Beneficial effect of farnesoid X receptor activation on metabolism in a diabetic rat model

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Abstract. Farnesoid X receptor (FXR) is an important regulator of glucose and lipid homeostasis. However, the exact role of FXR in diabetes remains to be fully elucidated. The present study examined the effects of chenodeoxycholic acid (CDCA), an agonist of FXR, on metabolism profile in a rat model of type 2 diabetes mellitus (T2DM). Male Wistar rats (8-week-old; n=40) were randomized into the following four groups (n=10): Untreated control, CDCA-treated, T2DM, and CDCA-treated T2DM. To establish the T2DM model, the rats were fed a high-fat diet (HFD) for 4 weeks and received a single low-dose intraperitoneal injection of streptozotocin (30 mg/kg), followed by an additional 4 weeks of HFD feeding. CDCA was administrated (10 mg/kg/d) intraperitoneally for 10 days. Reverse transcription-quantitative polymerase chain reaction and western blotting assays were performed to determine the RNA and protein expression of FXR, phosphoenolpyruvate carboxykinase, G6Pase, proliferator-activated receptor-γ coactivator-1 and short heterodimer partner in rat liver tissue. The results revealed that FXR activation by CDCA did not reduce body weight, but it lowered the plasma levels of fasting glucose, insulin and triglycerides in the T2DM rats. CDCA administration reversed the downregulation of the mRNA and protein expression of FXR in the T2DM rat liver tissue samples. Furthermore, treatment with CDCA reduced the mRNA and protein expression levels of phosphoenolpyruvate carboxykinase, glucose 6-phosphatase and peroxisome proliferator-activated receptor-γ coactivator-1 in the liver tissue samples of the T2DM rats. By contrast, CDCA treatment increased the mRNA and protein expression levels of short heterodimer partner in the liver tissue samples of the T2DM rats. In conclusion, FXR agonist treatment induces beneficial effects on metabolism in the rat T2DM model. In conclusion, the present study indicated that the FXR agonist may be useful for the treatment of T2DM and hypertriglyceridemia.

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Introduction

Type 2 diabetes mellitus (T2DM) presents a global epidemic with an estimated global prevalence of 8.3%, and with the World Health Organization predicting that the current number (170,000,000) of patients with diabetes diagnosed with T2DM are likely to more than double by 2030 (1). It is well-established that T2DM is a metabolic disorder, characterized by impaired pancreatic β-cell function and insulin resistance in target tissues (2). Abnormalities of glucose and insulin result in hyperglycemia, together with dysregulation of carbohydrate, fat and protein metabolism. Severe complications, including retinopathy (3), nephropathy (4,5) and cardiovascular disease (6,7), contribute to the deleterious consequences of T2DM. Although the progression of clinical complications of T2DM can be prevented or delayed by effective glycemic control (8), the current medical management for T2DM may yield unsatisfactory results due to the lack of comprehensive awareness with regards to the physiological basis of T2DM. Currently, an unhealthy lifestyle and high-fat diet (HFD), as well as genetic background, are considered to be important indicators of T2DM and T2DM-associated obesity (9). In animal models and in humans, T2DM and T2DM-associated obesity have been found to be correlated with a decrease in whole body insulin sensitivity, which is also referred to as insulin resistance (10). Improvements in current understanding of the development of insulin resistance and pathogenesis in T2DM may lead to the development of novel approaches for the prevention and control of this chronic disease.

Nuclear receptors are ligand-activated transcription factors, which govern aspects of major metabolic pathways by regulating gene expression via binding to specific response elements in the promoters of target genes. Farnesoid X receptor (FXR) is a member of the nuclear receptor family, and is expressed at high levels in the liver (11). FXR was found to serve as a receptor for physiological concentrations of several bile acids (BA), among which chenodeoxycholic acid (CDCA) is the most potent (12). FXR is important in maintaining BA, cholesterol, glucose and lipid levels by promoting BA efflux from the liver, inhibiting hepatic BA synthesis and intestinal absorption (11). Furthermore, FXR regulates lipid metabolism, insulin sensitivity and energy homeostasis, which are closely associated with T2DM. In diabetic liver tissue samples, FXR expression levels are significantly lower, compared with those of a normal control (13), and administration of FXR agonists has been shown to lower blood glucose and lipid levels (14-16). FXR activation suppresses hepatic gluconeogenic expression, and increases hepatic glycogen synthesis and glycogen content via a mechanism involving enhanced insulin sensitivity (14). Therefore, therapeutic strategies, which target FXR may to hold promise for the treatment of T2DM. However, the beneficial effects of an FXR agonist in metabolic disorders has been challenged in several previous studies. A study in obese FXR-knockout animals demonstrated that genetic ablation of FXR, as opposed to FXR activation, improved glucose intolerance (17). Another investigation reported that FXR knockout in mice gained less body weight in *ob*/*ob* mice (18). Therefore, the potential therapeutic value of FXR activation on metabolism and lipid profile remain in dispute.

