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Amyloid β oligomer-induced ERK1/2-dependent serine 636/639 phosphorylation of insulin receptor substrate-1 impairs insulin signaling and glycogen storage in human astrocytes

Qinghua Zhang, Shougang Guo, Xiao Zhang, Shi Tang, Lu Wang, Xiaojuan Han, Wen Shao, Lin Cong, Yifeng Du *

Department of Neurology, Provincial Hospital Affiliated to Shandong University, Jinan City, Shandong Province, PR China

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ABSTRACT

Aims: This study is to investigate the effect of amyloid β_{1-42} oligomers on insulin signaling in astrocytes.
Methods: Synthetic $A\beta_{1-42}$ oligomers were prepared and the oligomeric form of $A\beta_{1-42}$ was verified by an electron microscope. Normal human astrocytes were cultured in Dulbecco's Modified Eagle Medium. Western blotting was employed to measure the amount of lysate proteins. Immunofluorescence was performed to detect the distribution of phosphorylated insulin receptor substrate-1 and expression of P-GSK3β in astrocytes under confocal microscopy and fluorescent microscopy, respectively. Periodic Acid-Schiff staining was used to detect glycogen, the content of which was measured using glycogen assay.
Results: Our data showed that $A\beta_{1-42}$ oligomers inhibited insulin-induced serine phosphorylation of Akt at 473 and GSK3β at serine 9, as well as glycogen storage. However, the levels of phosphorylated GSK3β at tyrosine 216 were significantly increased in the presence of $A\beta_{1-42}$ oligomers. In addition, the levels of phosphorylated ERK1/2 and insulin receptor substrate-1 at serine 636/639 were significantly increased in response to treatment with $A\beta_{1-42}$ oligomers. Of note, the responses and inhibitory effects of $A\beta_{1-42}$ oligomers on insulin signaling were partially reversed by ERK1/2 upstream inhibitor PD98059.
Conclusions: Our results demonstrated that $A\beta_{1-42}$ oligomers impaired insulin signaling and suppressed insulin-induced glycogen storage in human astrocytes, probably due to ERK1/2-dependent serine phosphorylation of insulin receptor substrate-1 at 636/639 induced by $A\beta_{1-42}$ oligomers.

1. Introduction

Insulin signaling impairment is a pathological feature of Alzheimer's disease (AD) (Talbot et al., 2012). Insulin plays a crucial role in the regulation of brain glucose metabolism, neurotransmission, learning and memory, synapse plasticity and anti-apoptosis (McNay et al., 2010; McNay and Recknagel, 2011; Dixon-Salazar et al., 2014). Accumulating evidence indicates that hippocampi in brains of AD patients exhibit defective insulin signaling with altered levels and cellular distribution of insulin receptors and inhibitory serine phosphorylation of insulin receptor substrate-1 (IRS-1) (Steen et al., 2005; Moloney et al., 2010; Bomfim et al., 2012; Talbot et al., 2012; Lourenco et al., 2013).

Amyloid β_{1-42} ($A\beta_{1-42}$), the main component of Aβ plaques, is generated from amyloid precursor protein. $A\beta_{1-42}$ is aggregated into $A\beta_{1-42}$ oligomers and $A\beta_{1-42}$ fibrillars in brains of AD patients (Greenwald and Riek, 2010; He et al., 2012). In addition, it is known that $A\beta_{1-42}$ oligomers are the more toxic form that can induce insulin signaling impairment, synapse loss, and memory impairment. Moreover, results of animal experiments and clinical trials have demonstrated beneficial effects of insulin or anti-diabetes agents for AD animal models or patients (Bomfim et al., 2012; Freiherr et al., 2013). The evidence suggests that impairment of insulin signaling may contribute to the development of AD. However, insulin receptors are expressed not only on neurons but also on astrocytes (Dringen and Hamprecht, 1992; Hamai et al., 1999; Heni et al., 2011). Previous studies about insulin signaling impairment in AD were mostly focused on neurons. Astrocytes, a major component of brain, play crucial roles in neurotransmitter regulation, energy metabolism, and anti-oxidation in central nervous system (Belanger et al., 2011). However, effect of insulin signaling in astrocytes is rarely reported.

