Int J Clin Exp Med 2017;10(11):15658-15665
www.ijcem.com /ISSN:1940-5901/IJCEM0054307

Original Article

Jinlida ultrafine powder inhibits IRS-1-Akt-mTOR cascade and promotes expression of glycogen synthase in HL7702 cells

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Received March 31, 2017; Accepted August 13, 2017; Epub November 15, 2017; Published November 30, 2017

Abstract: *Objective:* To study the effects of the traditional Chinese medicine Jinlida on the insulin signalling pathway of hepatocytes. *Methods:* HL7702 cells at the logarithmic growth phase were treated with different concentrations of Jinlida (0.1 mg/ml, 0.2 mg/ml, 1 mg/ml, 2 mg/ml) for 24 h. The control group was treated with media. Total protein and phosphorylation levels involved in the insulin signalling pathway were detected by Western blot. *Results:* Compared with the control group, there was no statistically significant elevation in the phosphorylation of IRS-1 (Ser 307 and Ser 1101). However, the level of p-Akt (Thr 308 and Ser 473), p-GSK3α (glycogen synthase kinase, Ser 21), p-GSK3β (glycogen synthase kinase, Ser 9) and p-S6K1 (Thr 389) had a concentration-dependent decrease. In addition, total amounts of glycogen synthase protein as well as the phosphorylation level of Ser 641 had a concentration-dependent increase. *Conclusion:* Jinlida may ameliorate lipid metabolism disorder by inhibiting the activity of Akt and increasing the expression of glycogen synthase so as to improve glycogen synthesis.

Keywords: Jinlida ultrafine powder, type 2 diabetes, Akt, glycogen synthase

Introduction

Hyperlipidaemia is a major risk factor for the occurrence of type 2 diabetes. In lipid metabolism disorders, the adipose tissue releases large amounts of free fatty acids (FFA) into circulation, depositing them in the form of triglycerides (TGs) in the liver, skeletal muscles and pancreatic islet β-cells [1]. In skeletal muscles, the metabolites of TG such as diacylglycerol (DAG) and ceramides activate protein kinase Cs (PKCs). PKCs catalyse the phosphorylation at the Ser 307 residue and reduce the phosphorylation at the tyrosine residue of insulin receptor substrate-1 (IRS-1), thereby disrupting the normal insulin signalling pathway [2]. FFA have similar effects on hepatocytes. Moreover, the metabolites provide resources for gluconeogenesis [3]. The elevation of FFA can also promote the secretion of insulin and reduce insulin clearance in the liver, thus resulting in hyperinsulinemia [4, 5]. Prolonged hyperinsulinemia can further elevate the phosphorylation level of IRS-1 serine/threonine residues through the PI3K-Akt-S6K1 negative feedback loop. The activation of this negative feedback loop not only inhibits the transduction of this signalling pathway but also promotes the degradation of IRS-1, gradually leading to insulin resistance [6]. In addition, a large number of studies have found that prolonged high levels of FFA can lead to islet β-cell apoptosis and necrosis because of their toxic effects [7, 8].

The traditional Chinese medicine Jinlida consists of seventeen herbs, such as Rhizoma Polygonati, Rhizoma Atractylodis, Radix Rehmanniae, Radix Ophiopogonis, Poria, Rhizoma Coptidis, Radix Salviae Miltiorrhizae, and Radix Puerariae. Jinlida has been widely used in the treatment of type 2 diabetes in China. Clinical studies have demonstrated that Jinlida can reduce plasma TG levels, ameliorate insulin resistance and decrease urinary protein [9]. Additionally, animal experiments using rats have supported its effects on the reduction of TG accumulation in skeletal muscles and islet β-cells [10, 11] and the alleviation of hepatic oxidative stress [12]. Nevertheless, the underlying molecular mechanisms remain unclear.

Liver plays a main role in lipid metabolism. PI3K-Akt-mTORC signalling pathway is the molecular basis for this function. Inhibiting this pathway reduces lipid accumulation in hepatocytes and prevents steatosis [13, 14]. In this study, we choose the normal human liver cell line HL7702 to investigate the direct effects of Jinlida ultrafine powder on hepatocytes and to explore the underlying mechanisms of its function in reducing plasma TG levels.