The present study investigated the therapeutic effects of FXR activation by CDCA in a rat T2DM model, which was induced by feeding with a HFD for 8 weeks with a single streptozotocin (STZ) injection. The expression levels of several metabolism-associated proteins, including phosphoenolpyruvate carboxykinase (PEPCK), glucose 6-phosphatase (G6Pase), short heterodimer partner (SHP) and peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1α) were evaluated in liver tissue samples using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting. The study aimed to determine whether, in the HFD and STZ-injection induced diabetic model, FXR activation improved or impaired the metabolic profile in diabetes.

Materials and methods

Animals. Male Wistar rats (8-week-old; n=40) were purchased from the Animal Center of Tongji Medical College (Huazhong University of Science and Technology, Wuhan, China). The animals were housed in controlled conditions (temperature 23±2˚C, humidity 60±10% and lighting 8 a.m-8 p.m) with access to water *ad libitum*. All experiments were performed in accordance with the National Institutes of Health Guidelines on the Use of Laboratory Animals (19) and were approved by the Animal Care Committee of Hubei Integrated Traditional Chinese and Western Medicine Hospital, Hubei University of Chinese Medicine (Wuhan, China).

T2DM model and treatment. The rats were fed a HFD consisting of 30% fat, 50% carbohydrate, 18% protein and 2% fiber (Wuhan Feiyi Technology Co., Ltd., Wuhan, China) for 4 weeks. At the end of the fourth week, the rats were administered with a single low-dose (30 mg/kg) intraperioneal injection of STZ (Sigma-Aldrich, St. Louis, MO, USA). Rats fed a regular diet were administered with an intraperioneal injection of 0.1 mol/l sodium citrate buffer (Sigma-Aldrich) as a vehicle. The provision of the HFD and regular diet continued for a further 4 weeks, following which blood was collected from the femoral vein following 12 h fasting, and rats with plasma glucose >16 mmol/l were identified as having diabetes. The control and the diabetic rats were randomly separated into two subgroups (n=10) and treated for 10 days with either an intraperitoneal injection of CDCA (10 mg/kg body weight/day; Sigma-Aldrich) or saline. The 2 h postprandial blood glucose concentration was then assayed. The following day, subsequent to overnight fasting, the rats were anesthetized with intraperitoneal phenobarbital sodium (40 mg/kg; Sigma-Aldrich) and blood was collected from the tail for the determination of fasting plasma glucose, lipid and insulin concentrations. Blood glucose concentration was measured using the blood obtained from a tail vein at the indicated time points with a OneTouch Ultra Glucometer (Lifescan, Burnaby, Canada). Body weight was recorded following anesthetization. The biochemical assays were performed using a Hitachi-7060C Autoanalyzer (Hitachi, Ltd., Tokyo, Japan) using kits supplied by Roche Diagnostics (Basel, Switzerland; cat. no. 11213070201). Fasting plasma insulin was assayed using a commercially available radioimmunoassay kit obtained from the China Institute of Atomic Energy (Beijing, China; cat. no. 20120125). In addition, liver tissue samples were collected for subsequent experiments. Left lateral liver tissue was dissected and washed in ice-cold deionized water three times. Subsequently, the rats were injected with phenobarbital sodium (200 mg/kg) for sacrifice.

