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.

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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-associated protein (tau-protein), and senile plaques that result from the accumulation of amyloid β (Aβ) [5]. Tau-protein hyperphosphorylation is associated with brain insulin deficiency or disorders of insulin signal transduction [6].

Unlike its action in peripheral tissues, insulin has only limited effects on glucose metabolism in the central nervous system. However, insulin does have important effects on neuronal function, including neurotransmitter release and reuptake, neuronal synaptic plasticity, learning and memory [7]. Studies have suggested that plasma insulin levels in patients with AD are normal, but that cerebrospinal fluid (CSF) insulin levels are decreased [8]. This is thought to be due to an impairment of insulin transport across the blood-brain-barrier (BBB) of patients with AD, since it is known that intracerebral insulin is derived from circulating blood [9]. Consistent with this, intracerebroventricular injection of

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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. If this were the case, thiazolidinediones (agonists of the peroxisome proliferator-activated receptor-γ [PPARγ] that act as insulin sensitizers) would be expected to alleviate intracerebral insulin resistance 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, consistent with a decrease in the levels of advanced glycation end products that are associated with impaired insulin function [19]. However, it is currently not clear whether CSF insulin levels in rats with T2D are decreased in a manner similar to that seen in patients with AD.

In this study, we have established a model of T2D in rats, and investigated the effects of pioglitazone on plasma and CSF insulin levels, AKT/GSK-3β activity in the insulin signal transduction cascade, and phosphorylated levels of tau-protein.

Materials and Methods

Rat model of T2D

Establishing T2D

21 male Sprague-Dawley (SD) rats, weighing 150–180 g and aged 10-12 weeks, were purchased from the Experimental Center of Tongji Medical College, Huazhong University of Science and Technology, China. Rats were assigned randomly to one of 3 groups: control group (CTL, n = 7), untreated T2D group (T2D, n = 7) and pioglitazone-treated T2D group (PIO, n = 7). Rats in the T2D and PIO groups were fed with high calorie food (calorie percentage: carbohydrate 26.0 %, protein 15.2 %, fat [refined lard] 58.8 %), while those in the CTL group were fed on a standard diet. After 3 months of feeding, rats in the T2D and PIO groups received intraperitoneal injection of streptozotocin 30–35 mg/kg (Sigma; streptozotocin powder dissolved in 0.1 mol/L citrate buffer solution, pH 4.3), while rats in the CTL group received intraperitoneal injection of 0.1 mol/L citrate buffer solution. 72 hours later, blood samples were drawn from the caudal vein. Obtaining rats with a plasma glucose level ≥ 16.7 mmol/L was considered to be indicative of successful establishment of the T2D model. All rats in the T2D and PIO groups had plasma glucose levels ≥ 16.7 mmol/L.

Treatment with pioglitazone

Rats in the PIO group were intragastrically administered pioglitazone 20 mg/kg (Takeda Pharmaceutical Co.) for 4 weeks, while those in the T2D and CTL groups were intragastrically administered normal saline for 4 weeks. The dosage of pioglitazone chosen was based on that used in previous studies [20]. 3 days after the end of this 4-week administration period, the surviving rats were sacrificed by cervical dislocation; survival was 7/7 in the CTL group (100 %), 5/7 in the TD2 group (71.4 %) and 6/7 in the PIO group (85.7 %). Throughout the experimental period, all rats were housed in single cages at a constant temperature (25 °C) in a clean animal house. Illumination was provided for 12 h every day; food was provided in the evening, and water was available *ad libitum*.

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: Prior to sacrifice of the animal, a 1 mL blood sample was withdrawn from the heart of each rat, and stored at −20 °C after centrifugation. Blood insulin was determined with a radioimmunoassay (RIA) kit purchased from the Beijing Atomic Energy Research Institute, Beijing, China, using a DFM 96-type multi-tube radioimmunoassay counter (ZhongCheng Mechanical and Electronic Technology Development Co., Ltd, Hefei City, Anhui Province, China). The intra-array coefficient of variation was < 2.5 %, while the inter-array coefficient of variation was < 3.5 %.

Index of insulin resistance

Insulin resistance was expressed as the homeostasis model assessment–insulin resistance (HOMA-IR):

$$\text{HOMA-IR} = \text{insulin(mIU/L)} \times \text{glucose(mmol/L)}/22.5 \text{ [22].}$$

Determination of CSF insulin

CSF insulin level was determined according to the method of Hoistad et al. [23]. Rats were anesthetized with 20 % ethyl carbamate (urethane). The skin of the head was cut away, and the subcutaneous tissue dissected to unmask the cerebellum and medulla oblongata. The meninges were tapped with the needle of a syringe, through which a drainage catheter was inserted, and 20–50 µL CSF was collected from each rat.

Determination of hippocampal AKT/GSK3β and tau-protein levels

After the rat had been sacrificed, the hippocampus was removed and homogenized on ice (12 000 × g for 10 min at 4 °C) in a pro-

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

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.

