Original research article

Hypoglycemic effect of Chrysanthemum morifolium extract on alloxan-induced diabetic mice is associated with peroxisome proliferator-activated receptor α/γ-mediated hepatic glycogen synthesis

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ABSTRACT

Previous studies have indicated that polyphenol-rich Chrysanthemum morifolium extract (CME) may inhibit the formation of hyperlipidemic fatty liver in mice. But there has been no report about therapeutic effect on diabetes mellitus. In the present study, we investigated the action of CME and its potential mechanisms. A mouse model with diabetes mellitus was induced by alloxan. The results showed that after treatment of diabetic mice with polyphenol-rich CME 150 and 300 mg/kg for 6 weeks, the levels of fasting blood glucose (FBG) as well as water and food consumption were decreased (P < 0.05 or P < 0.01), while CME might reverse the changes (P > 0.01). These findings demonstrate that the reduction of PPARα/γ-mediated hepatic glycogen synthesis may involve in the alloxan-induced hyperglycemia, and the hypoglycemic mechanisms of CME may be mainly associated with the increment of hepatic glycogen synthesis via upregulation of PPARα/γ-mediated GS and Glut-2 protein expressions.

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Introduction

Diabetes mellitus is a metabolic disease and has become one of the most common chronic diseases in many countries. Its main clinical characteristics are hyperglycemia, polyphagia, polydipsia, polyuria, and weight loss. This disease can also cause a series of complications in late stage, including diabetic nephropathy, retinopathy, and vascular lesion (Joussen et al., 2009; Moore et al., 2009). The pathogenesis of the disease is very complex and still unclear, and may be the results of many factors. Peroxisome proliferator activated receptor (PPAR) α/γ are ligand-activated transcriptional factors that may regulate the expressions of numerous genes involved in glucose and lipid homeostasis (Desvergne and Wahli, 1999). Recent studies have indicated that PPARα/γ have an important role in the generation and development of diabetes mellitus (Barroso et al., 1999; Flavell et al., 2005; Jay and Ren, 2007). Though the main function of hepatic PPARα is to control lipid metabolism, its activation may also lead to increase in the synthesis of hepatic glycogen via increment of glycogen synthase (GS) (Ortmeier et al., 2005; Kersten, 2014), a rate-limiting enzyme during glycogen synthesis. Glucose transporter-2 (Glut-2), highly expressed in the liver, is the major glucose transporter of hepatic tissue, and the rate of glucose uptake is not limiting for the
process of glycogen synthesis (Mueckler and Thorens, 2013). Im et al. thought that hepatic Glut-2 might be a target of PPARγ agonists contributing to circulating glucose transport into liver (Im et al., 2005), indicating that its expression may be regulated by PPARγ.

Natural products derived from plants are generally considered to be less toxic and have fewer side effects than synthetic medicines. *Chrysanthemum morifolium* (CM) is from the dried anthodium of CM Ramat., a medicinal and edible plant. It contains a wide variety of natural active ingredients, such as polyphenols and three terpenes (Lin and Harnly, 2010; Miyazawa and Hisama, 2003; Yoshikawa et al., 2000; Ukiya et al., 2001), and has many beneficial functions and shows anti-inflammatory (Lee et al., 2009), antiviral (Lee et al., 2003), antioxidant (Lii et al., 2010; Kim and Lee, 2005), antitumor (Wang et al., 2010; Yang et al., 2011), antiallergic (Xie et al., 2012), and cardiovascular and hepatic protective activities (Jiang et al., 2005; Rusu et al., 2005). Our previous studies found that polyphenol-rich CM extract (CME) could decrease the hepatic lipid accumulation via the increment of PPARα expression in high fat milk-induced fatty liver mice (Cui et al., 2014). But there is no report of polyphenol-rich CME being used in the treatment of diabetes mellitus. In this study, the hypoglycemic effect of CME on alloxan-induced diabetic mice was evaluated, and its potential mechanism was also investigated.