RT-qPCR. RT-qPCR was performed, as previously described (20). The mRNA expression levels of FXR, PEPCK, G6Pase, SHP and PGC-1α were assayed using RT-qPCR on a 7300 Real-Time PCR Detection system (ABI; Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and normalized to β-actin. Tissues were homogenized in TRIzol® reagent. RNA was isolated from the liver tissue samples using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and 2 μg total RNA was reverse transcribed to cDNA using reverse transcriptase (Takara Bio, Inc., Dalian, China). The RT-qPCR reaction mixture contained 0.4 μM primers (5'-3'; Invitrogen; Thermo Fisher Scientific, Inc.) and 2 μl cDNA in SYBR® Green Supermix (Toyobo, Osaka, Japan) (21). The experimental mRNA expression levels were expressed as the percentage change relative to the control (β-actin). The thermocycling steps were as follows: 95˚C for 60 sec, 95˚C for 15 sec and 60˚C for 60 sec for 40 cycles. The primers used are listed in Table I. Data were normalized using β-actin as an internal control and calculated using the comparative Cq method ($2^{-\Delta\Delta Cq}$) as described previously (22).

Western blot analysis. Western blot analysis was performed, as previously described (23). The protein expression levels

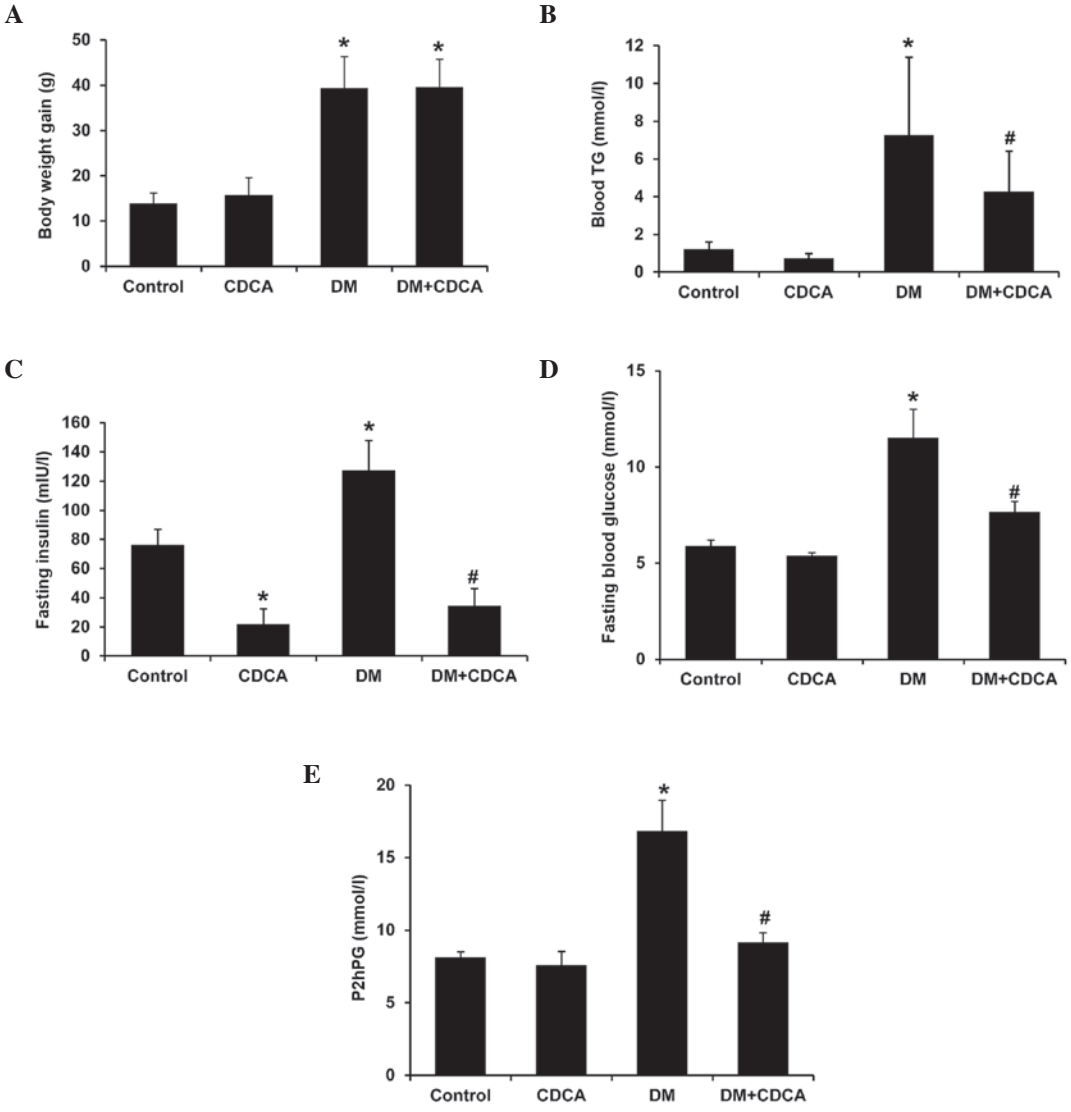

Figure 1. Effect of CDCA on body weight and blood parameters. (A) Body-weight gain. (B) Fasting blood TG levels. (C) Fasting plasma insulin levels. (D) Fasting blood glucose concentration. (E) 2 h postprandial blood glucose concentration. Results are expressed as the mean ± standard error of the mean (n=10). *P<0.05, vs. control; #P<0.05, vs. DM. TG, triglycerides; CDCA, chenodeoxycholic acid; DM, diabetes mellitus; P2hPG, 2 h postprandial blood glucose.

of FXR, PEPCK, G6Pase, SHP, and PGC-1α were assayed using western blot analysis. Liver tissues were homogenized in radioimmunoprecipitation assay lysis solution and centrifuged at 12,000 x g. The supernatant was collected and the protein concentration was determined using a BCA assay (Beyotime Institute of Biotechnology, Haimen, China). Liver proteins (30-50 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Sigma-Aldrich) and blotted onto nitrocellulose membranes (Millipore, Bedford, MA, USA). The