Pioglitazone Ameliorates Intracerebral Insulin Resistance and Tau-protein Hyperphosphorylation in Rats with Type 2 Diabetes

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Key words
- Alzheimer’s disease
- dementia
- glucose metabolism
- anti-diabetic drug

Abstract

Objective: To investigate intracerebral insulin resistance and its relationship with tau-protein hyperphosphorylation.

Methods: A rat model of type 2 diabetes (T2D) was established with streptozotocin (STZ). Diabetic rats received intragastric administration of pioglitazone (PIO group) or normal saline (T2D group) for 4 weeks. As a control, non-diabetic rats received intragastric normal saline (CTL group). The insulin concentrations in cerebrospinal fluid (CSF) and blood were determined with radioimmunoassay, and blood glucose concentration was determined using a glucose oxidation technique. Total and phosphorylated levels of protein kinase B (AKT), glycogen synthase kinase-3β (GSK-3β) and tau-protein in the hippocampus were analyzed using western blotting.

Results: The plasma insulin level in the T2D group was higher, and the CSF insulin level in the T2D group lower than in the CTL group. Hippocampal phosphorylated AKT and phosphorylated GSK-3β levels were significantly lower in the T2D group than in the CTL group. Hippocampal tau-protein in the T2D group was hyperphosphorylated at Ser199 and Ser396. Plasma insulin levels in the PIO group were lower than in the T2D group, with no differences in CSF insulin levels. Phosphorylated AKT and phosphorylated GSK-3β levels in the PIO group were significantly higher than in the T2D group. Hippocampal phosphorylated tau-protein (Ser199/Ser396) was lower in the PIO group than in the T2D group.

Conclusion: Hyperphosphorylation of tau-protein in pioglitazone-treated rats with T2D was improved. Rats with T2D have both cerebral insulin resistance and cerebral hypoinsulinism. Pioglitazone can ameliorate intracerebral insulin resistance and decrease tau-protein hyperphosphorylation, but cannot increase intracerebral insulin levels.

Introduction

Diabetes is the most common endocrine-metabolic disease, and the prevalence of type 2 diabetes (T2D) in China has now reached levels of 9.7% in people aged more than 20 years old [1]. With improvements in therapy, the lifespans of individuals with diabetes have been prolonged, such that cognitive disorders and dementia (Alzheimer’s disease, AD) have become more common, important complications. Indeed, epidemiologic data suggest that the risk of developing dementia is 1.4–4.3 times higher in people with T2D than in non-diabetic individuals [2–4]. The main clinical characteristic of AD is a progressive impairment of memory. The major pathologic features of AD are intraneuronal neurofibrillary tangles, comprised of paired helical filaments (PHF), that arise as the consequence of hyperphosphorylation of a microtubule-associ-
Insulin has been reported to enhance memory in rats, tested in a passive-avoidance task experiment [10]. Furthermore, insulin deficiency exacerbates cerebral amyloidosis and behavioral deficits in a transgenic mouse model of AD [11]. Together, these studies suggest that brain insulin deficiency is one of the risk factors for AD. However, non-diabetic patients with AD have been reported to show intracerebral insulin resistance [12]. Intracerebral insulin resistance is analogous to the situation of peripheral insulin resistance, and reflects reduced actions of insulin in the central nervous system [13,14]. Decreased activation of intracerebral insulin signaling cascades leads to the impairment of the normal functions of insulin in neurons; these functions include regulation of glucose metabolism, neural growth, survival and remodeling, and microtubule assembly. Interestingly, Li et al. (2011) have identified 8 proteins that are differentially expressed in type 1 diabetes mellitus, and have suggested that these may be involved in the pathogenesis of diabetic encephalopathy [15].

T2D is characterized by insulin resistance, and is associated with brain tau-protein hyperphosphorylation and increased activity of glycogen synthase kinase-3β (GSK-3β), a key kinase of tau-protein [16,17]. GSK-3β lies downstream of phosphatidylinositol-3-kinase (PI3K) and protein kinase B (AKT) in the insulin signal transduction cascade, and its activity is depressed by PI3K/AKT. Normal insulin signaling excites PI3K/AKT and suppresses GSK-3β, thereby inhibiting tau-protein hyperphosphorylation. It is possible that there is not only peripheral but also intracerebral insulin resistance in T2D, leading to decreased activity of PI3K/AKT, increased activity of GSK-3β and tau-protein hyperphosphorylation. One study has reported that pioglitazone, a thiazolidinedione anti-diabetic drug, can improve the cognitive function of patients with T2D and mild AD [18]. Furthermore, in a rat model of T2D, Kanazawa et al. (2011) reported that pioglitazone was able to reduce serum concentrations of pentosidine, a rat model of T2D, Kanazawa et al. (2011) reported that pioglitazone was able to reduce serum concentrations of pentosidine, a derivative of the Maillard reaction, as previously described [21].

**Materials and Methods**

**Experimental parameters measured**

**Common parameters**

**Plasma glucose:** Plasma glucose was determined using the glucose oxidation method (One Touch, Ultra Easy Glucometer, LifeScan), as previously described [21].