It is well known that alloxan may cause an increase in the blood glucose via the specific toxic effect on the pancreatic β-cells (Lenzen, 2008). However, whether or not alloxan affects the synthesis of hepatic glycogen is unclear. The aim of our present study was to observe whether PPARα/γ-mediated hepatic glycogen synthesis involved in the alloxan-induced diabetes.

**Materials and methods**

**Chemicals, reagents, CME preparation, and polyphenol determination**

Dried flowers of CM Ramat., were obtained from Tongxiang, in the Zhejiang Province of China, and authenticated by Ye Lu at the College of Pharmaceutical Sciences, Soochow University. The chrysanthemum flowers (3.0 kg) were extracted with 75% ethanol (30 L) under reflux for 1.5 h, and the procedure was repeated 2 times. The liquids obtained were then pooled, concentrated under vacuum to 10 L, filtered, and applied to a prepared AB-8 resin column (70 cm × 15 cm i.d.). After sample loading and adsorption equilibrium, desorption was performed successively with water (25 L), 10% ethanol (10 L), and 70% ethanol (25 L) at a flow rate of 10 L/h. The 70% ethanol effluent was collected and dried to produce CME (218 g).

The contents of 5 predominant polyphenol ingredients in the CME were determined by high-performance liquid chromatography on a Shimadzu Prominence LC-20A liquid chromatographic system (Shimadzu Instruments Co., Kyoto, Japan). The chromatographic separation was accomplished on a C18 column (250 mm × 4.6 mm, 5 μm) at a column temperature of 30 °C, and the sample load was 20 μL. The flow of the mobile phase consisted of acetonitrile (with the following gradient program: 0–10 min, 10–18%; 11–30 min, 18–20%; and 31–45 min, 20%) and 0.1% acetic acid solution (w/v) was set at a rate of 0.1 mL/min, and the diode-array detection wavelength was set at 348 nm. The contents of chlorogenic acid and luteolin-7-β-glucoside in the CME were calculated according to their peak areas of respective reference substances, and the contents of other 3 polyphenols were also calculated by comparisons of their peak areas as previous reports (Lin and Harnly, 2010; Jia et al., 2004; Shen et al., 2010). The prepared CME contained 8.15% luteolin-7-β-glucoside, 3.82% chlorogenic acid, 9.91% apigenin-7-O-glucoside, 10.37% 3, 5-di-cafeoylquinic acid, and 5.86% 4, 5-di-cafeoylquinic acid. The CME used in this study was suspended in 0.5% sodium carboxymethyl cellulose solution prior to the onset of the experiment.

The assay kit for fasting blood glucose (FBG) was purchased from Shanghai Rongseng Biological Pharmaceutical Co., Ltd. (Shanghai, China). The assay kit for glycogen was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The ELISA kit for mouse serum insulin was obtained from Shanghai Xitang Biotechnology Co., Ltd. (Shanghai, China). Alloxan was procured from Sigma-Aldrich Co. (Saint Louis, MO, USA) and dissolved in normal saline. Anti-PPARα and anti-GS antibodies were products of Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-PPARγ, anti-GS, and anti-β-actin antibodies were products of Cell Signaling Technology (Boston, USA).

**Animals**

Kunming mice (male, 22 ± 2 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The animals were maintained in regular cages in a temperature (22 ± 2 °C) and humidity (55–60%) controlled room with a 12-h light (8:30–20:30)/12-h dark (20:30–8:30) cycle and allowed free access to food and water. These mice were allowed to acclimatize to the laboratory environment for 2 days prior to the study. The animal care and handling procedures were performed according to the protocols approved by the Animal Experimentation and Ethics Committee of Soochow University.