membrane was blocked using 4% evaporated milk for 4 h. The membranes were then incubated with the following antibodies: Goat anti-FXR (cat. no. sc-1204; 1:200; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), mouse anti-PEPCK (cat. no. sc-271204; 1:200; Santa Cruz Biotechnology, Inc.), goat anti-G6Pase (cat. no. sc-33839; 1:200; Santa Cruz Biotechnology, Inc.), mouse anti-SHP (cat. no. sc-271470; 1:200; Santa Cruz Biotechnology, Inc.), rabbit anti-PGC-1α (cat. no. ab-54481; 1:1,500; Abcam, Cambridge, UK) or rabbit actin (cat. no. sc-1616R; 1:2,000; Santa Cruz Biotechnology, Inc.) polyclonal antibodies. Following being washed with phosphate buffered-saline with Tween 20 (PBST; 5 min, three times), the membranes were then incubated with corresponding HRP-conjugated goat anti-mouse IgG (cat. no. 074-1806) goat anti-rabbit IgG (cat. no. 074-1506) and biotinylated goat anti-rat IgG (cat. no. 71-00-31) secondary antibodies (1:3,000 dilution; KPL, Inc., Gaithersburg, MD, USA). Subsequently, the membranes were washed with PBST again (5 min, three times). The protein bands were visualized using enhanced chemiluminescence reagents (Millipore) and analyzed with Quantity One Software (version 4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA) (24). The membranes used in the western blot assays were re-probed with rabbit anti-β-actin polyclonal antibody (Santa Cruz Biotechnology, Inc.) to confirm equal loading of proteins for each sample.

Statistical analysis. All results are expressed as the mean ± standard error of the mean. The results were analyzed using either a two-tailed Student's t-test or one-way analysis

Table I. Sequences of the primers used for reverse transcription-quantitative polymerase chain reaction.

Gene	Accession number	Primer	Fragment length (bp)
FXR	NM_021745	5'-GCGAAAGTGCTGGGCTTTG-3' 5'-TGTGCTTCTGGGATGGTGGT-3'	118
PEPCK	NM_001108377.2	5'-ACCAGTGATGGCGGTGTGTA-3' 5'-AAAGCGAGAGTTTGGATGCG-3'	132
G6-Pase	NM_013098.2	5'-CAGCTCCGTGCCTCTGATAAA-3' 5'-CAATGCCTGACAAGACTCCAG-3'	281
SHP	NM_057133.1	5'-TCTCTTCCTGCTTGGGTTGG-3' 5'-GTGAGGGTTGTGGTGGGTCT-3'	146
PGC-1α	NM_031347.1	5'-ACAGGTCGTGTTCCCGATCA-3' 5'-CTTTCAGACTCCCGCTTCTCA-3'	259
β-actin	NM_031144	5'-CGTTGACATCCGTAAAGACCTC-3' 5'-TAGGAGCCAGGGCAGTAATCT-3'	110

