a selective dopaminergic neurotoxin, exists in the brain and urine of PD patients (Curtius et al., 1974). Since 6-OHDA accumulates in dopaminergic neurons, it is extensively used to induce an *in vitro* and *in vivo* model of PD (Hernandez-Baltazar et al., 2017). An *in vitro* model, commonly used in PD research is the human

neuroblastoma SH-SY5Y cell line due to similarity with dopaminergic neural cells (Xie et al., 2010; Esmaeili-Mahani et al., 2013).

While historically insulin was recognized to mediate peripheral glucose homeostasis, the existence of insulin receptors in some areas of the brain such as basal ganglia and substantia nigra, suggest a role for this peptide (Athauda and Foltynie, 2016). PD has been shown to be associated with a reduction in insulin receptor mRNA and an increase in insulin receptor resistance, in the substantia nigra pars compacta (Takahashi et al., 1996; Morris et al., 2014). Moreover, serine IRS (insulin receptor substrate) phosphorylation -that inhibits insulin signaling- increases in substantia nigra and basal ganglia (Moroo et al., 1994).

Akt, is assumed as an important down-stream of insulin signaling with the ability to phosphorylate more than 50 down-stream protein substrates, as well as an important regulator of cell function (Greene et al., 2011). Akt exists in the cytosol in an inactive conformation while its phosphorylation in serine 473 leads to its activation (Viniegra et al., 2005). Evidence suggests that Akt signaling alteration might play a key role in PD pathogenesis, supporting a possible connection between insulin signaling and PD neurodegeneration (Greene et al., 2011). A reduction in Akt was reported in post mortem studies of patients with PD (Malagelada et al., 2008). Moreover, single polymorphisms in AKT gene increases PD development (Xiromerisiou et al., 2008). In line with this, inhibition of Akt signaling was reported to induce dopaminergic cell death in experimental models (Canal et al., 2014; Xu et al., 2014). Recently, it was shown that intranasal insulin treatment in the 6-OHDA rat PD model delivered behavioral improvements and potent protection of dopaminergic neural cells of substantia nigra, proposing that insulin might have therapeutic effects in PD (Pang et al., 2016). Therefore, this study aimed to assess whether insulin prevents 6-OHDA-induced cell death and altered ratio of phosphorylated to total Akt in the human neuroblastoma cell line.

Materials and methods

Materials

Cell culture materials including Dulbecco's Modified Eagle Medium (DMEM/F12, #32500-035), fetal bovine serum (FBS, #10270-106) and penicillin-streptomycin (#15140-122) were purchased from Gibco® life technologies™. Insulin (#I9278) and 6-OHDA (#H4381) were purchased from Sigma-Aldrich. Western blot antibodies including beta-actin antibody (Rabbit mAb #4970), phospho-Akt (Ser473) antibody (Rabbit mAb #4060), Akt antibody (Rabbit mAb #4685) and secondary horseradish peroxidase-conjugated antibody (anti-rabbit IgG, HRP-linked antibody #7074) were purchased from Cell Signaling Technology. Halt™ protease and phosphatase inhibitor cocktail was from Thermo Scientific, USA and Amersham ECL select™ (RPN2235) reagent kit was purchased from GE health care. Bovine serum albumin (BSA, 1120180100) and PVDF membrane (IPVH00010) was purchased from Millipore. Other reagents were obtained from the usual commercial sources.

Cell culture

Human neuroblastoma (SH-SY5Y) cells, were purchased from national cell bank of Iran affiliated to Pasteur Institute (Tehran, Iran) and maintained with DMEM supplemented with 10% (v/v) of heat inactivated FBS and 1% of penicillin-streptomycin solution. For subcultures, SH-SY5Y cells were dissociated with trypsin-EDTA and split into a 1:3 ratio. The media was replaced every 2 days until the cells reached the total confluence (4–5 days of initial seeding). Cells were maintained in controlled conditions: 95% humidified atmosphere, 5% CO_2 and temperature of 37°C. Undifferentiated cells were used, since the differentiated SH-SY5Y cells were reported to have some Akt alteration and higher resistance to 6-OHDA (Cheung et al., 2009).