**Plasma insulin:** Plasma insulin was determined using the glucose oxidation method (One Touch, Ultra Easy Glucometer, LifeScan), as previously described [21].

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tein extraction solution (40 mmol/L Tris-HCl, pH 7.0, 1% Triton X-100, 0.2% sodium dodecyl sulfate [SDS], 1.0 mmol/L sodium deoxycholate, 1.0 mmol/L Na3VO4, 50 mmol/L NaF, 1.0 mmol/L phenylmethylsulfonyl fluoride [PMSF], 2.0 mg/L aprotinin, 2.0 mg/L leupeptin, 2.0 mg/L pepstatin, 1.0 mmol/L ethylene glycol tetraacetic acid [EGTA] and 1.0 mmol/L ethylene diamine tetraacetic acid [EDTA]). The supernatant was taken to determine protein concentration using the Bradford method, and the remainder was stored at −80°C for later use in western blot experiments. For western blot experiments, the frozen sample was thawed, mixed with 2× buffer solution and denatured at 100°C for 5 min. 10–30 μg protein was added into each lane of a vertical electrophoresis chamber for electrophoresis on a 10% SDS polyacrylamide gel. After electrophoresis, protein was transferred onto a nitrocellulose (NC) membrane, shaken with 5% bovine serum albumin (BSA) for 2 h, and then hybridized overnight (at 4°C) with the primary antibody (all antibodies used are listed in Table 2). The NC membrane was subsequently washed 3 times with phosphate buffered saline with Tween-20 (PBST), 10 min for each wash, and then hybridized with horseradish peroxidase-labeled secondary antibody (goat anti-rabbit IgG, goat anti-mouse IgG or rabbit anti-goat IgG, as appropriate; antibodies purchased from Pierce Co.) for 1 h, with shaking. Following this, the membrane was washed 3 times with PBST (10 min for each wash), and the bands visualized with enhanced chemiluminescence (ECL) and developed on film. Immunoreactive bands were quantitatively analyzed using BandScan v5.0 software.

Statistical analysis

Data were analyzed using the Prism 5.0 software package. Measured data are expressed as means ± standard deviations (SD). Comparison of the means between groups was carried out using analysis of variance (ANOVA) with a Bonferroni post-hoc test. A value of P < 0.05 was taken to be indicative of statistical significance.

Results

Blood glucose, insulin, insulin resistance index and CSF insulin levels in the 3 groups of rats

As shown in Table 1, the plasma glucose level and the plasma insulin level in the T2D group were both significantly higher than the corresponding values in the CTL group, whereas the CSF insulin level in the T2D group was significantly lower than that in the CTL group. The plasma insulin and glucose levels in the PIO group were significantly lower than the corresponding values in the T2D group, but not significantly different from those in the CTL group. The CSF insulin level in the PIO group was not significantly different from that in the T2D group, but was significantly lower than that in the CTL group. HOMA-IR in the T2D group was significantly higher than that in the CTL group, while HOMA-IR in the PIO group was significantly lower than that in the T2D group, and not significantly different from that in the CTL group (Table 1).

Hippocampal levels of total and phosphorylated AKT/GSK3β and tau-protein

As shown in Fig. 1 and Fig. 2, hippocampal levels of total AKT, total GSK-3β and total tau-protein in the T2D group were not significantly different from the corresponding values in the CTL group. However, the level of AKT phosphorylated at amino acid residue Thr308 in the T2D group was significantly lower than that in the CTL group. In addition, the level of GSK-3β phosphorylated at amino acid residue Ser9 in the T2D group was significantly lower than that in the CTL group, while the level of tau-protein phosphorylated at amino acid residues Ser199/Ser396 in the T2D group was significantly higher than that in the CTL group.

Hippocampal levels of total AKT, total GSK-3β and total tau-protein in the PIO group were not significantly different from the corresponding values in the T2D group. However, the levels of Thr308-phosphorylated AKT and Ser9-phosphorylated GSK-3β in the PIO group were significantly higher than those in the T2D group, while the level of Ser199/Ser396-phosphorylated tau-

<table>
<thead>
<tr>
<th>Group</th>
<th>Type 2 diabetes group (T2D)</th>
<th>Control group (CTL)</th>
<th>Pioglitazone group (PIO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>5</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Diet</td>
<td>High glucose, high fat, high protein diet</td>
<td>Normal diet</td>
<td>High glucose, high fat, high protein diet</td>
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<tr>
<td>STZ injection</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Weight before sacrifice (kg)</td>
<td>436.8 ± 11.82***</td>
<td>314.4 ± 4.45</td>
<td>444.6 ± 6.87***</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>21.5 ± 7.13***</td>
<td>6.54 ± 1.35</td>
<td>8.84 ± 0.73***</td>
</tr>
<tr>
<td>Plasma insulin (mlU/l)</td>
<td>27.54 ± 2.32***</td>
<td>9.78 ± 1.37</td>
<td>12.36 ± 2.48***</td>
</tr>
<tr>
<td>CSF insulin (mlU/l)</td>
<td>1.18 ± 0.78</td>
<td>2.82 ± 0.24</td>
<td>1.14 ± 0.42</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>28.8 ± 7.01***</td>
<td>2.76 ± 0.34</td>
<td>4.07 ± 2.11***</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD. Statistical comparisons were made using ANOVA with a Bonferroni post-hoc test. *P < 0.05 and ***P < 0.001 for the T2D or PIO group vs. the CTL group; ###P < 0.001 for the PIO group vs. the T2D group.

