Chronic Hyperinsulinemia Leads to the Development of Neuronal Insulin Resistance Model

อินซูลินขนาดสูงแบบเรื้อรังนำไปสู่ภาวะดื้อต่ออินซูลินของเซลล์ประสาทในหลอดทดลอง

Salinee Jantrapirom (สาลินี จันทราภิรมย์)* Dr.Nipon Chattipakorn (ดร.นิพนธ์ ฉัตรทิพากร)**
Dr.Siriporn Chattipakorn (ดร.สิริพร ฉัตรทิพากร)*** Dr.Saranyapin Potikanond (ดร.ศรัณยภิญ โพธิกานนท์)****

ABSTRACT

Insulin resistance appears to be a significant risk factor for neurodegeneration. In this study, we hypothesized that chronic hyperinsulinemia leads to the development of neuronal insulin resistance. Human neuroblastoma cell line (SH-SY5Y) was treated with either 100 nM insulin or solution control for two days. After that, the activities of neuronal insulin signaling, including the phosphorylation of insulin receptor (IRβ), Akt and GSK3 (tau kinase), were determined by western blot analysis. We found that chronic insulin treatment led to neuronal insulin resistance, indicated by decreased IRβ and Akt phosphorylation. In addition, the phosphorylation at Ser9 of GSK3 (tau kinase) was also reduced, which may finally lead to increase GSK3 activity. GSK3 plays a major role in the development of Alzheimer's disease. This study suggests that hyperinsulinemia can lead to neuronal insulin resistance and increased GSK3 activity. This *in vitro* neuronal insulin resistance model can be used as a model to test the potential therapeutic effect of anti-diabetic drugs in the brain.

บทคัดย่อ

ภาวะดื้อต่ออินซูลินเป็นปัจจัยเสี่ยงที่สำคัญในการเกิดโรคสมองเสื่อม การศึกษานี้ตั้งสมมุติฐานว่า การได้รับอินซูลินขนาดสูงเรื้อรังนำไปสู่ภาวะดื้อต่ออินซูลินในเซลล์ประสาท โดยใช้เซลล์มะเร็งประสาทของมนุษย์ (เอสเอช-เอสวายห้าวาย) โดยเซลล์ถูกแบ่งเป็น 2 กลุ่มคือ กลุ่มที่ได้รับอินซูลินความเข้มข้น 100 นาโนโมลาร์ และ กลุ่มที่ได้รับอาหารเลี้ยงเซลล์ปกติ เป็นเวลา 2 วัน หลังจากนั้นทำการวิเคราะห์โปรตีนเพื่อศึกษาการทำงานของสื่อสัญญาณอินซูลินในเซลล์ ซึ่งประกอบด้วย อินซูลินรีเซพเตอร์, โปรตีน Akt และโปรตีน GSK3 (tau kinase) จากการทดลองพบว่าเซลล์ที่ได้รับอินซูลินขนาดสูงเป็นเวลานานมีสื่อสัญญาณอินซูลินในเซลล์ลดลง โดยแสดงจากปริมาณการเติมฟอสเฟตที่อินซูนลินรีเซพเตอร์และโปรตีน Akt ที่ลดลง นอกจากนั้นการเติมฟอสเฟตที่ตำแหน่งอะมิโนเซอรีนที่ 9 ของโปรตีน GSK3 ก็ลดลงด้วย อาจส่งผลให้การทำงานของเอนไซม์ GSK3 เพิ่มมากขึ้น ซึ่งมีส่วนในการพัฒนาของโรคอัลไซเมอร์ ผลการวิจัยนี้แสดงให้เห็นว่าการได้รับอินซูลินขนาดสูงเป็นเวลานานทำให้เกิดภาวะดื้อต่ออินซูลินในเซลล์ประสาทและเพิ่มการทำงานของเอนไซม์ GSK3 แบบจำลองภาวะดื้อต่ออินซูลินในเซลล์ประสาทนี้ อาจใช้ทดสอบแนวโน้มของยาต้านเบาหวานในการรักษาโรคสมองเสื่อม

