Insulin resistance in Alzheimer’s disease (AD) mouse intestinal macrophages is mediated by activation of JNK

Y.-L. ZHOU1,2, Y.-F. DU1, H. DU1, P. SHAO3

1Department of Neurology, Provincial Hospital Affiliated to Shandong University, Jinan, Shandong, China
2Department of Neurology, Yantai Affiliated Hospital of Binzhou Medical University, Yantai, Shandong, China
3Department of Rehabilitation Medicine, Yantaishan Hospital, Yantai, Shandong, China

Corresponding Author: Yifeng Du, MD; e-mail: duyifeng2013@163.com
Heng Du, MD; e-mail: senyaheng@gmail.com

Abstract. – OBJECTIVE: Alzheimer’s disease (AD) has been considered as a metabolic disorder disease, which closely related to insulin signaling impairment. Therefore, identifying the potential mechanism of insulin resistance is important for AD treatment.

MATERIALS AND METHODS: An APP/PS1 double transgenic AD mouse model was introduced to study insulin resistance in gut. The expressions of AD markers and key elements of insulin signaling were detected in ileum and intestinal macrophages of AD mice by immunohistochemistry. Furthermore, mouse intestinal macrophage cell line RAW264.7 was treated by Aβ25-35 or Aβ25-35 + insulin to explore the mechanism of insulin resistance in vitro. The expression of IR-β and the activation of cell signaling related proteins (Insulin receptor substrate 1 (IRS1), protein kinase B (AKT) and c-Jun N-terminal kinase (JNK)) in Aβ25-35-stimulated macrophages were performed via Western blotting.

RESULTS: The expressions of IRS1, Aβ and Tuj in AD mice ileum were significantly different from WT mice (p<0.05). Also, there were significant discrepancies in the expressions of β2AR and eNOS in intestinal macrophages of two groups (p<0.05). After exposure to Aβ25-35, cell proliferation rate (p<0.01) of macrophage and the levels of TNF-α (p<0.01) and IL-6 (p<0.01) was significantly elevated and treatment with insulin could reverse these changes (p<0.05). The amount of IR-β and the p-AKT/AKT ratio significantly decreased in Aβ25-35-treated macrophages (p<0.01), while the ratios of p-IRS1/IRS1 and p-JNK/JNK significantly enlarged (p<0.01). Furthermore, all the changes caused by Aβ25-35 treatment were attenuated by insulin addition.

CONCLUSIONS: Activation of JNK pathway played an important role in insulin resistance of AD mice, suggesting that inhibition of JNK pathway might be a new strategy toward resolving insulin resistance related diseases, such as AD.

Key Words
Insulin resistance, Alzheimer’s disease, Macrophage, IRS1, JNK.

Introduction
Alzheimer’s disease (AD) is a neurodegenerative disease, occurring in 60% of senile dementia patients. This disease is characterized by the following signs: memory loss and problems with learning, judgment, communication, and daily life. Unfortunately, there is no cure for AD so far. FDA has approved four medications for AD, which aimed to break down acetylcholine or keep the level of glutamate in check. These drugs may slow down how fast symptoms get worse for about half of patients. The effect lasts on average 6 to 12 months. On the other hand, novel drugs screening focus on identifying both the inhibitors of amyloidogenic and the promoters of non-amyloidogenic pathways of amyloid β (Aβ), and clinical trials for the immunotherapies are in progress, detailed clinical studies on patients are yet awaited. Thus, more drug molecule targets and treatment strategies need to be explored based on pathological mechanism of AD. The patients with AD display symptoms that insulin signaling is decreased, glucose metabolism is impaired or hyperinsulinemia. Mounting data supports that AD is substantially one of metabolic syndrome which associated with brain insulin resistance and insulin deficiency. A critical role of insulin resistance in AD is related to Aβ production and aggregation, neurofibrillary tangles (NFTs) formation, synaptic transmission disorder and neuronal degeneration. Thus, effective measures to inhibit insulin resistance may be a novel drug target in AD. Studies indicate that phosphorylation of insulin receptor substrate 1 (IRS1), a key element of insulin signaling, blocks the transduction of insulin signaling in the brain of AD animal models and human autopsy samples. How does IRS1 successfully disturbing the
insulin signaling is still not quite clear. It has been reported that cultured hippocampal neurons with Aβ oligomers leads to high level of IRS1 serine phosphorylation, which is induced by activation of JNK pathway. The result is further verified in primate brain tissues. Interestingly, gut is the biggest immune system and a critical digestive organ in human and animal bodies, but the mechanism of insulin resistance in intestinal tract has not been well established. Besides, intestinal macrophages are main components of adipose tissue and appear to be major sources of inflammatory mediators that are linked to insulin resistance. According to these, this study concentrated on insulin resistance in the intestinal macrophages. Here, insulin resistance in gut was investigated by immunohistochemistry in an APP/PS1 double transgenic AD mouse model. The results suggested that AD mice acquired insulin resistance through a non-insulin receptor impaired way. Furthermore, Aβ_{25-35}, a well-known oligomer could induce insulin resistance in vivo and in vitro, was used to explore the potential mechanism of insulin resistance in AD intestinal macrophage cell line RAW264.7.

