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Abstract

In the present study, we examined the hypoglycemic properties and the effective mechanisms of a triterpenoid-rich extract from the *Euryale* shell (ES) in streptozotocin-induced (STZ) diabetic mice. The hydroalcoholic extract of ES (200, 400 and 600 mg/kg/day) was orally administered to STZ-diabetic mice for 4 weeks. We observed that in the liver of diabetic mice, the ES extract caused a sharp reduction in the gene expression of protein tyrosine phosphatase-1B (PTP1B) but induced the gene expression of phosphatidylinositol-3-kinase (PI-3K) and protein kinase B (PKB) compared with that of untreated diabetic mice. Additionally, a significant increase in the phosphorylation of the PKB protein was observed ($p < 0.01$). This was corroborated by the inhibition of PTP1B and by the regulation of glucose uptake via PI-3K activation, which together demonstrate that the reduction of PTP1B can modulate key insulin signaling events downstream of the insulin receptor.

Introduction

Type 2 diabetes mellitus (T2DM) is a very common endocrine disorder (King et al., 1998) characterized by persistent hyperglycemia, and the disease is also associated with abnormalities in carbohydrate, protein, and lipid metabolism caused by a failure of insulin secretion and/or increased cellular resistance to insulin (Brahmachari, 2011). Current evidence suggests that some plant medicines, especially soluble supplements, may improve glucose metabolism (Topping, 2007; Ali et al., 2006; Lima et al., 2012).

Euryale ferox belongs to the *Euryale* genus of the family Nymphaeaceae and was identified by Nath BK in 1985 (Nath et al., 1985). Originally produced in Southeast Asia and China, it has been cultivated for a long time, mainly for its mature seeds. The outer covering of the *Euryale ferox* seeds is a hard shell (Row et al., 2007), and a large proportion of the shells is discarded during processing. The hydroalcoholic extract of the *Euryale*

shell (ES) is composed of triterpenoids, flavonoids, saponins, steroids and other phenolic compounds (Lee et al., 2002).

Our previous research had confirmed the hypoglycemic activity of a triterpenoid-rich extract from the ES (Yuan et al., 2013). The results indicated that the ES extract could prevent the reduction in the body weight of diabetic mice and regulate fasting blood glucose. Therefore, the present study endeavored to evaluate the effects of the triterpenoid-rich extract from the ES on phosphorylation-related genes and to investigate the mechanisms of action.

Materials and Methods

Preparation of the triterpenoid-rich extract of the ES

ES were collected around Huainan City in China. A voucher specimen authenticated by the Department of



Botany at the Hefei University of Technology, was deposited at the College of Biotechnology and Food Engineering. The broken shells of the Euryale seed were thoroughly washed with water and dried in the shade. A total of 500 g of air-dried shells were ground into a fine powder and soaked in 75% ethanol at 60°C for 16 hours prior to the extraction. The extract was then concentrated with a vacuum rotary evaporator at 50°C, diluted with water, and the solution was extracted 3 times with petroleum ether, ethyl acetate and water-saturated butanol, in that order. The extracts were then pooled and concentrated using a rotary evaporator to yield triterpenoid-rich main fractions that were stored at -20°C and were suspended in water and administered orally as needed.

Materials and chemicals

Streptozotocin (STZ) was purchased from Sigma Chemical Co. (Nanjing, China). The citrate-citrate buffer solution (25 mg/kg, 1% solution of 0.1 mol/L, pH 4.4) and glimepiride were purchased from Pharmaceutical Group Co., Ltd. (Anhui, China). The remaining reagents and chemicals used were of analytical grade.

Selection of animals and induction of diabetes mellitus

Sixty male mice (weighing 18-20 g) (Anhui Medical University, Hefei) were maintained under standard environmental conditions (12:12 hours light/dark cycles) and fed with a standard diet (Pharmaceutical Technology Co., Ltd., Hefei) and water *ad libitum*.