Recent evidence indicates that insulin signaling pathway participates in the regulation of all the functions in astrocytes. Glutamate transporter subtype-1 (GLT-1) exclusively expressed on astrocytes accounts for more than 90% of glutamate uptake in the central nervous system (CNS) (Rothstein et al., 1996). Several studies indicate that insulin can increase the expression and membrane distribution of GLT-1 on astrocytes (Li et al., 2006; Ji et al., 2011). In addition, insulin signaling

Abbreviations: AD, Alzheimer's disease; $A\beta_{1-42}$, Amyloid β_{1-42}; CNS, the central nervous system; DMEM, Dulbecco's Modified Eagle Medium; GLT-1, Glutamate transporter subtype-1; IRS-1, insulin receptor substrate-1; PAS, Periodic acid-Schiff; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3 kinase.

* Corresponding author at: Department of Neurology, Provincial Hospital Affiliated to Shandong University, No. 324, Jingwuweiqi Road, Jinan City, Shandong Province, PR China.
E-mail address: neurology2014j@163.com (Y. Du).

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pathway is required for insulin-like growth factor-1-mediated antioxidative function in astrocytes (Genis et al., 2014). Glycogen, almost exclusively expressed in astrocytes (Brown and Ransom, 2007) is the main energy source of neurons during hypoglycemia or intense activation. The storage of glycogen is regulated by insulin presumably through phosphatidylinositol 3 kinase (PI3K)/Akt pathway. Therefore, it is necessary to investigate the effects of $A\beta_{1-42}$ oligomers on insulin signaling.

In the present study, we use human astrocytes to investigate the effect of $A\beta_{1-42}$ oligomers on insulin-induced phosphorylation of Akt at serine 473 and GSK3β at serine 9 and tyrosine 216, glycogen storage in astrocytes, and the possible underlying mechanisms.

2. Materials and methods

2.1. Preparation of $A\beta_{1-42}$ oligomers

$A\beta_{1-42}$ oligomers were prepared as described previously (Ryan et al., 2010). In brief, human synthetic $A\beta_{1-42}$ (American Peptide Company, Sunnyvale, CA, USA) was suspended in hexafluoroisopropanol (Sigma-Aldrich, St. Louis, MO, USA) to reach 1 mM and stored as a dried film at −20 °C after evaporation of solvent using a Speed-Vac. To form $A\beta_{1-42}$ oligomers, peptide films were re-suspended to 5 mM in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA), bath-sonicated for 10 min, diluted to 100 μM with cold PBS + 0.05% SDS and vortexed for 30 s. Samples generated above were allowed to proceed for 24 h at 4 °C and then further diluted with cold PBS to 11.1 nM before incubation for 2 weeks at 4 °C. The oligomeric form of $A\beta_{1-42}$ was verified by an electron microscope. Prior to using, the oligomer solution was centrifuged at 13,000 rpm for 10 min at 4 °C.

2.2. Cells

Normal human astrocytes (HA1800, Sciencell, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (1 g/L glucose, Hyclone, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in an atmosphere containing 5% CO_2. Prior to each experiment, cells were washed twice with phosphate-buffered saline (PBS) and starved in DMEM + 0.5% fetal bovine serum for 24 h and then exposed to $A\beta_{1-42}$ oligomers (1 nM) for indicated periods followed by stimulation with human insulin (100 nM, Novo Nordisk, Denmark) for 30 min. If needed, mitogen-activated protein kinase inhibitor PD98059 (Giannoni et al., 2012) (50 μM, Promega, USA) was added to cells 1 h before $A\beta_{1-42}$ oligomer treatment.

2.3. Western blotting

Equal amounts of lysate proteins were separated in 10% SDS-PAGE gels before being transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% milk and then immunoblotted with antibodies (P-ERK1/2 (Thr202/Tyr204), 1:6000; ERK1/2, 1:6000; P-IRS $ser^{636/639}$, 1:1000; IRS, 1:1000 (Cell Signal Technology, USA); P-Akt1 ser^{473}, 1:1000 (Sangon Biotech, China); and Akt, 1:1000 (Beyotime Institute of Biotechnology, China)) overnight at 4 °C. Afterwards, the membranes were blotted with horseradish peroxidase-linked secondary antibody. Then, protein bands were detected by electrochemiluminescence kit and the density of bands was quantified by densitometry. Protein content was normalized by α-tubulin (1:5000, Sigma-Aldrich, USA).