Key Words: Insulin, Neuronal insulin resistance, Alzheimer's disease
คำสำคัญ: อินซูลิน ภาวะดื้ออินซูลินในเซลล์ประสาท โรคอัลไซเมอร์

* *Student, Master of Science in Pharmacology, Faculty of Medicine, Chiang Mai University*
** *Professor, Department of Physiology, Faculty of Medicine, Chiang Mai University*
*** *Associate Professor, Department of Oral Biology and Diagnostic Sciences, Faculty of Dentistry, Chiang Mai University*
**** *Lecturer, Department of Pharmacology, Faculty of Medicine, Chiang Mai University*

Introduction

Insulin resistance is the pathological condition in which target tissues cannot response to the normal insulin levels (Ng et al., 2009; Zimmet et al., 2001). The characteristic of insulin resistance is hyperinsulinemia with euglycemia (Calles-Escandon et al., 1998). In addition insulin resistance in target organs can be determined by impairment of insulin signaling (Boucher et al., 2014). Generally, the insulin signaling consists of two main pathways; PI3K/Akt and Ras/MAPK pathways (Siddle et al., 2011). Binding of insulin to its receptor leads to autophosphorylation and many downstream molecules such as insulin receptor substrate-1 protein (IRS-1), Akt and GSK3 are further phosphorylated (Hemmings et al., 1997).

In the brain, insulin plays an important role in cognition and other aspects of normal brain abilities (Plum et al., 2005). Previous study demonstrated that peripheral insulin resistance in animal models appears to be a significant risk factor for neurodegeneration as well as Alzheimer's disease (AD) (Rivera et al., 2005; Craft et al, 2007; Watson et al., 2003; Lester-Coll et al., 2006). It has been shown that insulin resistance is the cause of beta amyloid and tau formation in the brain, which are the pathological markers of AD (Selkoe et al., 2001).

Insulin signaling impairments have been documented in both postmortem analysis and in animal models of AD (Talbot et al., 2012; Siddle et al., 2011; Frölich et al., 1998; Hölscher & Li 2010). Recent *in vitro* study in mouse neuroblastoma cells showed that chronic insulin application caused insulin resistance in neuronal cells and developed pathological conditions of neurodegeneration including marker of AD (Gupta et al., 2011). However, the association between hyperinsulinemia and neuronal insulin resistance in human neuronal cells has not been investigated. Therefore, this study was design to demonstrate that chronic hyperinsulinemia leads to the development of neuronal insulin resistance in the human neuroblastoma cell line.

Objectives of the study

To investigate whether chronic insulin application on human neuroblastoma cell line can lead to neuronal insulin resistance

Methodology

Cell culture

The human neuroblastoma cell line SH-SY5Y was obtained from American Type Culture Collection (ATCC No. CRL-2266) and maintained in DMEM/Ham F12 medium (Sigma-aldrich, USA), 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 atmosphere at 37°C. The cells were sub-cultured at 80-90% confluent by 0.25% trypsin. The mediums were changed every 3-4 days. In chronic insulin treatment, SH-SY5Y was incubated with 100 nM insulin for 2 days and medium was changed every 24 hours. Then, the cells were incubated with medium at 37°C, overnight. On the experimental day, the cells were stimulated with or without 10 nM insulin for 30 minutes at 37°C.

Cell viability assay

SH-SY5Y were seeded at a density of 2 x 10^4 cells per well on 96-well plate for 24 hours and treated with 1 nM, 10 nM, 20 nM, 30 nM, 50 nM and 100 nM insulin concentrations for 48 hours medium was changed every 24 hours). Cellular viability was

determined by measuring colorimetric absorbance at 490 nm and read with a microplate reader. Experiments were done in triplicate.