FXR, farnesoid X receptor; PEPCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose 6-phosphatas; SHP, short heterodimer partner; PGC-1α, proliferator-activated receptor-γ coactivator-1.

of variance, followed by a Newman-Keuls post-hoc test. Statistical analyses were performed using Prism 4 (GraphPad software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of FXR activation on blood biomedical parameters in T2DM rats. Body weight, blood TG levels, fasting insulin levels, fasting glucose levels and 2 h postprandial plasma glucose levels in the T2DM rats were significantly higher, compared with those in the rats fed regular diets (Fig. 1A-E). CDCA treatment had no effect on body weight gain (Fig. 1A). However, CDCA treatment decreased the levels of blood TG (Fig. 1B), fasting insulin (Fig. 1C), fasting glucose (Fig. 1D) and 2 h postprandial plasma glucose (Fig. 1E). These results indicated that FXR activation improves glucose and lipid metabolism in the T2DM rats.

Effects of FXR activation on the expression levels of FXR in liver tissue samples. The mRNA and protein expression levels of FXR were significantly lower in the liver tissues of the T2DM rats, compared with those of the control rats (Fig. 2). FXR activation by CDCA treatment significantly increased the mRNA and protein expression levels of FXR in the liver tissue samples of rats with T2DM (Fig. 2).

Effects of FXR activation on hepatic gluconeogenesis in the liver. PEPCK and G6Pase are two important enzymes catalyzing the rate-limiting steps of hepatic gluconeogenesis (25). The mRNA and protein expression levels of PEPCK and G6Pase were significantly increased in the liver tissue samples of the T2DM rats (Fig. 3). FXR activation lowered the upregulated expression levels of PEPCK and G6Pase in the T2DM rats (Fig. 3). These results indicated that CDCA treatment repressed hepatic gluconeogensis in diabetes.

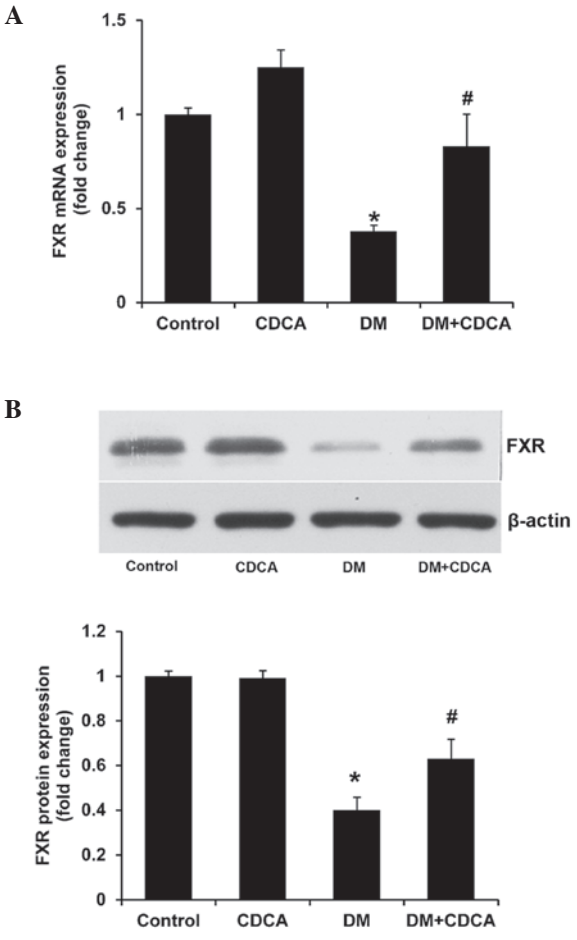

Figure 2. mRNA and protein expression levels of FXR in rat liver tissues. (A) mRNA expression levels of FXR were determined using reverse transcription-quantitative polymerase chain reaction. (B) Protein expression levels were determined using western blotting. The results are presented as the mean ± standard error of the mean (n=10). *P<0.05, vs. control; #P<0.05, vs. DM. FXR; farnesoid X receptor; CDCA, chenodeoxycholic acid; DM, diabetes mellitus.

