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Borapetoside C from *Tinospora crispa* improves insulin sensitivity in diabetic mice

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ABSTRACT

Diabetes mellitus (DM) often leads to disability from vascular complications and neurological complications. *Tinospora crispa* has been widely used in Asia and Africa as a remedy for diabetes and other diseases. In this study, we investigated the hypoglycemic actions of borapetoside C isolated from *T. crispa*, and the mechanisms underlying its actions. Acute treatment with borapetoside C (5 mg/kg, i.p.) attenuated the elevated plasma glucose induced by oral glucose in normal and type 2 DM (T2DM) mice. Compared to the effect of injected insulin (0.5 IU/kg), borapetoside C caused a more prominent increase of glycogen content in skeletal muscle of T2DM mice, but a less increase in type 1 DM (T1DM) mice. Combined treatment of a low dose borapetoside C (0.1 mg/kg, i.p.) plus insulin enhanced insulin-induced lowering of the plasma glucose level and insulin-induced increase of muscle glycogen content. Continuous treatment with 5 mg/kg borapetoside C (twice daily) for 7 days increased phosphorylation of insulin receptor (IR) and protein kinase B (Akt) as well as the expression of glucose transporter-2 (GLUT2) in T1DM mice. Combined treatment of a low dose borapetoside C (0.1 mg/kg, twice daily) plus insulin for 7 days enhanced insulin-induced IR and Akt phosphorylation and GLUT2 expression in the liver of T1DM mice. This study proved that borapetoside C can increase glucose utilization, delayed the development of insulin resistance and enhanced insulin sensitivity. The activation of IR-Akt-GLUT2 expression and the enhancement of insulin sensitivity may contribute to the hypoglycemic action of borapetoside C in diabetic mice.

Introduction

Diabetes mellitus (DM) is associated with the pathological progression in various organs such as the liver and skeletal muscle. The epidemiological studies characterize obesity to be highly associated with DM. About 80% of individuals with type 2 DM (T2DM) are overweight (Bays et al. 2007). Since the onset of metabolic disease starts to develop earlier in obese people, studies have focused on the development of pharmaceuticals that can be used in patients with obesity and DM (Bays 2009).

There are several studies showing that diabetes is associated with abnormal insulin secretion and insulin sensitivity. Since insulin is the most important substance in regulating glucose metabolism, impaired insulin secretion results in an increase in hepatic glucose production and reduction of glucose uptake in muscle (Kahn et al. 2006). On the other hand, increased insulin resistance is a key feature in T2DM. It is characterized with a remarkable decrease in tissue glucose utilization in response to insulin (Granberry et al. 2007).

Tinospora crispa (family Menispermaceae) has been widely used in Asia and Africa as a herbal remedy for a long time. In traditional medicine, a decoction from the stems of *T. crispa* has been used for anti-inflammation, reducing thirst, increasing appetite, antipyretics, and maintaining good health (Messmer 1961; Zafinindra et al. 2003). The chemical constituents of *T. crispa* extracts have been extensively studied since the 1980s. The major active ingredients of *T. crispa* are identified as terpenoids and terpenoid glycosides. The terpenoid glycosides are mainly composed of borapetosides A, B, C, D, E and F (Cavin et al. 1998; Choudhary et al. 2010a,b; Kongkathip et al. 2002; Martin et al. 1996; Pachaly et al. 1992; Pathak et al. 1995; Ragasa et al. 2000; Yonemitsu et al. 1993). Most of these substances are yet to be investigated for their pharmacological activities.

Our previous study showed that borapetoside C from *T. crispa* could decrease serum glucose via enhancing insulin secretion in both normal and T2DM mice, whereas it reduced glucose level without changing the insulin level in type 1 DM (T1DM) mice (Lam et al. 2012). The molecular mechanism for the increase of glucose utilization, inhibition of hepatic gluconeogenesis, and associated lowering of plasma glucose by borapetoside C in diabetic mice remain to be investigated. In this study, we demonstrated that borapetoside C attenuated the elevation of plasma glucose

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induced by oral glucose in normal and diabetic mice. Continuous treatment with borapetoside C at 5 mg/kg (twice day) for 7 days induced phosphorylation of insulin receptor (IR), protein kinase B (Akt) and resulted in enhancement of glucose transporter subtype 2 (GLUT2) expression in the liver of T1DM mice. Combined treatment with insulin and low dose of borapetoside C (0.1 mg/kg) increased insulin sensitivity and enhanced insulin induced IR/Akt/GLUT2 signaling in the liver of T1DM mice. On the basis of the study, we propose that borapetoside C is a potential therapeutic agent that can be further explored and developed as an alternative remedy for managing diabetic disorders in the future.

Material and methods

Animals and treatment protocol

This study was conducted by following the University ethical guidelines on animal experimentation and complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85–23, revised 1996). The animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the National Taiwan University (IACUC No. 20070004). The animal facility was well controlled for temperature ($22 \pm 1\,^{\circ}C$), and humidity ($60 \pm 5\%$) and a 12 h/12 h light-dark cycle was maintained with access to food and water *ad libitum*. Four-week-old male ICR mice were acquired from BioLasco Taiwan Co., Ltd. and maintained at College of Medicine Experimental Animal Center, National Taiwan University. The study was conducted on 8–10 week-old male ICR mice.