## Materials and Methods

### Mice Experiment

Animal experiments were approved by the Institutional Animal Care and Use Committee and were performed in accordance with the guidelines of the Laboratory Animal Science Association. Twenty APP/PS1 double transgenic AD model mice and twenty WT mice were respectively purchased from Jackson Laboratory (Bar Harbor, ME, USA) and Guangdong Medical Laboratory Animal Center (GDMLAC, Shanghai, China). All mice (4-5 mice in each cage) were maintained in specific pathogen-free condition in 20-24°C, 12 h of light with 12 h of darkness every day, with food and water supplied. Adaptive fed for one week.

### Immunohistochemistry Analysis

Immunohistochemistry analysis of murine ileal tissue samples was conducted as previously described with modifications. Briefly, ileal specimens in 20 cm from *Helicobacter pylori* obtained from mice were washed with normal saline and fixed in 4% paraformaldehyde (MACKLIN Biochemical Co, Ltd, Shanghai, China), embedded in paraffin (MACKLIN, Biochemical Co, Ltd, Shanghai, China), and cut into 3-mm sections. Slides were dewaxed and rehydrated. Antigen (Ag) retrieval was subsequently performed with citrate antigen retrieval solution (Yopebio, Shanghai, China). Endogenous peroxidase was abolished by 3% H₂O₂ and slides were blocked with goat serum (Haoranbio, Shanghai, China). Samples on slides were detected by different antibodies (IR, IRS1, Aβ, Tuj, KIBRA, β2AR and eNOS), followed by a Polink-2 plus Polymer HRP Detection System (GBI, Mukilteo, WA, USA), according to the manufacturer’s instructions (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China). The colorimetric reaction was developed by diaminobenzidine (R&D Systems, Minneapolis, MN, USA).

### Cell Culture and Treatment

Mice gut macrophages (RAW264.7) were purchased from Shanghai Institute of Biochemistry (Shanghai, China). Cells were cultured at 37°C in Dulbecco’s modified eagle medium (DMEM) (Shanghai Srkbio Co., Ltd, Shanghai, China) supplemented with 10% fetal calf serum (FCS) (Shanghai Srkbio Co., Ltd, Shanghai, China), 100 units/ml penicillin and 100 μg/ml streptomycin (Shanghai Bioleaf Biotech Co., Ltd, Shanghai, China) in a humid incubator with 5% CO₂. RAW264.7 cells in Aβ group were incubated with different concentrations of Aβ_{25-35} (2, 4 or 6 μmol/L) (Sangon Biotech, Shanghai, China) for 24 h. The cells in Aβ + insulin group were treated with 2 μmol/L Aβ for 24 h. Then, the culture was refused and the cells were exposed to insulin at the final concentration of 200 μmol/L for further 1 h, while the control cells received an equal volume of saline.

### Cell Viability Assay

Cell viability was determined by the conventional 3-(4,5-dimethyl-2-thiazolyl)- 2,5-diphenyl-2-H-tetrazolium bromide (MTT) reduction assay. RAW264.7 cells were plated at a density of 5×10⁴ cells/well in 96-well plates. After the indicated treatments, cells were co-incubated with MTT solution (a final concentration of 0.5 mg/ml) (Fortune Bio-Tech, Shanghai, China) for 4 h. The medium was removed and 150 μl dimethyl sulfoxide (DMSO) was added to each well. The formazan dye crystal was solubilized for 15 min and absorbance was measured at 570 nm using a microplate reader (Bio-Tek, Winooski, VT, USA).