Treatments

Cell treatments were done one day after seeding the cells in 96 or 6 well plates. Dose response experiments and MTT assay were performed to obtain effective concentrations of insulin between doses 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1µM based on our previous works (Moosavi et al., 2008; Ghasemi et al., 2015). The results showed the best protection doses to be 0.9 and 1µM ($F(11, 36) = 4.881$, $P=0.0001$). Accordingly, the doses 0.9 and 1µM of insulin were selected for further studies. 6-OHDA was dissolved in 0.1% ascorbic acid and then diluted in culture medium to obtain 50µM

concentration (Amiri et al., 2016; Moosavi et al., 2018). Insulin and 6-OHDA were added to cell culture media simultaneously. Following 24h incubation, the cell viability test, morphological observation and biochemical assessments were performed.

Assessment of cell viability

Cell viability was evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay. The cells were plated into 96-well plates at a density of 1×10^4 cells/well. The cells were exposed to 6-OHDA and/or insulin. After 24h incubation, the media was replaced with 0.5mg/ml MTT in cell culture media and incubated for 4h for formazan formation. Then, the media was replaced with 100µl DMSO to dissolve the formazan crystals and the OD values were spectrophotometrically determined at 570nm (Synergy HT, Biotek®).

Western blot analysis

Protein extraction from cultured cells

SH-SY5Y cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold RIPA lysis buffer containing protease and phosphatase inhibitor cocktail. The resulting homogenate was centrifuged at 13000 rpm for 30min at 4°C. The supernatant was removed and analyzed as the total protein extract. The supernatant was then kept for western blot analysis. Protein concentrations were determined using Lowry method (Lowry et al., 1951).

Western blotting

Western blotting was performed as previously described (Moosavi et al., 2014; Negintaji et al., 2015). The supernatants were mixed with sample buffer. Afterward, equal amounts of protein (20µg/well) were separated by 10% polyacrylamide gel electrophoresis and transferred to PVDF membrane at 100V 1h at 4°C in transfer buffer. After blocking for 60min with 5% BSA in tris-buffered saline, the membrane was incubated overnight at 4°C with the primary antibodies (p-Akt, t-Akt and beta-actin). Afterward, the membrane was incubated in HRP-conjugated secondary antibody at room temperature for 120min. The bands were then revealed using ECL (enhanced chemiluminescence) select kit, which were then transferred to radiographic films in a darkroom. The radiographic films were then scanned and the density of bands was calculated using Image-J software.

Data analysis

The MTT experiments and western blot analysis were repeated 3-4 times. The data was analyzed by one-way ANOVA followed by post-hoc Tukey's test.

Results

The neuroprotective effect of insulin on SH-SY5Y cells exposed to 6-OHDA

MTT assays enumerate the ratio of live and dead cells in a cell population via assessing cell health attained by measuring its metabolic activity (ability to reduce tetrazolium salts in MTT assay). Since, 6-OHDA decreases the ratio of live cells in MTT assay, it can be concluded that this toxin induces cell death. Based on the results from the preliminary dose-response test (Fig. 1), the doses 0.9 and 1µM were selected as the protective doses of insulin. When SH-SY5Y cells were incubated with insulin (0.9 and 1µM), it partially prevented the toxicity induced by 6-OHDA (Figs. 1 and 2A, $F(5, 17)= 13.80$, $P<0.0001$). Post-hoc Tukey's test revealed that insulin prevented 6-OHDA induced cell death. Insulin by itself had no effect on cell survival in comparison with the control group.

Cell morphology

Morphological results of SH-SY5Y cells are shown in Figure 2B. As it was shown, 6-OHDA exposure led to cell body shrinkage, decrease in the number of alive cells and increase in the cell debris. These morphological changes attenuated following insulin co-treatment.

Insulin inhibits 6-OHDA-induced p-Akt/t-Akt changes in SH-SY5Y cells

Total Akt (t-Akt) is assumed as the total amount of Akt while its phosphorylated form (p-Akt) is considered as the active conformation (Viniegra et al., 2005). As shown in Figure 3, the analysis of the phosphorylation statuses of Akt revealed significant differences. The treatment with 50µM 6-OHDA decreased p-Akt/t-Akt ratio while insulin treatment in 0.9 and 1µM partially reversed the decrement of p-Akt/t-Akt ratio ($F(5, 12)= 4.197$, $P=0.0194$).