After 1 week of dietary manipulation, a subset of the mice (52) were injected intraperitoneally with a low dose of STZ (100 mg/kg BW) dissolved in 0.1 mol/L citrate buffer (Maiti et al., 2005). The 8 mice used as the normal control (NC) were intraperitoneally injected with the buffer solution. Three days after the STZ injection, diabetic mice were defined as having a plasma glucose level greater than 11.0 mmol/L.

Experimental design

Except for the normal control group (NC), the 40 mice with the closest body weights that had developed T2DM were chosen. They were randomly divided into five groups of 8 animals each: the model diabetic mice (MD) group, positive control (PC) group and the extract from ES (Low-dose, LG; Middle-dose, MG; and High-dose, HG) groups (Yoshida et al., 2009).

The NC and MD mice were fed the standard diet, whereas the LG, MG and HG groups received the triterpenoid-rich extract of ES suspended in distilled water orally at doses of 200, 400 and 600 mg/kg, respectively. The PC group received 5 mg/kg of glimepiride, used as a reference standard drug. Each treatment lasted for 4 weeks, during which period the mice had free access to food and water.

Sampling and sample processing procedures

Blood samples from each sacrificed mouse were collected for obtaining serum samples, and the processed serum samples were then stored at -20°C until analysis of various biochemical and hormonal indices. Liver tissue samples were immediately collected, wrapped in aluminum foil, frozen in liquid nitrogen, and then stored at -80°C until molecular analysis.

RT-PCR of mRNA of PTP1B, PI-3K and PKB

Total RNA was isolated from the liver tissues using the Trizol total RNA extraction kit (Shanghai Sangon Biological Engineering Technology & Service Corporation Ltd., Shanghai, China). The A260/A280 of total RNA was between 1.8 and 2.0.

The primers were designed by Primer Premier 5 (Premier Biosoft, Canada) based on the gene sequences of PTP1B, PI-3K and PKB and the housekeeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) logged in the gene bank. Primers for PTP1B (Forward 5'-CCTA CCTG GCTG TCAT CG-3', Reverse 5'-CCAC CATC CGTC TCCT AAC-3'; 365 bp), PI-3K (Forward 5'-GCAC AACA GAGG CGAT GA-3', Reverse 5'-AGCG ACAC CCAG GCTA CA-3'; 518 bp), PKB (Forward 5'-TCGC CAAC AGTC TGAA GC-3', Reverse 5'-CCGG ATCC TCCG TGAA-3'; 483 bp), and GAPDH (Forward 5'-CAAG GTCA TCCA TGAC AACT TTG-3', Reverse 5'-GTCC ACCA CCCT GTTG CTGT AG-3'; 496 bp) were synthesized by the Shanghai Sangon Biological Engineering Technology & Service Corporation Ltd.

Total RNA was reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Shanghai Sangon Biological Engineering Technology & Service Corporation Ltd., China) and amplified by PCR. Each 25 µL of the RT reaction contained 11.4 µL of sterile de-ionized H₂O, 2.5 µL of 1 × PCR Buffer, 2.5 µL of 0.2 mmol/L dNTP mixture, 1.3 µL of each 0.5 µmol/L primer for either PTP1B or PI-3K PKB, 0.1 µL of 0.5 µg/25 µL Taq DNA polymerase, 1.5 µL of 1.5 mmol/L MgCl₂, and 2 µL of 10 pg/µg cDNA. The amplification profile was as follows: 1 cycle of 95°C for 5 min, 30 cycles of 94°C for 30 sec, the PTP1B primer at 57°C or the PI-3K primer at 57°C or the PKB primer at 55°C for 30 sec, 72°C for 30 sec, and a final elongation step of 72°C for 10 min.

Each PCR product was mixed with 2 µL of the loading dye (25% bromophenol blue, 25% glycerol) and electrophoresed on a 1.5% agarose gel containing ethidium bromide (0.5 µg/mL) for 1 hour at 100 V. A low DNA mass ladder was used as the molecular weight marker. The DNA bands were visualized and densitometric analysis was performed using a UV transilluminator.