Materials and methods

Reagents

Jinlida ultrafine powder was purchased from Yiling Pharmaceutical Co. Ltd. (Shijiazhuang, Hebei, China). The primary antibodies used were as follows: anti-Phospho-IRS-1 (Ser 307) rabbit mAb, anti-Phospho-IRS-1 (Ser 1101) rabbit mAb, anti-IRS-1 rabbit Ab, anti-Phospho-Akt (Thr 308) rabbit mAb, anti-phospho-Akt (Ser 473) rabbit Ab, anti-Akt rabbit Ab, anti-Phospho-GSK-3α (Ser 21) rabbit mAb, anti-GSK-3α rabbit Ab, anti-Phospho-GSK-3β (Ser 9) rabbit mAb, anti-GSK-3β rabbit Ab, anti-Phospho-glycogen synthase (Ser 641) rabbit Ab, anti-Glycogen Synthase (15B1) Rabbit mAb, anti-Phospho-S6K1 (Thr 389) rabbit mAb, (Cell Signalling Technology), anti-actin mouse mAb (Abcam), anti-S6K1 rabbit Ab (Millipore). The secondary antibodies for Western blot HRP-linked anti-rabbit/mouse IgG (Cell Signalling Technology).

Stock solution preparation

The main chemical constituents in Jinlida ultrafine powder was identified by Ultra performance liquid chromatography (UPLC) fingerprint. Ginsenoside Rb2, ginsenosideRc, ginsenoside Rb1, icariin, epimedin C, epimedin B, salvianolic acid B, sodium dashensu and puerarin were the quality control markers [11, 15]. The appropriate amounts of Jinlida experimental ultrafine powder were dissolved in serum-free RPMI1640 media (Gibco, USA) to prepare 38 mg/ml Jinlida ultrafine powder stock solution. The stock solution was stored at -20°C.

Cell culture and treatment

The human normal liver cell line HL7702 (purchased from the Cell Bank of the Chinese Academic of Science) was cultured in RPMI 1640 media (Gibco) supplemented with 10% new-born bovine serum (Zhejiang Tianhang Biotechnology) at 37°C in a humidified atmosphere containing 5% CO_2. For treatment, cells at the logarithmic phase were allowed to attach and grow for 24 h before the media were substituted with fresh media containing different concentrations of Jinlida ultrafine powder (0.1 mg/ml, 0.2 mg/ml, 1 mg/ml, 2 mg/ml). The control group was treated with fresh culture media.

Western blot analysis

For Western blot, cells in each group were lysed with RIPA buffer (50 mM Tris (pH 7.4), 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, protease inhibitors) for extracting total protein. The protein concentration was quantified by BCA assay. Equal amounts of protein from each sample were heated in boiling water for 5 min. Subsequently, the denatured proteins were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose (NC) membranes. Then, the membranes were blocked with TBST containing 5% fat-free milk for 3 h, followed by incubating with the corresponding primary antibodies overnight at 4°C. The membranes were subsequently washed with TBST three times for 5 min and incubated with secondary antibodies for 1 h at room temperature. The target bands were analysed by the chemiluminescence imaging system and quantified by ImageJ software.

Statistical analysis

One-way ANOVA was applied to analyze date. The analysis was performed by SPSS (17.0).The data were shown as the mean ± SD from three independent experiments (n = 3). $P<0.05$ was defined as statistically significant.

Results

Jinlida ultrafine powder decreases the phosphorylation of Akt

After being exposed to various concentrations of Jinlida ultrafine powder for 24 hours, the phosphorylation at the Ser/Thr sites of Akt in HL7702 cells were detected. As shown in **Figure 1**, the phosphorylation of Akt at Thr 308 and Ser 473 were significantly decreased at the concentrations of 1 mg/ml and 2 mg/ml of Jinlida ($p<0.01$). The total levels of Akt did not show any significant change.