### Inflammatory Cytokines Assay

Cells were harvested and washed three times with ice-cold phosphate buffer solution (PBS) and centrifuged at 1000 rpm for 5 min. The superna-
Insulin resistance in Alzheimer’s disease (AD) was used to measure the levels of TNF-α and IL-6, using Enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer’s protocols (Nanjing Jiancheng Co., Nanjing, China). The plates were determined at 450 nm with microplate reader.

**Western Blotting**

Cells were harvested in lysis buffer with the protease inhibitor cocktail as described previously. Equal amounts of reducing protein were fractionated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Roche, Basel, Switzerland) using the Bio-Rad microassay system (Bio-Rad, Hercules, CA, USA). Immunoreactive bands were visualized by enhanced chemiluminescence (Pierce, Rockford, IL, USA). Densitometric analysis of the film was performed using a HP LaserJet 3055 scanner and Bio-Rad Discovery software (Quantity One) (Bio-Rad, Hercules, CA, USA).

**Statistical Analysis**

Analyses were performed with SPSS19.0 software (IBM, Armonk, NY, USA). Data was representative of at least three independent experiments and presented as means ± standard deviation (SD). The statistical significance of correlations was determined by the Spearman test. The p-values <0.05 were considered statistically significant.

**Results**

**Expression Difference of IR, IRS1, Aβ and Tuj in AD Mice and WT Mice Detected by Immunohistochemistry**

Given the fact that neurofibrillary tangles and Aβ aggregation have been considered as characteristic pathological changes of AD, the expressions of Aβ and Neuron-specific class III beta-tubulin (Tuj), and basic elements of insulin signaling, insulin receptor (IR) and IRS1 in AD mice and WT mice gut were determined using immunohistochemical methods. As shown in Figure 1A, the expressions of IRS1, Aβ and Tuj in AD mice significantly increased (p<0.05 for these determinations, Figure 1B). Amounts of IRS1 and Tuj increased 36.68% and 16.16% respectively, compared with WT mice. Especially the Aβ expression quantity of AD mice is 44.25% higher than that of WT mice. However, there is no obvious difference in IR expression between AD and WT mice. Thus, insulin resistance occurred in AD mice gut might not dependent on the amount change of IR but through other ways.

**Immunohistochemical Detection of Expression Difference of KIBRA, β2AR and eNOS for Gut Macrophages in AD Mice and WT Mice**

Macrophages in gut are main components of adipose tissues and appear to be major sources of inflammatory mediators that are linked to insulin resistance. Therefore, the expressions of protein KIBRA (KIBRA), beta-2 adrenergic receptor (β2AR) and endothelial nitric oxide synthase (eNOS) in gut macrophages were detected. The results (Figure 1A) showed that expressions of β2AR and eNOS in AD mice were much higher than those in WT mice (19.00% and 8.76%, respectively; p<0.05 for these determinations, Figure 1B). The amount of KIBRA was just slightly declined in AD mice compared to WT mice, and there was no statistical significance between these two groups.

**The Effect of Aβ on RAW264.7 Cell Proliferation**

According to the results of the MTT assay (Table I), Aβ promoted RAW264.7 cells proliferation in a dose-dependent manner, which showed significant difference with control group (p<0.01). Insulin significantly decreased cell proliferation rate of RAW264.7 cells (p<0.05).

**The effect of Aβ on Inflammatory Cytokines in RAW264.7 Cells**

As compared with untreated cells, the levels of TNF-α and IL-6 in macrophages were significantly increased in a dose-dependent manner after Aβ25-35 treatment (p<0.01, Figure 2). In insulin-treated groups, TNF-α and IL-6 levels were significantly reduced (p<0.05, Figure 2).