Western blot analysis

The cells were washed with 1% cool PBS buffer followed by addition of cell lysis buffer (20 mM Tris-HCl, 1mM Na_3VO_4, 5mM NaF and cocktail protease and phosphatase inhibitor) and extracted in 2x sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The protein samples were measured by protein assay (Bio-Rad, USA). The protein preparations were separated by 10%SDS-PAGE. The separated proteins were transferred to PVDF membranes (Immobilon-P; Millipore, Bedford, MA) for 1 hour at 100V. The membranes were blocked in 5% non-fat milk in TBS containing 0.1% tween 20 for 1 hour at 25°C and then incubated with primary antibodies against phosphorylation of insulin receptor (Tyr 1162/1163) (1:1000, Santa Cruz, USA), insulin receptor β-subunit (1:1000, Santa Cruz, USA), phosphorylation of Akt (Ser 473) (1:1000, Cell Signaling, USA), Akt (1:1000, Cell Signaling, USA), phosphorylation of GSK3β (Ser9) (1:1000, Cell Signaling, USA), GSK3β (1:1000, Cell Signaling, USA) and β-actin (1:2000, Santa Cruz, USA) at 25°C for overnight and followed by the incubation of horseradish peroxidase-conjugated antirabbit IgG (1:2000, Santa Cruz, USA). The protein bands were visualized by ECL western blotting detection reagent and quantitated using Image J software.

Statistical analysis

All data was expressed as mean ± SEM. One-way ANOVA followed by LSD post-hoc analysis was used to determine the difference between each groups. $P<0.05$ was considered statistically significant.

Results

Effects of chronic insulin application on human neuroblastoma cell line

Before chronic insulin application, we used cytotoxic assay to determine toxic which dose of insulin (varies from 1-100 nM) on neuronal cells (Fig. 1). After 2 days of insulin treatment, insulin increased cell growth in a concentration dependent manner. Insulin at high dose (100 nM) did not increase cell growth and toxic to the cells as floating cells were not observed in the medium (data not shown). The percentage of cell survival incubated in 100 nM insulin was not different from that of control (0 nM, Fig.1). Therefore, we chose insulin at dose 100 nM to mimic hyperinsulinemia. We next tested the effect of chronic presence of 100 nM insulin on insulin receptor. We found that in the absence of chronic insulin application, phosphorylation of insulin receptor was significantly increased after 30 min stimulation with insulin 10 nM (Fig. 2A). In chronic insulin treatment, insulin was not able to stimulate phosphorylation of insulin receptor (Fig. 2A). The ratio of tyrosine phosphorylation of insulin receptor-β (IRβ) was reduced 31.16% in chronic insulin application (Fig. 2B). We next tested phosphorylation of Akt protein, the downstream signal molecules and found that the signal was decreased in chronic insulin application (Fig. 3A). Insulin-mediated Akt phosphorylation was markedly impaired (50.64%) under chronic insulin conditions (Fig. 3B).

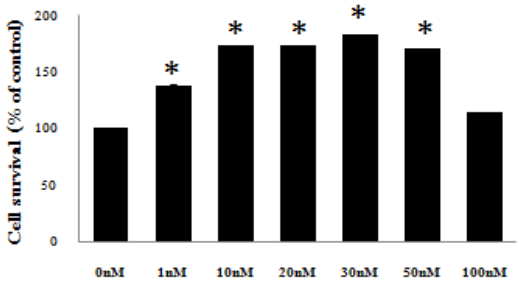

Figure 1 Cytotoxicity assay at different concentrations of insulin in neuroblastoma cell line (SH-SY5Y). Values are mean ± SEM. * *P<0.05* compared with (0 nM insulin)