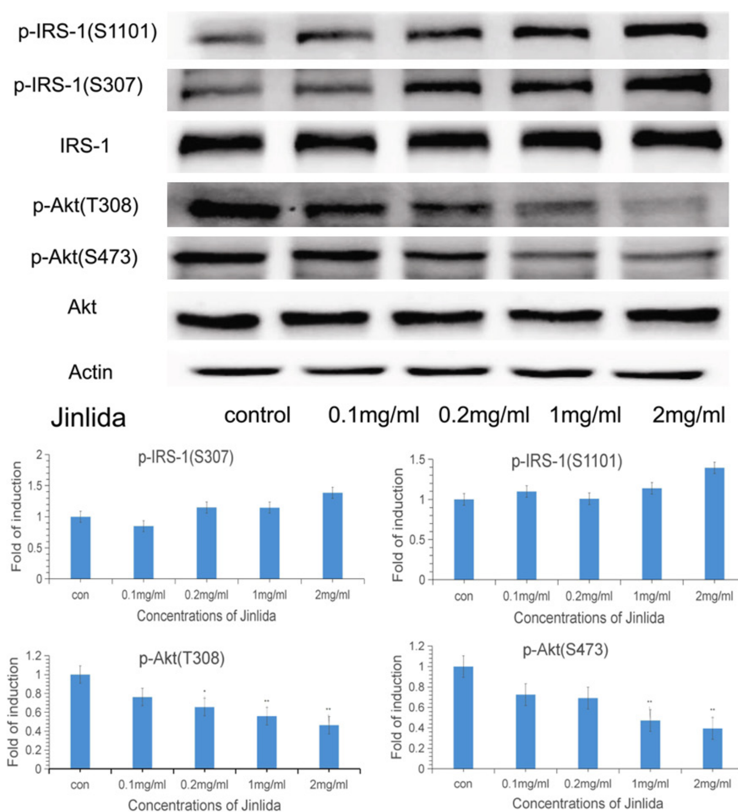

Figure 1. After being exposed to various concentrations of Jinlida ultrafine powder for 24 hours, phosphorylation of IRS-1 at Ser 307 and Ser 1101 was increased, but these results failed to achieve statistical significance. The phosphorylation of Akt at Thr 308 and Ser 473 was significantly decreased at the concentrations of 1 mg/ml and 2 mg/ml ($p<0.01$).

Jinlida ultrafine powder induces phosphorylation of IRS-1 at serine residues

The phosphorylation of IRS-1 at Ser/Thr residues is mediated by the Akt-mTORc1-S6K1 negative feedback loop. As shown in **Figure 2**, the phosphorylation level of S6K1 (Thr 389) was decreased, thus revealing that this cascade was inhibited. As shown in **Figure 1**, however, phosphorylation at multiple serine sites of IRS-1 were detected, and phosphorylation of IRS-1 at Ser 307 and Ser 1101 were increased by Jinlida ultrafine powder in HL7702 cells. Although these results failed to achieve statistical significance, they still indicate that Jinlida ultrafine powder can promote phosphorylation at serine residues of IRS-1.

Jinlida ultrafine powder promotes glycogen synthase expression

We found that Jinlida ultrafine powder inhibited Akt by reducing phosphorylation at Ser 473 and Thr 308. Glycogen synthase kinase (GSK), one of the important substrates of Akt, was detected after the HL7702 cells were exposed to various concentrations of Jinlida ultrafine powder. As shown in **Figure 2**, the levels of phosphorylated GSK3α (Ser 21) and GSK3β (Ser 9) were decreased, showing that the GSK3 was still activated. The activated GSK3 was further able to phosphorylate GS. We also detected that the phosphorylation of GS at the Ser 641 site was increased, but this increase failed to achieve statistical significance. Interestingly, the total level of GS was also increased ($p<0.05$, **Figure 3**). These results might indicate that Jinlida ultrafine powder promotes glycogen synthase expression.