**Western Blotting for the Expressions of IR-β, IRS1, p-IRS1 Ser307, AKT, p-AKT Ser473, JNK and p-JNK Tyr185 in Intestinal Macrophage Cell Line RAW264.7**

The expressions of IR-β, IRS1, p-IRS1 Ser307, AKT, p-AKT Ser473, JNK and p-JNK Tyr185 in macrophages RAW264.7 were determined by Western blotting. As shown in Figure 3, treated RAW264.7 cells with 2 μmol/L (low), 4 μmol/L (mid) or 6 μmol/L (high) of Aβ25-35 could significantly reduce the expression of IR-β, compared to untreated cells (p<0.01).
Figure 1. Immunohistochemical staining of IR, IRS1, Aβ, Tuj, KIBRA, β2AR and eNOS for AD mice and WT mice gut. The ileal specimens in 20 cm from helicobacter pylori obtained from AD and WT mice were isolated, and tissues were detected by immunohistochemistry. The colorimetric reaction was developed by the addition of diaminobenzidine and counterstained with Hematoxylin and Eosin (H&E). A, expressions of IR, IRS1, Aβ, Tuj, KIBRA, β2AR and eNOS in AD and WT mice gut were detected by immunohistochemistry; B, optical density analysis of immunohistochemistry. Data are mean ± SD of three independent experiments. *p<0.05 were considered statistically significant.
Table I. Effect of Aβ on cell proliferation of macrophages RAW264.7 determined by MTT assay.

<table>
<thead>
<tr>
<th>Group</th>
<th>Proliferation rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Control</td>
<td>0.00 ± 0.09</td>
</tr>
<tr>
<td>Aβ (low)</td>
<td>9.29** ± 0.13</td>
</tr>
<tr>
<td>Aβ (mid)</td>
<td>14.33** ± 0.16</td>
</tr>
<tr>
<td>Aβ (high)</td>
<td>18.13** ± 0.14</td>
</tr>
<tr>
<td>Aβ+insulin</td>
<td>6.25# ± 0.13</td>
</tr>
</tbody>
</table>

Note: Control, untreated cells; Aβ (low, mid, high), cells treated with Aβ25-35 of 2 μmol/L, 4 μmol/L and 6 μmol/L respectively; Aβ + insulin, cells firstly treated with 2 μmol/L Aβ25-35 for 24 h, then treated with 100 μmol/L insulin for 1 h. Data are mean ± SD of three independent experiments. **p<0.01 vs. control group, #p<0.05; ##p<0.01 vs. Aβ (low) group.

Figure 2. Effect of Aβ on the inflammatory cytokines in macrophages RAW264.7. A, TNF-α level; B, IL-6 level. Control, untreated cells; Aβ (low, mid, high), cells treated with Aβ25-35 of 2 μmol/L, 4 μmol/L and 6 μmol/L respectively; Aβ + insulin, cells firstly treated with 2 μmol/L Aβ25-35 for 24 h, then treated with 100 μmol/L insulin for 1 h. Data are mean ± SD of three independent experiments. **p<0.01 vs. control group, #p<0.05; ##p<0.01 vs. Aβ (low) group.

Figure 3. Effect of Aβ25-35 alone, Aβ25-35 followed by insulin on the expressions of IR-β, IRS1, p-IRS1 Ser307, AKT, p-AKT Ser473, JNK and p-JNK Tyr185 of Macrophages RAW264.7. Control, untreated cells; Aβ (low, mid, high), cells treated with Aβ25-35 of 2 μmol/L, 4 μmol/L and 6 μmol/L respectively; Aβ + insulin, cells firstly treated with 2 μmol/L Aβ25-35 for 24 h, then treated with 100 μmol/L insulin for 1 h. Data are mean ± SD of three independent experiments. *p<0.05 and **p<0.01 were considered statistically significant between control group and Aβ group; #p<0.05 and ##p<0.01 were considered statistically significant between Aβ + insulin group and Aβ (low) group.
After 24 h treatment of 2 μmol/L Aβ25-35, 100 μmol/L insulin was added to macrophages for 1 h. The expression of IR-β increased significantly (p<0.05, compared to 2 μmol/L Aβ25-35 treatment). Also, Aβ25-35 decreased the ratio of p-AKT/AKT (p<0.01), while the ratio increased when cells treated by insulin following 2 μmol/L Aβ25-35 (p<0.05). The ratio of p-IRS1/IRS1 and p-JNK/JNK also could be significantly increased by Aβ25-35 treatment (p<0.01), but the phosphorylation of IRS1 and JNK caused by Aβ25-35 treatment were attenuated by sequential insulin treatment (p<0.01).

