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Gossypol ameliorates liver fibrosis in diabetic rats induced by high-fat diet and streptozocin

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ABSTRACT

11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) inhibitors have been shown to treat type 2 diabetes (T2D). Since gossypol is an 11β-HSD1 inhibitor, the objective of the present study was to treat T2D and T2D-related liver fibrosis in rat model using low-dose gossypol. T2D was induced by feeding with high fat diet plus injection of streptozocin (30 mg/kg). Diabetic rats were treated with either vehicle control or racemic gossypol with a dose of 15 mg/kg/day for 4 weeks followed by 15 mg/kg/week for additional 8 weeks. Blood glucose, cholesterol, LDL, and triglycerides were measured. Messenger mRNA levels of glucocorticoid receptor (*Nr3c1*), phosphoenolpyruvate carboxykinase (*Pck1*), glucose-6-phosphatase (*G6pc*), collagen I (*Col1a1*), collagen III (*Col3a1*), fibronectin (*Fn1*), tissue inhibitor of metalloproteinase 1 (*Timp1*), and 2 (*Timp2*) were measured. T2D rats had higher serum glucose, cholesterol, LDL, and triglyceride levels compared to control. Liver *Nr3c1*, *Col1a1*, *Col3a1*, *Fn1*, *Timp1*, and *Timp2* were increased in T2D rats. T2D liver showed significant fibrosis with the increases of α-smooth muscle actin and fibronectin. After gossypol treatment, serum glucose level was lowered by 64%. Liver fibrosis was significantly ameliorated. *Nr3c1*, *Col1a1*, *Col3a1*, *Fn1*, *Timp1*, *Timp2*, *Pck1* as well as *G6pc* levels were significantly reduced. In conclusion, low dose gossypol is effective for the treatment of T2D and T2D-related fibrosis.

1. Introduction

The metabolic syndrome is a cluster of disorders including type 2 diabetes (T2D) and hyperlipidaemia that are caused by insulin resistance in important target tissues such as liver, muscle, and adipose tissue [26]. Although many hypotheses have been proposed for the causes of the metabolic syndrome, the exact mechanism is unknown. One of the hypothesis is that the metabolic syndrome is caused by the excessive glucocorticoid levels both systemically (such as in Cushing's syndrome) or locally (increased expression of 11β-hydroxysteroid dehydrogenase 1, 11β-HSD1) [5,15]. 11β-HSD1 is an oxidoreductase, primarily catalyzing the interconversion of active glucocorticoid cortisol (in humans) or corticosterone (in rats) and biologically inactive cortisone (in humans) and 11-dehydrocorticosterone (in rats). It is abundantly located in liver, muscle, and adipose, where it acts predominantly as a reductase to activate cortisone to cortisol, thus amplifying glucocorticoid action in a tissue-specific manner [31].

Correction of glucocorticoid excess or blocking glucocorticoid receptor (NR3C1) has been proven to be an effective way to treat the metabolic syndrome. For example, NR3C1 antagonist RU486 ameliorated diabetic and dyslipidemic profiles of patients with Cushing's syndrome [10,23] or 11β-HSD1 inhibitor carbenoxolone attenuated symptoms of metabolic syndrome in a mouse model [24] or human subjects [1]. NR3C1 blocking clinically is not acceptable because it activates hypothalamus-pituitary-adrenal axis [21]. Apparently, suppression of 11β-HSD1 has been proven a good approach to treat the metabolic syndrome [3].