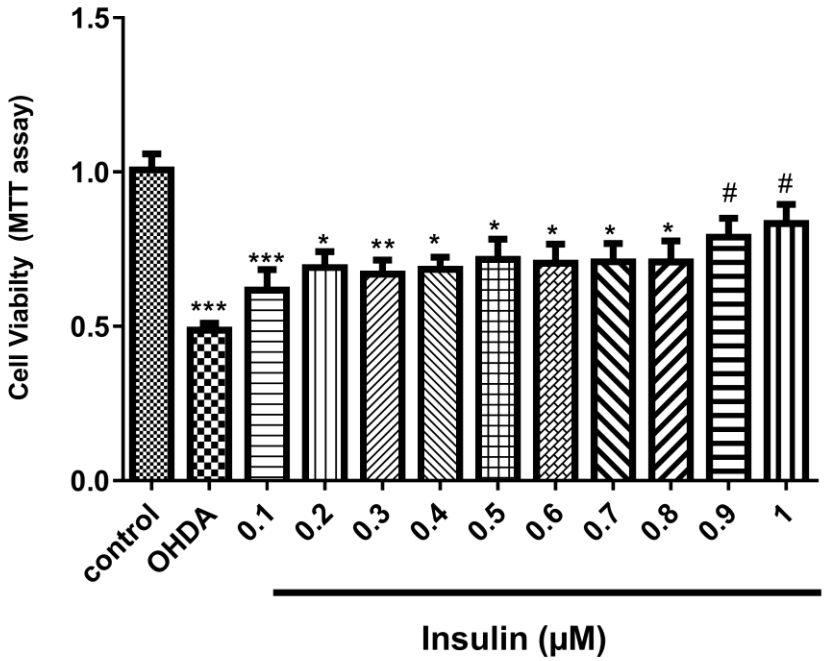

Fig.1. The effect of different insulin doses (0.1-1μM) against 6-OHDA (50μM) induced cell death in MTT assay. The effect of insulin exposure at doses 0.9 and 1μM with/without 6-OHDA. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ represent the differences between the control and other groups. $^{\#}P<0.05$ represents the differences between OHDA treated cells and insulin+OHDA exposed cells.

Discussion

The data showed that insulin can partially restore the inhibitory effects of 6-OHDA on cell viability and p-Akt in SH-SY5Y cell line. PD primarily occurs due to loss of substantia nigra dopamine neurons (Forno, 1996). The SH-SY5Y cell line provides a cell supply of human origin with identical characteristics to dopaminergic neural cells (Xie et al., 2010). The 6-OHDA that was shown to promote apoptotic cell death in dopaminergic neurons (Gomez-Lazaro et al., 2008) can induce toxicity in SH-SY5Y cells (Storch et al., 2000; Amiri et al., 2016). Insulin significantly attenuated the effect of 6-OHDA on cell viability in MTT assay. This neuroprotective effect of insulin is in line with previous studies that showed the protective effect of insulin against hydrogen peroxide in SH-SY5Y cells (Ramalingam and Kim, 2014b; Ramalingam and Kim, 2014a).

Evidence indicates that the impairment of insulin signaling might elevate the risk of PD (Morris et al., 2008; Bosco et al., 2012; Ashraghi et al., 2016; Pang et al., 2016). While insulin is well known for its role in glucose cell entry, within the brain, it appears to have neuroprotective effects (Yu and Pei, 2015). Insulin receptors exist in the substantia nigra and supposed to affect neuronal survival and dopaminergic transmission (Bassil et al., 2014). Furthermore, it seems that an event comparable to peripheral insulin resistance develops in the brains of patients with PD (even in non-diabetic patients), suggesting that the defect of insulin signaling might contribute to the pathological events of PD (Athauda and Foltynie, 2016).

Binding of insulin to its receptor results in phosphatidylinositol 3-kinase (PI-3K) and Akt activation (van der Heide et al., 2006). Phosphorylated (active) Akt level decreases in the striatum of patients with PD (Greene et al., 2011). In this study, 6-OHDA exposure resulted in Akt inactivation, which is in line with previous reports (Chen et al., 2004; Moosavi et al., 2018). Although, some evidence imply that strong Akt activation leads to oxidative stress and cell death (Nogueira et al., 2008), p-Akt is supposed to have a pro-survival effect against neuronal apoptosis (van der Heide et al., 2006). Consistently, reports have shown that Akt pathway activation results in apoptosis inhibition and therefore attenuate neural degeneration in PD models (Shimoke and Chiba, 2001; Nakaso