Evaluation of the activating phosphorylation of PI-3K and PKB proteins in the liver

Protein extraction and processing were carried out as previously reported, with some modifications (Vascon-

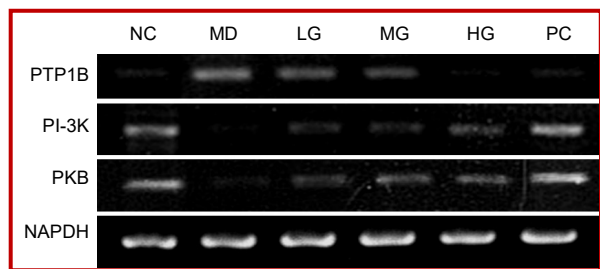


Figure 1: Treatment of the hyperglycemic mice with the ES extract resulted in PTP1B, PI-3K and PKB mRNA expression in the liver. NC: normal control group (physiological saline); MD: model diabetic mice group (physiological saline); LG: low-dose ES extract group (200 mg kg⁻¹ BW); MG: middle-dose ES extract group (400 mg kg⁻¹ BW); HG: high-dose ES extract group (600 mg kg⁻¹ BW); and PC: positive control group (glimepiride solution)

celos et al., 2011). Fragments of liver tissues were obtained from animals and homogenized in PBS lysis buffer. PI-3K and PKB protein concentration in the liver was determined by using a mouse PI-3K and PKB enzyme linked immunosorbent kit (Jiancheng Technology & Service Corporation Ltd., Nanjing, China), which used purified recombinant mouse protein as the standard. Protein levels in the liver were measured by enzyme linked immunosorbent assay and determined according to the manufacturer's instructions.

Statistical analysis

The statistical software Origin 6.1 (Origin Lab Corporation, USA) was used by the first author in this study. All experimental data were expressed as the mean \pm SD. The differences between the test and control groups were evaluated by Student's *t*-test.

Results

Following agarose gel electrophoresis, a UV image scanning analytical system was used to detect the electrophoresis strips, and GAPDH was the designated internal reference to calculate the relative amount of mRNA. The ratio of the PTP1B, PI-3K or PKB strips to the GAPDH strips was used as a quantitative indicator of PTP1B, PI-3K and PKB mRNA levels (Figure 1).

At 4 weeks subsequent to the treatment with the ES extracts, mRNA expression level of each group was evaluated. The mRNA expression level of PTP1B in the MD group was significantly higher than that in the NC group; however the expression reduced following treatment with the hydroalcoholic extract from the ES and treatment with glimepiride. Compared with the MD group, the differences between the LG group and the MG group were not significant. A significant reduction of the PTP1B mRNA was observed in both the HG and PC groups. This result indicated that

feeding the mice with ES extracts could inhibit the expression of PTP1B in diabetic mice.

Additionally, the effect of the hydroalcoholic extract of ES on the mRNA expression of PI-3K and PKB in diabetic mice is also shown in Figure 1. The present study suggests that supplementation with the ES extracts could enhance the expression of PI-3K and PKB at the mRNA and subsequently at the protein level, to improve glucose metabolism in diabetic mice. The expression of PI-3K was induced on treatment of mice with the ES extracts. The liver of mice that was administered glimepiride in their diet had the most apparent level of PI3K mRNA expression compared with the MD group. The mRNA expression level of PKB in the liver was higher for the mice fed the glimepiride and the high-dose diet, followed by the MG group, while the LG group had the least expression.

At 4 weeks following treatment with the ES extract, the protein levels of PI-3K in each group were evaluated. Analysis of the experimental data yielded the following results (Figure 2).

In the liver of type 2 diabetic mice, PI-3K promotes the tyrosine phosphorylation of the insulin receptor substrate, which is a key enzyme in the regulation of insulin-stimulated glucose transport. We therefore analyzed the impact of the stimulation of PI-3K activity by the ES extracts. PI-3K expression was severely impaired in the MD group when compared to the NC group (60% reduction). However, treatment of these diabetic mice with the ES extract led to an enrichment in the expression of the PI-3K protein by 22 and 30% for the LG and MG groups, respectively ($p > 0.05$). Strikingly, compared with the diabetic controls, there was significant improvement ($p < 0.01$) in the expression of this kinase in both the HG and PC groups, which may result in a recovery of the liver function.