Discussion

Studies have revealed that multifarious mechanisms contribute to the onset and advance of Alzheimer’s disease. Growing data demonstrate that the aggregation of the Aβ plays a necessary role in the onset of AD, especially in the preclinical phase of AD. In the central nervous system, Aβ has been reported to impair neuronal synaptic function in early AD by compromising insulin signaling. In this study, an APP/PS1 double transgenic AD mouse model was introduced to study insulin resistance in gut, with no transgenic wild mice as control. The expressions of IRS1, Aβ and Tuj were significantly increased in AD mice ileum, though IR expression displayed a similar level between AD and WT mice. The results suggested that Aβ aggregated in ileum of AD mice, and AD mice ileum exhibited insulin resistance. Also, there was a significant up-regulation of β2AR and eNOS in AD mice intestinal macrophages, demonstrating that the insulin resistance and sequential Aβ aggregation in intestinal tract of AD mice were related to those two proteins. Abnormal activation of β2AR contributes to Aβ accumulation in AD pathogenesis. Autopsy results of human brain indicate that the expression of eNOS increases in AD patient cerebral microvascular. Besides, high nitration level of serine has been found in AD patient neuron, suggesting that the expressions and activities of NOS and NO are up-regulated. Our results, expressions of β2AR and eNOS, were up-regulated in AD mouse, and were consistent with these studies. In vitro, Aβ promoted RAW264.7 cells proliferation and insulin showed an excellent anti-proliferation effect on RAW264.7 cells. To reveal the potential mechanism of insulin resistance in AD mice intestinal macrophages, Aβ25-35 was added to mouse intestinal macrophages RAW264.7. The amount of IR-β and the p-AKT/AKT ratio dramatically decreased, while the ratios of p-IRS1/IRS1 and p-JNK/JNK were significantly enhanced. All the changes caused by Aβ25-35 treatment were attenuated by addition of insulin. In insulin signaling, phosphorylation of IRSs proteins leads to the activation of an important signaling pathway: the phosphatidilinositol 3-kinase-AKT/protein kinase B (PI3K-AKT/PKB) pathway, which is responsible for most of the metabolic actions of insulin. The phosphorylated-AKT/total AKT (p-AKT/AKT) ratio decline in this study indicated that activation of AKT was at least partially blocked. Various inflammatory factors, such as TNF-α and IL-6 abnormally activate IRS1 by phosphorylating serine site, lead to decrease of PI3K activity. Our results suggested that Aβ oligomer might induce insulin resistance through abnormal activation of IRS1, subsequently decline of AKT pathway. c-Jun N-terminal kinase 1 (JNK1) phosphorylates the adapter protein IRS1 at an inhibitory site that can block signal transduction by the insulin receptor. Jun N terminal kinase 1 (JNK1) may, therefore, directly induce insulin resistance. JNK1 may act in hematopoietic cells to regulate the expression of cytokines that can influence insulin sensitivity. Thus, abnormal activation of JNK pathway might play an important role in the onset and progress of insulin resistance in AD. Indeed myeloid cells, including macrophages, may be critical. “Metabolic syndrome” or “insulin resistance syndrome”, are terms for a still evolving disorder that clusters insulin resistance, impaired glucose regulation, dyslipidemia, abdominal obesity, and hypertension with risk for cardiovascular disease and T2DM. There is growing recognition that insulin resistance, T2DM and other features of the metabolic syndrome are also associated with brain disorders including AD and other neurodegenerative dementias. Researchers consider that diabetes, the most common outcome of insulin resistance, is onset at intestinal mucus. Together, further studies on intestinal macrophages about insulin resistance would supply more information to AD and other insulin-linked diseases research and treatment strategies.

Conclusions

This work investigated insulin resistance on intestinal macrophages in an APP/PS1 double transgenic mouse model. The potential mechanism of that was discussed in vitro by Aβ25-35 oligomers-stimulated intestinal macrophages. It turned out that activation of JNK pathway played...
an important role in insulin resistance of AD mice, which might disturb insulin signaling by abnormally phosphorylating IRS1. This study suggests that inhibition of JNK pathway might be a new strategy toward resolving insulin resistance related diseases, such as AD.

Conflict of Interest

The authors declare no conflicts of interest.

References