Discussion

Diabetes has become a major public health problem in China. According to a national study conducted in China between 2007 and 2008, the total prevalence of diabetes in adults was 9.7%, and that of prediabetes (including impaired levels of fasting glucose and impaired glucose tolerance, IGT) was 15.5%. What is more troubling is that the majority of cases of diabetes remain undiagnosed [16]. Because the cost of diabetes treatment would eventually become a heavy financial burden for both patients and society, the primary prevention of diabetes should be a priority for China [17]. Obesity and hyperlipidaemia are important factors in the occurrence and development of diabetes. If the levels of obesity induced by changes in diet and lifestyle remain unchanged, the "diabetes epidemic" cannot be suppressed [18]. Lifestyle intervention is the first step in the treatment of diabetes. On this basis, if glucose control cannot meet a certain criterion, then oral antidiabetic drugs have to be added. The first line of antidiabetic drugs is Western medicine such as metformin and dipeptidyl peptidase-4 inhibitors, and so far, Chinese traditional medicines have not been

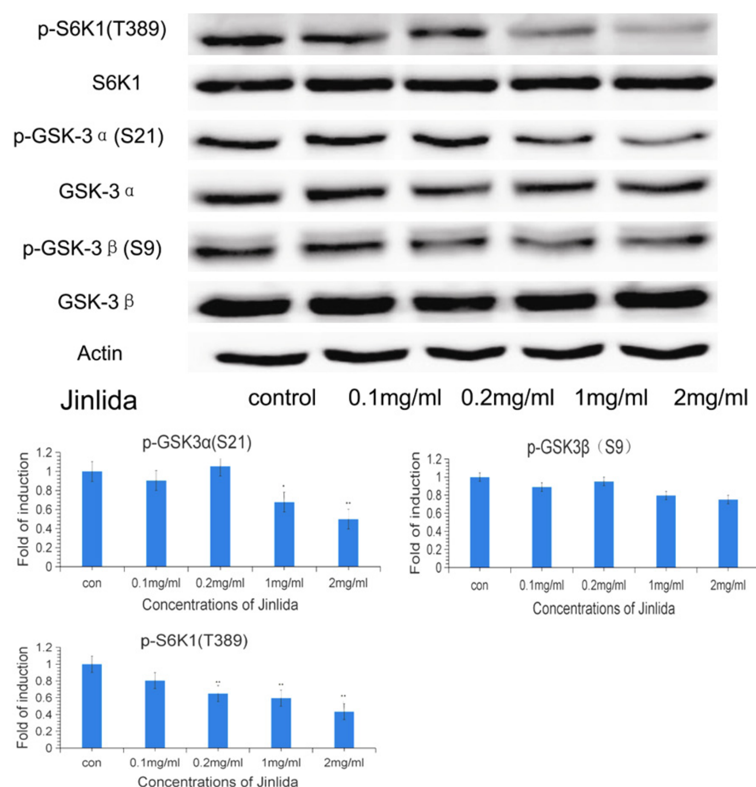

Figure 2. After being exposed Jinlida ultrafine powder for 24 hours, phosphorylation of GSK3α at Ser 21 was decreased at the concentrations of 1 mg/ml (p<0.05) and 2 mg/ml (p<0.01). The phosphorylation of GSK3β at Ser 9 was also decreased, but the result failed to achieve statistical significance. The phosphorylation of S6K1 at Thr 389 was also significantly decreased at concentrations 0.2 mg/ml, 1 mg/ml and 2 mg/ml (p<0.01).

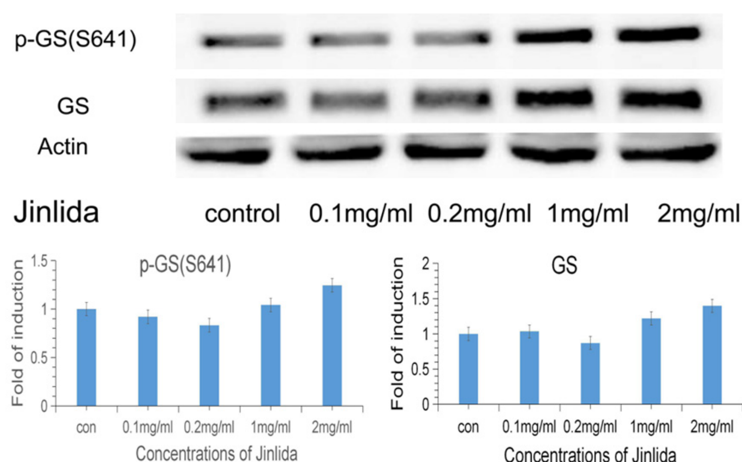

Figure 3. After being exposed to various concentrations of Jinlida ultrafine powder for 24 hours, phosphorylation of GS at Ser 641 was increased, but the results failed to achieve statistical significance. The total amount of GS protein was also increased (p<0.05).