The expression of total and phosphorylated levels of PKB in the liver of the treated and control groups are presented in Figure 3. At the end of the experiment, the NC group without the STZ injections grew well. At the same time, PKB concentrations in the MD group were significantly lower than that in the NC group. The liver of mice in the MG group treated with the ES extract at 400 mg/kg/day displayed a significant 73% increase ($p < 0.05$) in the expression of PKB when compared to the diabetic control group. Similarly, the HG group showed a significant 79% increase ($p < 0.01$) in PKB when compared to the diabetic control group. However, there was no difference in PKB expression between the livers of mice in the LG group and the MD group.

Together, these results suggest increased glucose uptake because kinase activity was markedly improved after the administration of the triterpenoid-rich extract from ES. The changes in the PKB expression suggested that a high-dose of the ES extract might restore the

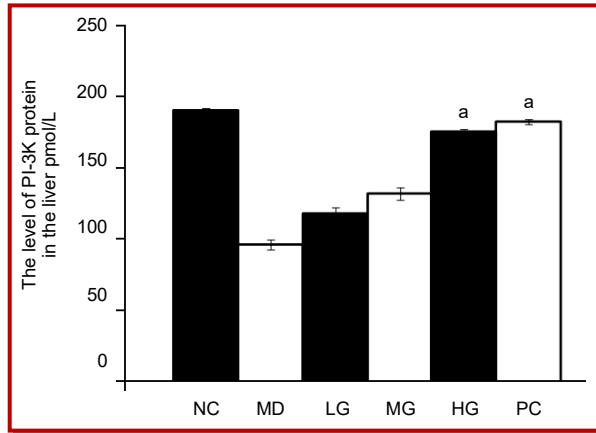


Figure 2: Enzyme linked immunosorbent analysis of PI-3K in the liver of STZ-diabetic mice. NC: normal control group (physiological saline); MD: model diabetic mice group (physiological saline); LG: low-dose ES extract group (200 mg kg⁻¹ BW); MG: middle-dose ES extract group (400 mg/kg BW); HG: high-dose ES extract group (600 mg/kg BW); and PC: positive control group (glimepiride solution). The data are presented as the mean \pm SD., n = 5. Compared with the MD group, 'b' means that the difference is significant ($p < 0.05$), 'a' means that the difference is higher ($p < 0.01$) while for the other groups the difference is not significant ($p > 0.05$)

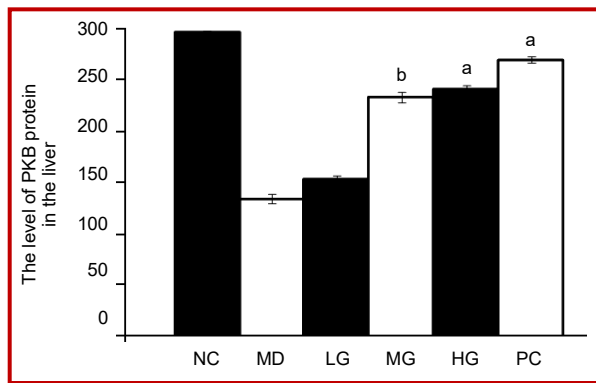


Figure 3: Enzyme linked immunosorbent analysis of PKB in the liver of STZ-diabetic mice. NC: normal control group (physiological saline); MD: model diabetic mice group (physiological saline); LG: low-dose ES extract group (200 mg kg⁻¹ BW); MG: middle-dose ES extract group (400 mg kg⁻¹ BW); HG: high-dose ES extract group (600 mg kg⁻¹ BW); and PC: positive control group (glimepiride solution). The data were presented as the mean \pm SD., n = 5. Compared with the MD group, 'b' means that the difference is significant ($p < 0.05$), 'a' means that the difference is higher ($p < 0.01$) while for other groups the difference is not significant ($p > 0.05$)

function of the islet beta-cells of the liver (Cousin et al., 2001) and reduce the insulin resistance by regulating the level of insulin secretion. Thus the ES extract could help improve the status of the diabetic mice albeit not being as effective as glimepiride.