**Establishment of mouse diabetic model and treatment**

The experimental mice were randomized to either a control group or a model group. The latter was intravenously injected once with alloxan 58 mg/kg to induce diabetes mellitus. After 72 h, the FBG was measured and 30 diabetic mice of FBG level between 10 mmol/L and 25 mmol/L were selected. These diabetic mice were then divided into three groups (n = 10): diabetic model group, CME 150 mg/kg group, and CME 300 mg/kg group. The mice in the medicine-treated groups were orally given 0.2 mL/10 g (body weight per day) of CME by gavage based on different doses for 6 weeks. The control and diabetic model mice were treated with an equivalent volume of 0.5% sodium carboxymethyl cellulose solution in the same manner. The food and water consumption was recorded daily, and the body weight was measured twice per week. After 6 weeks of treatment with CME, all of these mice were euthanized by stunning followed by cervical dislocation. Blood, liver, and skeletal muscle of right hindlimb were collected for parameter measurements, and partial livers were quickly obtained and frozen in liquid nitrogen and stored at −80 °C for Western blot assay.

**Measurements of FBG and fasting serum insulin**

Mouse blood was collected into Eppendorf tubes after 10 h of overnight fasting and centrifuged at 3500 × g, at 4 °C for 5 min. The FBG was determined by colorimetric method according to the procedure provided, and the fasting serum insulin level was measured with ELISA.

**Measurements of hepatic and muscular glycogens**

Partial hepatic tissues and skeletal muscles were taken, mixed with an aqueous alkali according to a ratio of 1:3 (w/v), and then boiled for 20 min. The tissue concentration of the hydrolyzed solution was finally adjusted to 1% for hepatic glycogen test and 5% for muscular glycogen test, respectively. The sample solution obtained was used for glycogen measurement according to the manufacturer’s instruction.
Western blot analysis for protein expression

Hepatic protein was extracted using a commercial kit (Keygen Biotech, China) and the protein concentration was determined by the bicinchoninic acid kit (Beyotime Institute of Biotechnology, China). An aliquot of 50 μg protein from each sample was loaded onto 10% SDS-polyacrylamide gel subjected to electrophoresis, and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk at room temperature for 2 h. Afterwards, the membranes were incubated with the primary antibodies for PPAR-α (1:500 dilution), GS (1:500 dilution), and Glut-2 (1:200 dilution), PPARγ and β-actin (1:1500 dilution) at 4 °C overnight and then incubated with fluorescent secondary antibody of goat anti-rabbit at room temperature for 1 h. The reaction products were quantified by densitometry, using Odyssey infrared imaging system and Image J software. The ratio of the protein of interest was subjected to β-actin, which acted as the internal control in the experiments.

Statistical analysis

The data are expressed as the means ± SD, the one-way ANOVA followed by a post hoc LSD test was used for comparisons between groups. The statistical analysis was conducted using SPSS 20.0, with P < 0.05 considered statistically significant.

Results and discussion

In the present experiment, the results showed that the FBG level and food and water consumption in the model group were higher than those in the control group (P < 0.01) (Tables 1–3), while the body weight was lower in the model group than in the control group (P < 0.05 or P < 0.01) (Table 4), suggesting that a model of mouse diabetes induced by alloxan was successfully developed, and was in accordance with previous reports (Mordes and Rossini, 1981). After treatment of diabetic mice with 150 and 300 mg/kg CME for 6 weeks, the FBG level was reduced, the decreased percentages were gradually increased with therapeutic time prolonging, and the maximal percentages were 26.2% and 28.5% at the 6th week, respectively (P < 0.05) (Table 1), indicating that the hypoglycemic effect of CME exhibited a good time-effect relationship. Also, the food and water consumption was decreased by 14.9%–17.4% and 21.2%–23.7% at the 6th weekend, respectively (P < 0.01) (Tables 2 and 3). These results suggested that CME might exert a therapeutic effect on alloxan-induced diabetes in mice. However, the mouse’s body weight gain between the model and the CME-treated groups were insignificantly different (Table 4). The potential reason might be from the lipid-lowering effect of CME (Cui et al., 2014), and it is a valuable issue to research further.