Gossypol is a yellow lipid soluble polyphenolic compound purified from cotton seeds and has been demonstrated to be very effective as a male contraceptive [34]. We and others have shown that racemic gossypol significantly inhibited rat, guinea pig and human 11β-HSD1 [9,20,27]. Although gossypol also inhibited 11β-HSD2 (an isoform of 11β-HSD is mainly located in kidney) might cause the hypokalemia at higher dose (20–30 mg/kg for rats), low dose (15 mg/kg for rats) of regimen of gossypol has been proven to effective for male contraceptive without causing hypokalemia [11]. Therefore, low dose gossypol could also be effective way to treat the metabolic syndrome. The object of

Abbreviation: 11β-HSD1, 11β-hydroxysteroid dehydrogenase 1; 11β-HSD2, 11β-hydroxysteroid dehydrogenase 2; ALT, alanine aminotransferase; Cat, catalase; Colla1, collagen; Col3a1, collagen III; Fn1, fibronectin 1; FN, fibronectin; G6pc, glucose-6-phosphatase; Gpx1, glutathione peroxidase 1; Gsta, glutathione S-transferase; HE, hematoxylin-eosin; LDL, low density lipoprotein; Nr3c1, glucocorticoid receptor; Pck1, phosphoenolpyruvate carboxykinase; Rps16, ribosome protein S16; α-SMA, α-smooth muscle actin; STZ, streptozocin; T2D, type 2 diabetes; Tg, triglyceride; Timp1, tissue inhibitor of metalloproteinase 1; Timp2, tissue inhibitor of metalloproteinase 2.

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the present study was to use low dose racemic gossypol to treat diabetic rats induced by high-fat diet and streptozocin (STZ).

2. Materials and methods

2.1. Chemicals and animals

Racemic gossypol (purity 99%) was the gifts from Dr. Samuel S. Koide in Population Council (New York, NY). Male Sprague-Dawley rats (140–180 g) were purchased from Wenzhou Medical College Animal Center (Wenzhou, China). Racemic gossypol was suspended in 2% carboxymethyl cellulose for gavage. STZ was purchased for Xiamen Xinglongda Chemical Reagent Co., Ltd. (Xiamen, China) and dissolved in 0.1 mmol/L citrate buffer for injection. High fat diet was prepared by Wenzhou Medical College Animal Center and is composed of 10.0% lard, 20.0% sucrose, 2.5% cholesterol, 1.0% bile salt and 66.5% of conventional diet.

2.2. Type 2 diabetes (T2D) rat model and gossypol treatment.

Thirty male Sprague Dawley (body weight 140–180 g) were randomly divided into three groups: vehicle control, T2D and T2D with gossypol treatment, with 10 rats in each group. T2D rats were established by feeding with high fat diet for 4 weeks, followed by i.p. injection of STZ (once, 30 mg/kg) according to the previously reported method [20]. 72 h after injection of STZ, serum glucose levels were measured. If the serum glucose level was over ≥8 mmol/L, the rat was considered to be diabetic. Then these T2D rats were received 15 mg/kg/day gossypol for 4 weeks followed by 15 mg/kg/week gossypol for additional 8 weeks or vehicle control (2% carboxymethyl cellulose). The dose of gossypol was adopted according to that for its male contraception application [16]. Normal control rats received an injection of citrate buffer at the same period and then received 2% carboxymethyl cellulose through the experimental treatment. The fresh liver was taken and weighed to calculate the liver index [liver index (%) = liver weight/body weight × 100%]. The right liver lobe was fixed in 10% formalin for immunohistochemical staining.

2.3. Serum glucose, lipid and ALT analysis

After treatment, rats were sacrificed by CO2 and bloods were taken. Serum was collected after placing the bloods at room temperature for 25 min and being centrifuged at 1500 *g*/min for 20 min. Serum glucose, total cholesterol, low density lipoprotein (LDL), triglyceride (Tg), potassium, and sodium levels, as well as alanine aminotransferase (ALT) levels were measured using a Hitachi 7600 biochemical analyzer (Hitachi, Japan).

2.4. Primer selection

All primers in the present study were designed and synthesized by Shanghai Jikang Inc (Shanghai, China). Forward and reverse primers were in different exons to minimize the effects of possible DNA contamination. The primers of the following genes were designed: glucocorticoid metabolism and action including 11β-HSD2 (Hsd11b2) and glucocorticoid receptor (Nr3c1); tissue remodeling and fibrosis-related genes including tissue inhibitor of metalloproteinase 1 (Timp1) and 2 (Timp2), collagen type I α 1(Col1a1) and III α 1(Col3a1) as well as fibronectin 1 (Fn1); glucose metabolism genes including phosphoenolpyruvate carboxykinase 1 (Pck1) and glucose-6-phosphatase (G6pc); anti-oxidative genes including glutathione peroxidase 1 (Gpx1), glutathione S-transferase cluster (*Gsta*) and catalase (*Cat*); internal control ribosome protein S16 (*Rps16*). The selection of *Pck1* and *G6pc* genes as glucose metabolism was adopted as previously described [14]. The primers were listed in Table 1.