widely accepted in diabetes treatment. Recently, several clinical studies have demonstrated that Chinese patented drugs, such as Jinlida granule and tianqi capsule, can safely achieve anti-diabetic effects [19]. More importantly, tianqi capsule can decrease the incidence of type 2 diabetes in subjects with IGT [20, 21]. The best therapy is prevention. At present, there is no Western medicine that can be used in patients with prediabetes. However, patients with type 2 diabetes often have irreversible damage in islet β-cells. Determining how to reduce the incidence of type 2 diabetes, rather than merely control glucose homeostasis in diabetic patients, is a problem that could be solved by Chinese traditional medicine.

Akt, also referred to as protein kinase B, is critical in human physiology and controls diverse cellular functions, including survival, proliferation and metabolism. The Akt family consists of three extremely homologous isoforms: Akt1, Akt2 and Akt3 [22]. In earlier studies, it was found that Akt plays a critical role in the occurrence of tumours. The abnormal increase in Akt activity has been shown to be related to the occurrence of many kinds of tumours [23]. Recently, many studies have demonstrated that Akt also plays an important role in metabolism and the occurrence of cardiovascular diseases. Insulin promotes glucose transporter 4 (GLUT4) translocation to increase glucose uptake through the activation of PI3K-Akt-mTORC cascade and subsequently activates glycogen synthase to inhibit hepatic glucose output [22]. According to traditional opinion, the deficiency of this pathway is a major determinant of the occurrence of type 2 diabetes [24]. However, recent research has illustrated that high doses

of insulin are sufficient to inhibit hepatic glucose production of the liver in Akt-knockout mice and has further demonstrated that this effect is dependent on the reduction of serum FFA by insulin. Moreover, Akt is critical to lipogenesis of the liver. The absence of Akt will hinder the synthesis of triglycerides in the liver. As is commonly accepted, obesity and hypertriglyceridaemia are the typical characteristics of patients with insulin resistance or type 2 diabetes, as well as areas that are dependent on an intact insulin signalling pathway [14]. However, the hepatic insulin signalling pathway in patients with type 2 diabetes is not usually inhibited. In addition, the plasma FFA can promote insulin secretion. Persistently high FFA levels can lead to hyperinsulinemia in the long term [25]. Hyperinsulinemia will result in hepatic insulin signalling pathway hyperactivation and increase TG synthesis. In addition, the excessive activation of the insulin signalling pathway induced by hyperinsulinemia in multiple organs and cells has been shown to play an important role in the occurrence of diabetic nephropathy, atherosclerosis and tumours [26-28]. Therefore, decreasing the level of plasma FFA could be key to the treatment of diabetes and associated complications.

In this work, a reduction in phosphorylation level of AktThr 308 and Ser 473 was found in HL7702 cells incubated with Jinlida ultrafine powder compared to the control group, which demonstrates decreased activity of Akt. This result is further illustrated by the decrease in the levels of GSK-3 phosphorylation. Previous studies have demonstrated that Akt is required for hepatic lipid accumulation in an insulin-resistant state [29]. Inhibiting the activity of Akt reduces lipogenesis and promotes lipolysis and β-oxidation [30, 31]. Several studies have shown that Jinlida granule can decrease TG accumulation in many types of cells and can ameliorate insulin resistance and reduce the apoptosis of islet β-cells [10, 11]. Moreover, Jinlida granule can inhibit renal hypertrophy and mesangium expansion by blocking the PI3K/Akt/mTOR cascade, thereby delaying the development of diabetic nephropathy [32]. Therefore, we think that the effects of Jinlida granule-induced reduction of TG levels and improvement of diabetic complications may be related to the inhibition of Akt activity.