Alloxan-induced experimental diabetes is generally acknowledged as a conventional model. In the present study, we observed that toxic alloxan treatment might decrease the fasting serum insulin and hepatic glycogen levels (P < 0.05 or P < 0.01) (Fig. 1A and C), and the latter was consistent with the levels of hepatic PPARα, GS, and Glut-2 protein expressions (P < 0.05 or P < 0.01) (Figs. 2 and 3). But the muscular glycogen level was only present in a decreasing tendency (Fig. 1B). As such, we believed that the reduction of PPARα and Glut-2-mediated hepatic glycogen synthesis might involve in the pathogenesis of alloxan-induced diabetes. In order to explore the possible hypoglycemic mechanisms of CME in treating alloxan-induced diabetes, we also examined these indexes. The results showed that after CME administration for 6 weeks, the level of hepatic glycogen was increased, especially in the 300 mg/kg group (P < 0.05), and next to the control level (Fig. 1A), but there was no significant variation in the content of fasting serum insulin...
Fig. 1. Hepatic glycogen, muscular glycogen, and fasting serum insulin levels after treatment with CME for 6 weeks in alloxan-induced diabetic mice. Values indicate the means ± SD of n = 10 mice per group. *P < 0.05, **P < 0.01 vs. control group; *P < 0.05 vs. model group.

Fig. 2. Hepatic PPARα and GS protein expressions after treatment with CME for 6 weeks in alloxan-induced diabetic mice. Values indicate the means ± SD of n = 3 mice per group. Protein bands were quantified relative to β-actin. *P < 0.05, **P < 0.01 vs. control group; **P < 0.01 vs. model group.
though with an increasing tendency (Fig. 1C). The CME treatment did not affect the level of muscular glycogen (Fig. 1B). These results revealed that the hypoglycemic effect of CME on diabetic mice might be mainly associated with the increment of hepatic glycogen synthesis.

Glycogen is a branched polysaccharide formed by the junction of numerous glucoses, and plays an important role in regulating glucose homeostasis (Roach et al., 2012). Several studies have indicated that the activation of PPARα/γ can inhibit the enhancement of blood glucose through interference with the glycogen synthesis (Im et al., 2005; Ortmeyer et al., 2005). In the present study, we examined the effects of CME on the hepatic PPARα/γ and their target genes involved in synthesis of hepatic glycogen and transport of glucose into liver. The results showed that after 6 weeks of CME administration, we observed an expected increase in the PPARα, PPARγ, GS, and Glut-2 protein expressions in hepatic tissue (P < 0.01) (Figs. 2 and 3), and these effects were dose-dependent in some extent. The GS is a key enzyme and controls the rate of glycogen synthesis (Bezborodkina et al., 2014). An increase in the expression of PPARα may increase the GS expression (Ortmeyer et al., 2005), and lead to the glycogen synthesis. Likewise, the Glut-2 is a main glucose transporter on hepatocytes and responsible for uptake and transport of glucose (Mueckler and Thorens, 2013). The increment of PPARγ can increase the Glut-2 expression and promote the transport of glucose for glycogen synthesis.

Recent studies show that, among the polyphenols in the CME, chlorogenic acid, dicaffeoylquinic acid, and apigenin can reduce the blood glucose in diabetic animals and glucose absorption in cultured Caco-2 cells via the inhibition of glucose transporters in vitro (Wang et al., 2008; Karthikesan et al., 2010; Ren et al., 2016), while luteolin may also improve the insulin resistance in obese mice (Kwon et al., 2015). So, we thought that these polyphenols in the CME might exert a synergic hypoglycemic effect via the PPARα/γ-mediated mechanisms, but the exact interaction between them is necessary to research further.

In conclusion, this study demonstrates that the reduction of PPARα/γ-mediated hepatic glycogen synthesis may involve in the alloxan-induced diabetes, and the hypoglycemic effect of polyphenol-rich CME on the diabetic mice may be related to increasing the synthesis of hepatic glycogen via increments of PPARα/γ-mediated GS and Glut-2 protein expressions. But it is necessary to investigate the exact effects of CME on PPARα/γ and related target genes by using specific PPARα/γ antagonists or the siRNA method in vivo and in vitro.

Conflicts of interest

The authors declare no conflicts of interest.

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