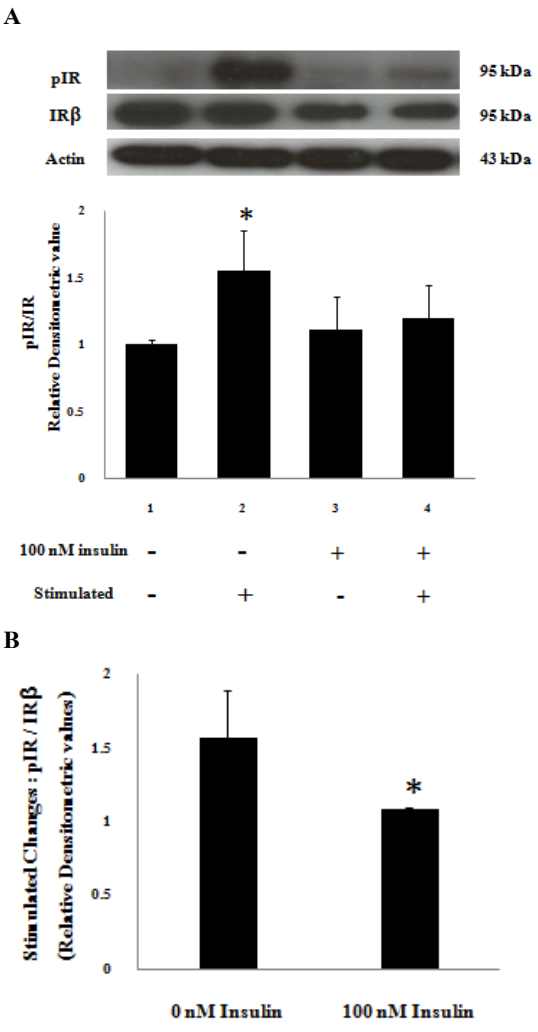

Figure 2 Effects of prolonged insulin condition on insulin receptor. The SH-SY5Y cells were either treated with or without 100 nM insulin for 2 days. Then cells were stimulated with or without 10 nM insulin for 30 minutes. (A) Western blots and bar graphs (below) represent densitometric values of each lane (B) Bar graphs represent the insulin-stimulated change of phosphorylated IR (pIR) after normalizing with IRβ expression (pIR/IRβ). All the experiments were repeated at least three times. Values are mean ± SEM. * *P<0.05* compared with control (0 nM insulin)

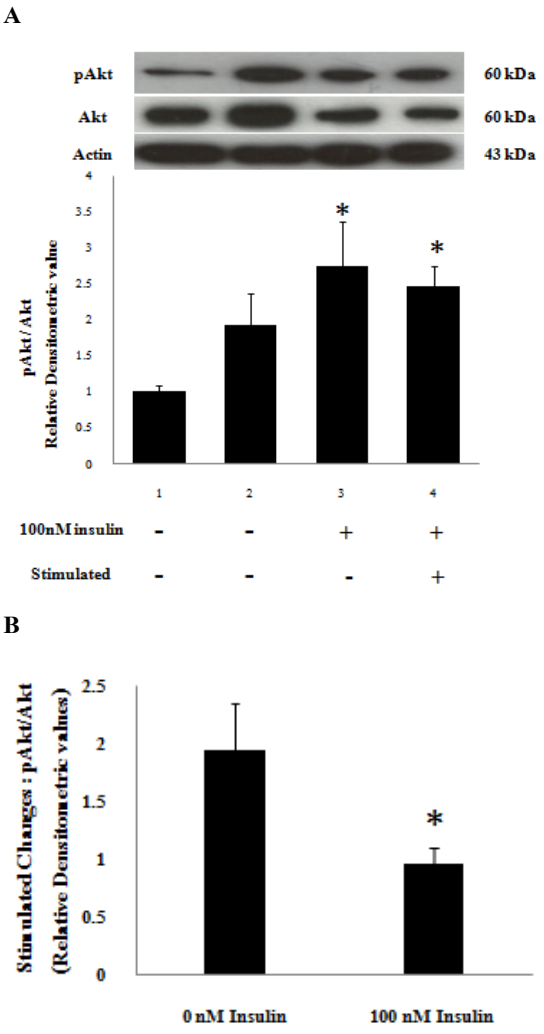

Figure 3 Effects of prolonged insulin condition on Akt protein. The cells were treated either with or without 100 nM insulin for 2 days and stimulated with or without 10 nM

insulin for 30 minutes. (A) Western blots and bar graphs (below) represent densitometric values of each lane of pAkt and Akt. (B) Bar graph represents the insulin-stimulated change of phosphorylated Akt (pAkt) after normalizing with Akt. All the experiments were repeated at least three times. Values are mean ± SEM. * *P<0.05* compared with control (0 nM insulin)

Next, we determined the effect of prolonged insulin application-induced neuronal insulin resistance on glycogen synthase kinase (GSK3β). We observed that insulin-induced phosphorylation of GSK3β at Ser9 sites was also markedly impaired by 51.66% under chronic insulin conditions as compared to normal conditions (Fig.4)