IRS is also important in the insulin signalling pathway, which includes two major isoforms: IRS-1 and IRS-2. Many papers have proposed that different IRS proteins mediate different branches of the insulin signalling pathway. The IRS-1 regulates lipid metabolism, but IRS-2 regulates hepatic glucose production [33]. However, recent data suggest that both IRS-1 and IRS-2 are necessary for the daily regulation of glucose metabolism in the liver. IRS-2 is abundantly expressed in low-insulin conditions, whereas high levels of insulin have been shown to suppress its expression. After the start of feeding, IRS-2 protein levels decrease immediately following the peak insulin secretion, while the IRS-1 protein levels remain unaffected. Sufficient expression of hepatic IRS-2 during fasting conditions appears to be critical for glucose homeostasis [34]. Over the past few decades, accumulating evidence has supported the concept that hepatocytes can be divided into the perivenous zone and periportal zone. The periportal hepatocytes, where IRS-2 expression is relatively high, are involved in gluconeogenesis, while the perivenous hepatocytes, where IRS-1 expression is relatively high, are involved in lipogenesis and glucose uptake. In the presence of hyperinsulinemia, insulin signalling mediated by IRS-2 is impaired, but insulin signalling mediated by IRS-1 is hyperactive. After the start of feeding, the elevated glucose level fails to decrease promptly, but lipogenesis is enhanced. This paradoxical condition is referred to as "selective insulin resistance" [35].

In this work, increases in phosphorylation level of IRS-1 Ser 307 and Ser 1101 were found compared to the control group, but the change failed to achieve statistical significance. As commonly accepted, the phosphorylation of IRS-1 at Ser/Thr residue mediated by the Akt-mTORC1-S6K1 negative feedback loop inhibits its activity. Our results show that as Akt activity was inhibited, the level of S6K1 (Thr 389) phosphorylation was also decreased, indicating that this signalling pathway was inhibited. Conversely, the phosphorylation levels of IRS-1 at Ser/Thr residue increased in a concentration-dependent manner. These results illustrated that Jinlida ultrafine powder may have promoted IRS-1 Ser/Thr residue phosphorylation through other pathways. Thus, it is possible that Jinlida granule reduced lipogenesis by counteracting the hyperactive insulin signalling mediated by IRS-1 in patients with hyperinsulinemia in clinics.

In addition to lipid metabolism, the liver also plays an important role in glucose metabolism. In hepatocytes, the process of glucose metabolism includes glucose uptake and glycogen synthesis. According to previous reports, glucose transport is unrestricted in liver and is not regulated by insulin[36]. Therefore, the activity of enzymes related to glycogen synthesis is critical in glucose metabolism. The enzymes involved in hepatic glucose metabolism include glycogen synthase (GS) and glycogen phosphorylase. In this research, we found that the expression of GS was enhanced ($p<0.05$). The reduction of Akt activity failed to inhibit GSK-3 and promoted GS phosphorylation, thus inhibiting the activity of GS. However, changes in the phosphorylation level of GS (Ser 641) did not reach statistical significance in this research. Additionally, intracellular glycogen levels and glucose 6-phosphate can regulate glycogen synthase activation [37, 38]. The glucose 6-phosphate allosterically activates glycogen synthase and overcomes inactivation induced by phosphorylation, restoring its full activity [39]. At present, there have been no clinical or animal trials that support the point that Jinlida promotes the expression of GS. Thus, whether it has the same effect in the human body as seen in cell culture needs to be further confirmed.

In summary, this study shows that inhibition of the hepatic IRS-1-Akt-mTOR cascade by Jinlida ultrafine powder may be the underlying mechanism by which Jinlida granule ameliorates lipid metabolism disorders in the body. Moreover, it is possible that Jinlida granule promotes glycogen synthase expression to increase glycogenesis.

Acknowledgements

The Jinlida ultrafine powder was provided by Shijiazhuang Yiling Pharmaceutical Company Limited. This work was supported by the Department of Biochemistry, School of Medicine, Zhejiang University, Hangzhou, China. We express our appreciation to all that have collaborated with us.

Disclosure of conflict of interest

None.

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