2.5. RNA extraction and RT-PCR

Total RNAs were isolated from liver samples using Trizol according to manufacturer's instructions (Invitrogen, USA). RNA pellets were resuspended in sterile ribonuclease-free water. Total RNAs were used as the template for cDNA synthesis by MMLV reverse transcriptase (Promega, USA) and primed with random hexamers. The reaction system for PCR was shown in Table 2. PCR products were then electrophoresed at 2% agarose gel and analyzed for intensity using Gel-pro31 gel analysis system (Media Cybernetics, Des. Moines, IA). The PCR application reaction for each gene was linear within 30 cycles. The efficiency

Table 1
Primers for liver and kidney genes.

Gene name	Symbol	Accession#	Primers and Probes	Function
11β-hydroxysteroid dehydrogenase 2	*Hsd11b2*	NM017081	5′-TTCCGCGAATGTATGG-3′ 5′-ACGGCCCGTGTAGTAGC-3′	Glucocorticoid action in liver and kidney
Glucocorticoid receptor	*Nr3c1*	NM_012576	5′-CCCTGGGTTGGAGATCATACA-3′ 5′-TCAGAGGAGACAAACAGCATGTG-3′	
Tissue inhibitor of metalloproteinase 1	*Timp1*	NM053819	5′TTTCCGGTTCGCCTACA-3′ 5′-GGAAGGCTTCGGGTCAT-3′	Tissue modeling and fibrosis in liver
Tissue inhibitor of metalloproteinase 2	*Timp2*	NM-021989	5′-CAAAGCAGTGAGCGAGAAGGAGGT-3′ 5′-CGTGTCCCAGGGCACAATAAAGTCA-3′	
Collagen, type I α 1	*Col1a1*	Z78279	5′-CTGGATGCCATCAAGGTCTACTGC-3′ 5′-CAAGCGTGCTGTAGGTGAATCGAC-3′	
Collagen, type III α 1	*Col3a*	AJ005395	5′-CTGGATGCCATCAAGGTCTACTGC-3′ 5′-CAAGCGTGCTGTAGGTGAATCGAC-3′	
Fibronectin 1	*Fn1*	NM019143	5′-CCATTCCTGCGCCAACCAATC-3′ 5′-GAGAGCTCCGGGCATTGTCGT-3′	
Phosphoenolpyruvate carboxykinase 1	*Pck1*	NM198780	5′-GCCGACCTCCCTTAGAAATAG-3′ 5′-CGAACTTCGGAGAACAAGACGTGA-3′	Glucose metabolism in liver
Glucose-6-phosphatase	*G6pc*	NM_013098	5′-AGAGACTGTGGGCATCAATCT-3′ 5′-CCGGAATCCATACGTTGATT-3′	
Glutathione peroxidase 1	*Gpx1*	NM_030826	5′-CACAGTCCACCGTGTATGCC-3′ 5-CACCATTCACCTCGCACTTC-3′	Anti-oxidative proteins
Glutathione S-transferase cluster	*Gsta*	J03752	5′- GGACTGACGAGAAGGTGGAA-3′ 5′-AAGGGTAGAGCCTGTGGATG-3′	
catalase	*Cat*	NM_012520	5′-TATTGCCGTCCGATTCTC-3′ 5′-ATGCCCTGGTCAGTCTTG-3′	
Ribosome protein S16	*Rps16*	X17665	5′-AAGTCTTCGGACGCAAGAAA-3′ 5′-TTGCCCAGAAGCAGAACAG-3′	Internal control