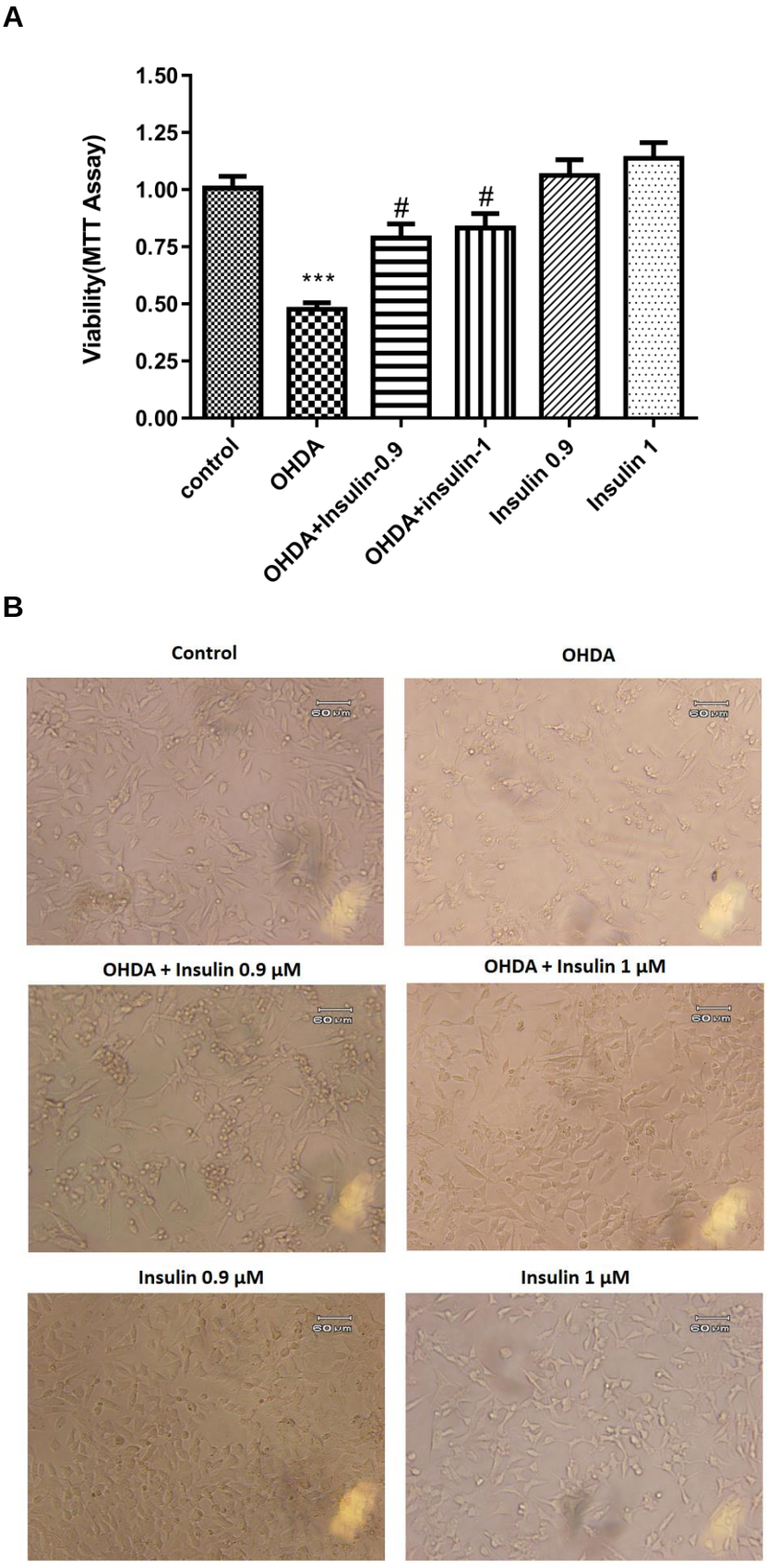

Fig.2. The effect of insulin exposure at doses 0.9 and 1µM with/without 6-OHDA on cell viability (A). $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ represent the differences between the control and other groups. $^{\#}P<0.05$ represents the differences between OHDA treated cells and insulin+OHDA exposed cells. The microscopic images of SH-SY5Y cells in different groups (B). Images were magnified 200 times. After 6-OHDA exposure, the cell bodies shrunk, alive cells decreased while there was an increment of cell debris, shown by black arrows. These morphological changes attenuated when insulin was added.