Discussion

Folk medicines such as medicinal plants are important

modulators of insulin action on glucose metabolism. However, not much is known about the impact of the *Euryale* shell on insulin resistance and the associated molecular mechanisms (Kim et al., 2007). The experiments in this study were designed to investigate the hypoglycemic activity of the triterpenoid-rich extract of the ES, as well as to assess the possible role of the enzymatic pathways of PTP1B, PI-3K and PKB (Xu et al., 2011).

Among several proteins in the insulin signaling pathway that are targeted for diabetes therapy, PTP1B is a typical non-receptor type that belongs to the PTP family (Gum et al., 2003). PTP1B has been implicated as a negative regulator of tyrosine kinases, including those associated with insulin signaling, and the expression of PTP1B is elevated in various genetic models of insulin resistance that are associated with diabetes. On knocking down the PTP1B gene in wild-type mice, we found that insulin sensitivity was greatly increased and the blood glucose levels were normal (Clampit et al., 2003).

In our studies, we treated STZ-induced diabetic mice with the ES extract to analyze the effect of PTP1B on insulin signaling. Our results are the first to demonstrate that the inhibition of PTP1B expression in vivo markedly increased insulin-stimulated PI-3K and PKB phosphorylation in liver cells, with a marked stimulation of tyrosine phosphorylation of the insulin receptor and IRS proteins. These results confirmed that PTP1B is indeed a potent negative regulator of insulin signaling in liver cells.

Additionally, we investigated the activation of the PI-3K and PKB protein kinases. The activation of PKB is essential for the regulation of glycogen synthesis in muscles, adipocytes, and in the liver and for the inhibition of gluconeogenesis. The increase in hepatic glycogen in treated animals, as a consequence of the activation of Akt, confirms the action of PKB on glycogen synthase as described by elsewhere (Farese et al., 2005). The activation of Akt in insulin-resistant rats was improved by treatment with polysaccharides from *Astragalus membranaceus* (Liu et al., 2010).

Our results showed that the administration of the triterpenoid-rich extracts from the ES stimulated the expression of the phosphorylated PKB enzyme in the liver. The activation of this enzyme is related to an increased glucose uptake in the liver, thereby reducing the gluconeogenic activity, and promoting greater glycogenesis through the activation of glycogen synthase (Sancho et al., 2005). These data could explain our previous results that showed an increase in liver glycogen storage, as well as the increased absolute and relative mass of the liver found in diabetic rats treated with the extract of the ES. Moreover, the increased activation of PKB that was observed in the muscle tissue of animals treated with the ES extract may assist muscle protein synthesis and consequently reduce the

proteolysis produced by dia-betes.

Several provocative issues arose in association with the effect of the ES triterpenoid-rich extract on blood glucose levels. The primary concern was the dose-response, with the low dose having little or no effect on the treatment of diabetic mice and a high dose causing potentially unadaptable changes. Another issue was that the IR mechanism is also regulated by other protein kinases such as the mitogen activated protein kinases and insulin receptor substrates (IRS-1/2), which needs further investigation (Sugimoto et al., 2008; Ostman et al., 2001; Xiao et al., 1993).

In summary, the inhibition of PTP1B mRNA expression in liver tissue positively modulates key insulin signaling events in the metabolic pathway. These results suggest that reducing PTP1B activity by using small molecule inhibitors or by antisense treatment may improve insulin signaling in the liver and may help understand the mechanism of insulin resistance in type 2 diabetic patients. PKB is activated by a variety of stimuli including growth factors, cytokines and hormones, which act via the PI3K receptor, suggesting that PKB plays an important role in the maintenance of glucose homeostasis. Our study is the first to propose a molecular mechanism for the supposed hypoglycemic activity of the ES, and our results demonstrate that the administration of the triterpenoid-rich extract from the ES can regulate glucose metabolism disorders in diabetes, thereby confirming its ethnomedicinal use.

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Ethical Issue

The experimental protocol was approved by the Animal Experimentation Ethics Committee of the UFPE (Process No. 012974), in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Conflict of Interest

Authors declare no conflict of interest

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