T1DM mice were induced by following a modified protocol after the acclimatizing period (Hayashi et al. 2006). In brief, an intraperitoneal injection of streptozotocin (STZ; Sigma Chemical Co.; St. Louis, MO) at 150 mg/kg was performed in mice that were fasted for 48 h. The induction of T1DM was assessed and confirmed when the mice had plasma glucose levels ≥350 mg/dl, accompanied with polyuria, hyperphagia and decreased body weight. The control mice group received an injection of vehicle and then carried out for 4 weeks. T2DM mice were induced by maintaining on a fat-rich chow diet and fructose-sweetened water for 4 weeks from the age of 4–5 weeks according to previous methods (Huang et al. 2004; Weng et al. 2010). The induction T2DM mice were assessed by measuring fasting plasma glucose levels and confirmed when plasma glucose level was ≥150 mg/dl after a 4-week induction.

Measurement of plasma glucose and insulin levels

Blood samples were collected from the orbital vascular plexus of mice under anesthesia with sodium pentobarbital (80 mg/kg, intraperitoneal, Sigma Chemical Co., St. Louis, MO, USA). Blood samples were then centrifuged at 13,000 rpm for 5 min, and the plasma was kept on ice prior to the assay (Park et al. 2005). The plasma glucose concentration was measured using commercial kits following manufacturer's instructions (BioSystems S.A., Barcelona, Spain).

Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT)

To perform OGTT, mice were fasted overnight, divided into 2 groups and then administered a vehicle control and 5 mg/kg borapetoside C. This was followed by administration of a glucose solution at 2 g/kg via tube feeding. Blood samples were withdrawn from the orbital vascular plexus at intervals of 30, 60, 120, and 150 min after glucose administration (Kuftinec and Mayer 1964). For the ITT, mice were fasted for 3 h. Human insulin (Insulin Actrapid® HM; Novo Nordisk, Denmark) was injected intraperitoneally after an intraperitoneal administration of 0.1 mg/kg borapetoside C for 30 min. Blood samples were collected from the orbital vascular plexus at the timed intervals mentioned above (Kuftinec and Mayer 1964).

Glycogen content assay

Glycogen content of skeletal muscles was measured according to an earlier established method (Sadasivam and Manickam 1996). In brief, mice were injected with borapetoside C (5.0 mg/kg, interperitoneal) for 60 min or insulin (0.5 IU/kg, interperitoneal.) for 30 min, and the soleus muscle was isolated from anesthetized mice. About 40 mg of muscle sample was dissolved in 1 N KOH at 75 °C for 30 min. The dissolved homogenate was neutralized by glacial acetic acid and then incubated overnight in acetate buffer (0.3 M sodium acetate, pH 4.8) containing amyloglucosidase (Sigma, St. Louis, MO). The mixture was then neutralized with 1 N NaOH to stop the reaction (Chou et al. 2005). The glycogen contents in the tissue samples were determined as μg of glucose per mg of tissue (wet weight).

Collection of liver tissue

After treatment, the mice were sacrificed by cervical dislocation under anesthesia. The liver was immediately frozen in liquid nitrogen. The liver tissue was preserved at −80 °C before they were used for further assays.

Western blot analysis

Tissues were homogenized in T-PER® Tissue Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL) with Halt™ Protease Inhibitor Single-Use Cocktail (Pierce Biotechnology, Rockford, IL). Liver homogenates were prepared by mechanical homogenization (Polytron PT3100, Luzernerstrasse, Switzerland). After centrifuging the homogenates at $10{,}000 \times g$ for 30 min, the supernatants were collected and frozen at −80 °C for further use. The protein concentrations were determined by using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). For western blot analysis, about 60 μg of protein preparations were applied on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were blocked with 5% (w/v) non-fat dry milk in phosphate buffered saline (PBS) containing 0.1% Tween 20 (PBS-T). After blocking, the blotted membrane was incubated with anti-phospho-insulin receptor β (IRβ) (Tyr^{1345}), phospho-Akt (Ser^{473}) (Cell Signaling Technology, Beverly, MA), anti-IRβ, Akt, β-actin (Santa Cruz Biotechnology, Santa Cruz, California), and anti-GLUT2 antibodies (Abcam, Cambridge, UK) in presence of 3% bovine serum albumin (BSA) in PBS-T buffer. Following the incubation, the membranes were washed 3 times with PBS-T for 15 min each and then incubated with the appropriate peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, USA) in PBS-T. After removal of the secondary antibody, blots were washed and developed using the enhanced chemiluminescence (ECL) western blotting system (Millipore, Billerica, MA). The density of the protein bands were quantified using ImageQuant (Chi et al. 2007).

Statistical analysis

Results were presented as mean ± SEM for the number (n) of animals in the group as indicated in the tables and figures. Statistical difference between the means of the various groups were analyzed using one way analysis of variance (ANOVA) followed by Turkey's multiple test with Prism 5.0 demo software (GraphPad Software Inc., La Jolla, CA). Data were considered statistically significant at $^{*}p < 0.05$.