2.4. Immunofluorescence

Human astrocytes, grown on coverslips under the condition described above, were fixed with 4% paraformaldehyde (20 min at room temperature), treated with 0.5% triton for 20 min at room temperature, blocked with 5% normal goat serum (1 h at room temperature), and then labeled with P-IRS (1:50), IRS (1:50), P-GSK (Ser9) (1:50) (Cell Signal Technology, USA) and P-GSK (Tyr216) (1:50) (Sangon Biotech, China) overnight at 4 °C. Afterwards, the cells were immunoblotted with Alexa Fluor® 488-conjugated affinipure goat anti-rabbit IgG-labeled goat anti-rabbit secondary antibody (1 h at room temperature, Zhongshan Golden Bridge Biotechnology, China) and mounted with 4,6-diamino-2-phenylindole for 5 min at room temperature (Beyotime Institute of Biotechnology, China). Finally, the slides detecting P-GSK (Ser9) and P-GSK (Tyr216) were viewed under a fluorescent microscope (TE2000-U, NIKON, Japan). The slides detecting P-IRS were viewed under a confocal microscope (LSM780, ZEISS, Germany).

2.5. Periodic acid-Schiff (PAS) staining and glycogen assay

Cells growing on coverslips or 6-well plates were pretreated with $A\beta_{1-42}$ oligomers in the presence or absence of PD98059 for 24 h. Then, the cells were washed with PBS for two times and incubated with DMEM (4.5 g/L glucose, Hyclone, USA) supplemented with 100 nM insulin for 30 min. Cells growing on coverslips were stained using PAS (Nanjing Jiancheng, China) following the manufacturer's instructions. Subsequently, the glycogen content of cells growing on 6-well plates was measured using glycogen assay kit (Bioassay System, USA) following the manufacturer's instructions.

2.6. Statistical analysis

The results were analyzed using SPSS 17.0 software (IBM, USA). Data were expressed as means ± SEM. One way ANOVA was used to compare differences between multiple groups and *t*-test was used to compare differences between two groups. Differences were considered significant when $P < 0.05$.

3. Results

3.1. $A\beta_{1-42}$ oligomers inhibit insulin-induced serine phosphorylation of Akt at 473 in astrocytes

To determine the most suitable concentration of insulin for Akt activation, astrocytes were exposed to DMEM supplemented with different concentrations of insulin (0, 5, 25, 50 and 100 nM). The levels of phosphorylated Akt at serine 473 stimulated by insulin started to increase at the concentration of 25 nM and reached a significantly higher level at 100 nM ($P < 0.05$) (Fig. 1A), with no changes in the levels of total Akt being observed. As a result, the concentration of 100 nM was used in the following experiments. Subsequently, the effect of $A\beta_{1-42}$ oligomers on insulin-induced serine phosphorylation of Akt at 473 was tested. In addition, the level of phosphorylated Akt at serine 473 was significantly reduced after treatment with $A\beta_{1-42}$ oligomers for 6, 12 and 24 h compared with the insulin-treated group ($P < 0.05$) (Fig. 1B), and 24 h of treatment with $A\beta_{1-42}$ oligomers was used in the following experiments. These data suggested that $A\beta_{1-42}$ oligomers inhibited insulin-induced serine phosphorylation of Akt at 473 in astrocytes.

3.2. $A\beta_{1-42}$ oligomers induce ERK1/2-dependent phosphorylation of IRS-1 at serine 636/639 that disturbs subsequent binding of IRS-1 and PI3K

To determine if $A\beta_{1-42}$ oligomers induced phosphorylation of ERK, astrocytes were treated with $A\beta_{1-42}$ oligomers for different time periods (3, 6, 12 and 24 h) and the levels of phosphorylated and total ERK1/2 were determined using western blotting. The results showed that the level of phosphorylated ERK1/2 was peaked after 3 h of treatment with $A\beta_{1-42}$ oligomers ($P < 0.05$), and returned to basal levels within 24 h (Fig. 2A). However, the levels of total ERK1/2 were not affected by $A\beta_{1-42}$ oligomers.

In order to clarify the association between the phosphorylation of ERK1/2 and the phosphorylation of IRS-1 at serine 636/639, the levels of phosphorylated ERK1/2, phosphorylated IRS-1 at serine 636/639