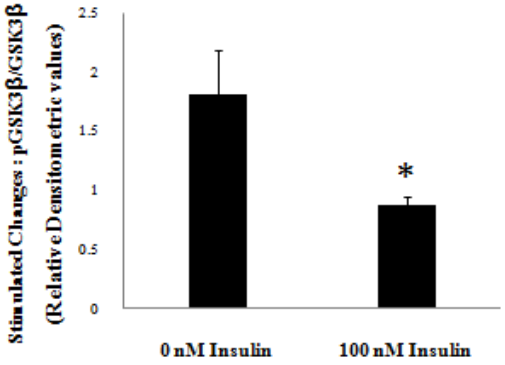

Figure 4 Effect of prolonged insulin conditions on glycogen synthase kinase (GSK3β) signaling protein. The cells were chronically treated with or without 100 nM insulin for 2 days and stimulated with or without 10 nM insulin for 30 minutes. Phosphorylated GSK3β (pGSK3β), Bars represent relative densitometric values of pGSK3β after normalizing with GSK3β expression (pGSK3β/GSK3β). All the experiments were repeated at least three times. Values are mean ± SEM. * *P<0.05* compared to control (0 nM insulin)

Discussion and conclusion

Major findings of this study is that 1) 100 nM insulin application onto neuron cells for 2 days, was a proper in vitro model of chronic hyperinsulinemia, to the impairment of neuronal insulin signaling. 2) chronic hyperinsulinemia also led to a decrease in insulin-mediated GSK-3 phosphorylation. It has been shown that hyperinsulinemia leads to the impairment of insulin signaling in several target organs such as liver muscle and fat cells (Ng et al., 2009; Zimmet et al., 2001). Excess of circulating insulin in the blood is the sign of pre-diabetes found in early onset of diabetes (Aponte et al., 2013). Prolonged hyperinsulinemia, eventually develops into type 2 diabetes (Gupta et al., 2011). Several studies are reported that chronic treatment of insulin induces peripheral insulin resistance shown as reduction of the insulin receptor signaling in many cell types such as muscle cells (Kumar & Dey, 2003), adipocytes (Sinha et al., 1987) and neurons (Gupta et al., 2011). In the present study, we provided evidence that chronic insulin applications in human neuroblastoma cells led to development of insulin resistance by impairing phosphorylation of IRβ and down-stream signal molecule; Akt protein, decreased Akt phosphorylation may link to cell survival in the future. Consistent with our findings, it has been shown that prolonged insulin conditions in mouse neuroblastoma cell line leads to cellular insulin resistance. Both results suggest that exposure of neurons to prolonged hyper-insulin conditions can cause neuronal insulin resistance.

We also identified the downstream target of Akt which is GSK3β, a type of tau kinase enzymes. GSK3β is involved in tau formation. Phosphorylation of Akt to GSK3β leads to inhibition of its kinase activity. Once GSK3 phosphorylates tau protein to become hyperphosphorylated tau, it contributes to tau aggregation which predominates in neurofibrillary tangle (NFT), a marker of AD. NFT accumulation disrupts the neuronal function and finally induce neuronal cell death (Iqbal et al., 2009). In this study, we found that the chronic insulin treatment of neuroblastoma cells led to impairment of GSK3β at Ser9 residue, an inhibitory residue in the phosphorylation of GSK3β (Jope et al., 2007). Decreased phosphorylation of GSKβ at Ser 9, finally resulted in an increase of overall GSK activity. Similarly, the impairment of GSK3β phosphorylation was also found in chronic insulin condition of mouse N2A cell line (Gupta et al., 2011). Our findings suggest that increased GSK3 activity could induce hyperphosphorylation of tau and finally increase aggregation of NFT. These findings confirm *in vitro* neuronal insulin resistance model which may lead to increased AD-like change.

In conclusion, the present study suggests that hyperinsulinemia can lead to neuronal insulin resistance and increased GSK3 activity. Therefore, this chronic hyperinsulinemia neuronal model can be used as a model of neuronal insulin resistance to test the potential therapeutic effect of anti-diabetic drugs in the brain.

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