Antibody	Type	Specificity	Phosphorylation sites	Reference/Source
GSK-3β	Poly	Total GSK-3β		Biovision, Mountain View, CA
p-GSK-3β	Poly	p-GSK-3β	Ser9	Cell Signaling, Danvers, MA
AKT (ab8805)	Poly	Total AKT		Abcam, Cambridge, MA
p-AKT (ab38449)	Poly	p-AKT	Thr308	Abcam, Cambridge, MA
H-71	Poly	α fragment of insulin receptor		Santa Cruz, Santa Cruz, CA
H-70	Poly	β fragment of insulin receptor		Santa Cruz, Santa Cruz, CA
Actin (I-19)	Poly	β-actin		Santa Cruz, Santa Cruz, CA

Table 2 Antibodies employed in this study.

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 Na_3VO_4, 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-

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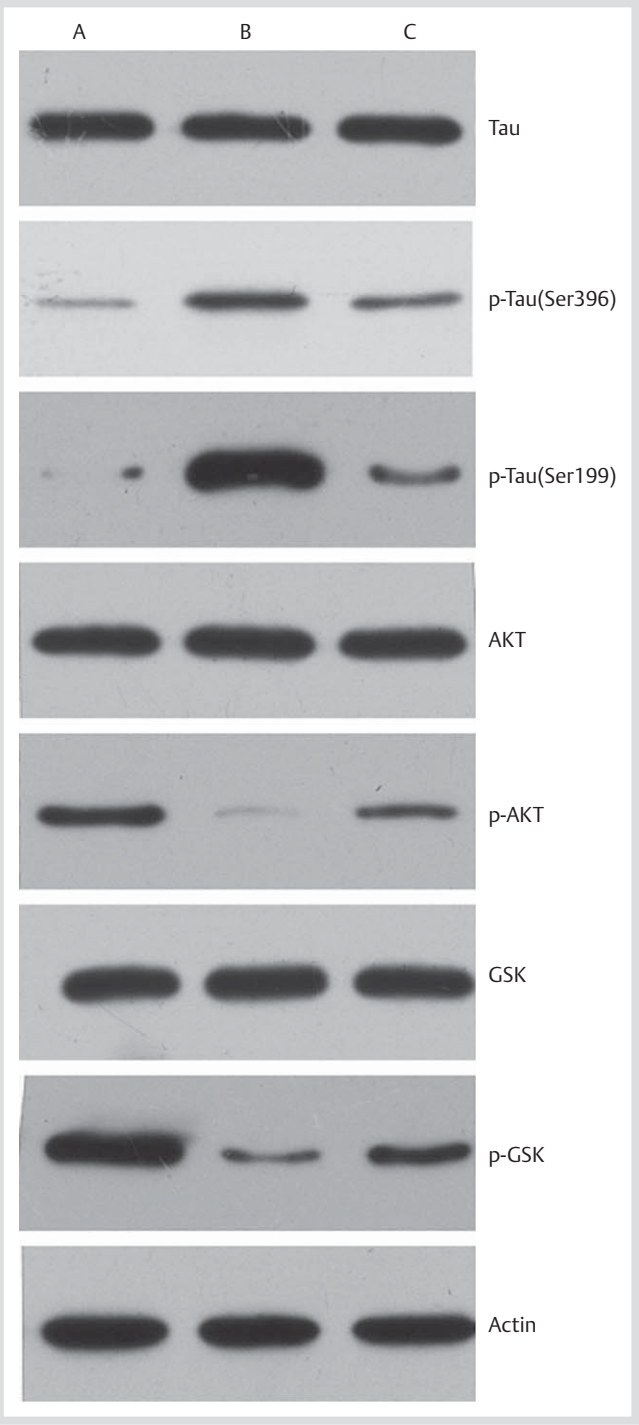

Fig. 1 Western blot analyses of tau-protein, AKT and GSK. Crude hippocampal extracts (10–30 µg/lane) were analyzed using the western blot technique. An actin blot was included as a loading control. A, CTL group; B, T2D group; C, PIO group.

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.

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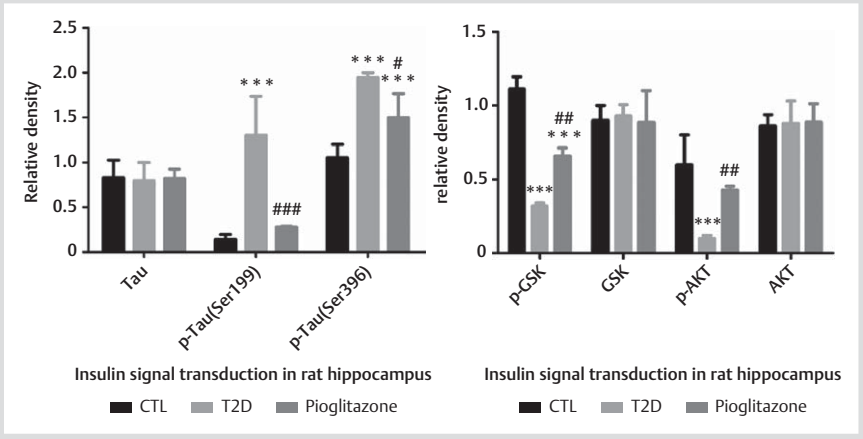

Fig. 2 Western blot analyses of kinases, in the rat hippocampus, that are involved in the insulin signal transduction pathway. Blots such as those shown in **Fig. 1** were quantitated densitometrically. All data are presented as the means ± SDs of the relative immunoreactivities. $^{***}P<0.001$ for the T2D or PIO group vs. the CTL group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ for the PIO group vs. the T2D group.

Conflict of interest: None.

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