Table 1 Characteristics of the rats in the 3 experimental groups.

Table 2 Antibodies employed in this study.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Specificity</th>
<th>Phosphorylation sites</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSK-3β</td>
<td>Poly</td>
<td>Total GSK-3β</td>
<td>Biovision, Mountain View, CA</td>
<td></td>
</tr>
<tr>
<td>p-GSK-3β</td>
<td>Poly</td>
<td>p-GSK-3β</td>
<td>Cell Signaling, Danvers, MA</td>
<td></td>
</tr>
<tr>
<td>AKT (ab8805)</td>
<td>Poly</td>
<td>Akt</td>
<td>Abcam, Cambridge, MA</td>
<td></td>
</tr>
<tr>
<td>p-AKT (ab38449)</td>
<td>Poly</td>
<td>p-AKT</td>
<td>Abcam, Cambridge, MA</td>
<td></td>
</tr>
<tr>
<td>H-71</td>
<td>Poly</td>
<td>α fragment of insulin receptor</td>
<td>Santa Cruz, Santa Cruz, CA</td>
<td></td>
</tr>
<tr>
<td>H-70</td>
<td>Poly</td>
<td>β fragment of insulin receptor</td>
<td>Santa Cruz, Santa Cruz, CA</td>
<td></td>
</tr>
<tr>
<td>Actin (I-19)</td>
<td>Poly</td>
<td>β-actin</td>
<td>Santa Cruz, Santa Cruz, CA</td>
<td></td>
</tr>
</tbody>
</table>

Table 2 Antibodies employed in this study.
protein in the PIO group was significantly lower than that in the T2D group.

Discussion

Insulin has different effects in the brain to those it has in peripheral tissues, but as in the periphery, it exerts effects through actions at the insulin receptor (IR) [24]. After insulin binds with the IR, the IR is autophosphorylated at tyrosine residues, leading to a signaling cascade that involves phosphorylation of numerous intracellular targets, including insulin receptor substrate (IRS), PI3K/AKT (activation) and GSK-3β (depression). The outcome of insulin binding to the IR is the promotion of insulin-sensitive GLUT (glucose transporter) translocation and a series of physiologic processes in favor of normal neural function, including inhibition of tau-protein hyperphosphorylation and Aβ accumulation, both of which are risk factors for AD.

Our previous study suggested that rats with either type 1 or type 2 diabetes demonstrated increased hippocampal GSK-3β activity and tau-protein hyperphosphorylation [25]. This indicated that both insulin deficiency and insulin resistance are associated with tau-protein hyperphosphorylation. However, it was not clear whether the intracerebral insulin level was altered in rats with type 2 diabetes, and whether an insulin sensitizer could normalize the intracerebral insulin level and insulin signal transduction pathway, and thus decrease tau-protein hyperphosphorylation.

In the present study, we found that rats with T2D showed decreased CSF insulin levels, decreased hippocampal AKT activity, increased GSK-3β activity and hyperphosphorylation of tau-protein. Taken together, these data suggest that the brains of rats with type 2 diabetes show both insulin resistance and insulin deficiency, resulting in tau-protein hyperphosphorylation. Since brain insulin is derived from peripheral blood, its deficiency may be the result of disruption of the BBB by chronic hyperglycemia.

Pioglitazone is an oral anti-diabetic agent that acts primarily by decreasing insulin resistance through its action at PPARγ receptors in tissues such as adipose tissue, skeletal muscle and the liver. Activation of PPARγ nuclear receptors modulates the transcription of a number of insulin responsive genes involved in the control of glucose and lipid metabolism [26]. It was interesting to note that, in our study, treatment with pioglitazone resulted in a reduction in the plasma insulin concentration. We speculate that the pioglitazone-mediated improvement in the insulin sensitivity led to a restoration of plasma glucose to normal (control) levels that, in turn, resulted in a fall in plasma insulin to a level similar to that of the control. This is consistent with previous studies reporting that pioglitazone was able to reduce fasting plasma insulin levels, both in rats fed a high-fat diet [27], and in patients with AD [18] or T2D [28–30]. After pioglitazone treatment, the CSF insulin level was not altered in rats with T2D, but hippocampal AKT activity was increased, GSK-3β activity was decreased, and tau-protein hyperphosphorylation was reduced. This implies that an insulin sensitizer can ameliorate intracerebral insulin resistance and reduce tau-protein hyperphosphorylation, despite a lack of effect on the CSF insulin level.

Acknowledgments

The study was supported by the Natural Science Foundation of China (NSFC) (81100582), China International Medical Foundation (CIMF) – Novo Nordisk China Diabetes Young Scientific Talent Research Funding (2012041), and the General Project of Hubei Province Health Department (JX5B04).
Conflict of interest: None.

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