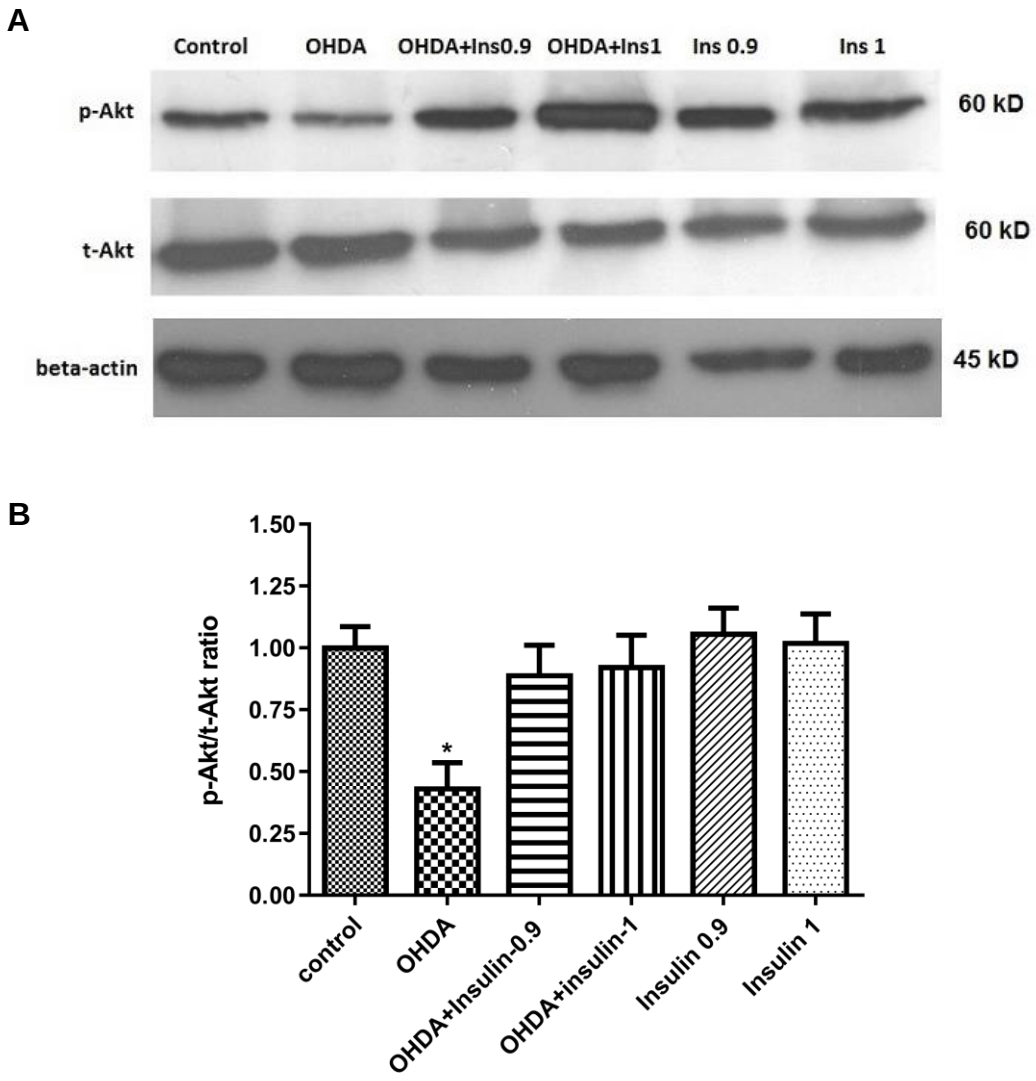

Fig.3. Western blot results showing the level of p-Akt, t-Akt and beta actin in SH-SY5Y cells after different treatments (A). The p-Akt/t-Akt ratio in those treated cells (B). $^{*}P<0.05$ represents the differences between the control and 6-OHDA treated cells.

et al.,2008). In line with our findings, number of *in vitro* researches have revealed that Akt activation is essential in maintaining the survival of different neuron types (Greene et al., 2011).

Conclusion

In conclusion, the present study revealed that insulin can significantly reduce human neuroblastoma cell death induced by 6-OHDA and showed that this action was associated with restoration of p-Akt/t-Akt level. Findings, suggest that insulin can be regarded as a protective agent for dopaminergic neurons.

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Conflict of interest

The authors declare that they have no conflict of interests.

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