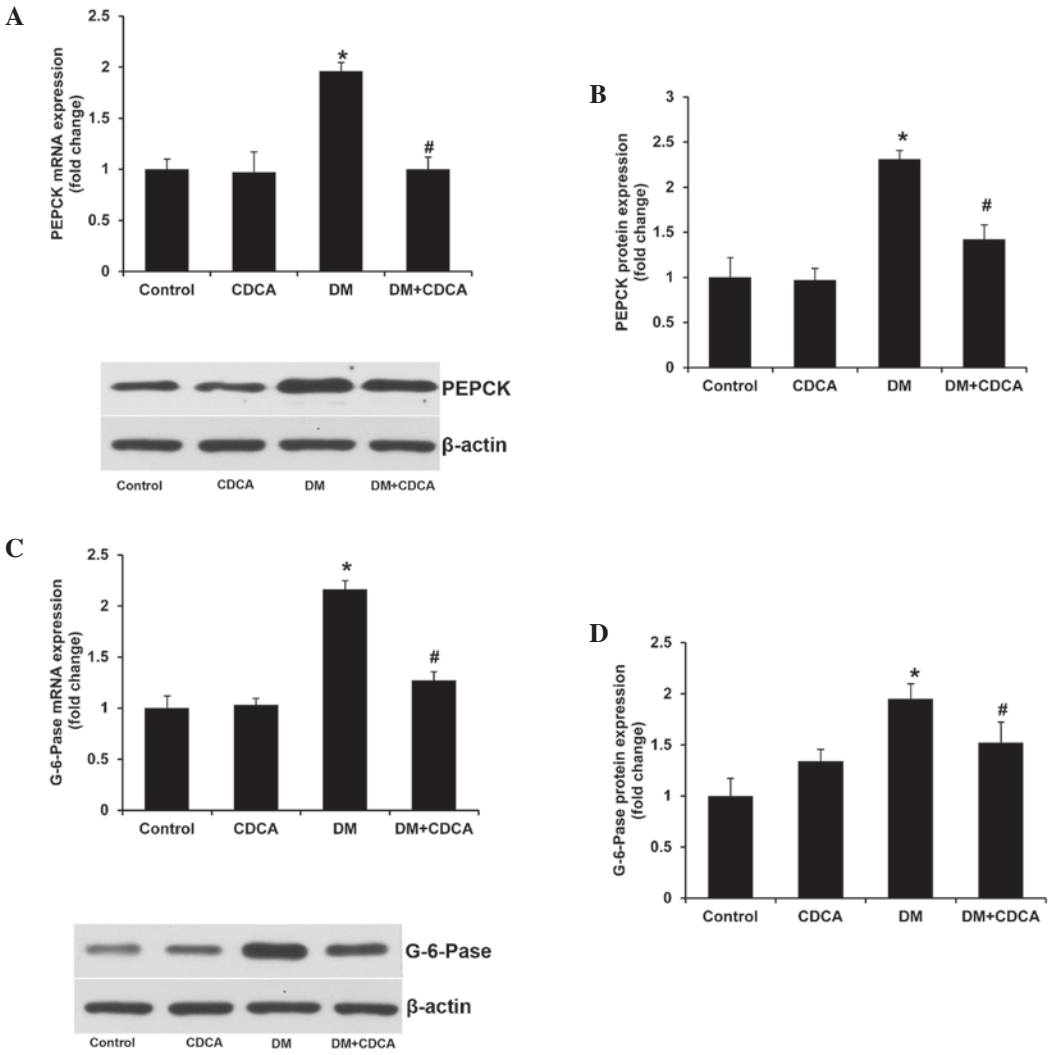

Figure 3. mRNA and protein expression levels of PEPCK and G6Pase in rat liver tissues. (A) mRNA expression levels of PEPCK were determined using RT-qPCR. (B) Protein expression levels were determined using western blotting. (C) mRNA expression levels of G6Pase were determined using RT-qPCR and (D) protein expression levels were determined by western blotting. Results are expressed as the mean ± standard error of the mean (n=10). *P<0.05, vs. control; #P<0.05, vs. DM. PEPCK, phoenolpyruvate carboxykinase; G6Pase, glucose 6-phosphatase; CDCA, chenodeoxycholic acid; DM, diabetes mellitus; RT-qPCR. reverse transcription-quantitative polymerase chain reaction.

Effects of FXR activation on FXR-associated gene expression levels in the liver. PGC-1α and SHP are two FXR-associated genes, and PGC-1α activates FXR-mediated transcription in a ligand-dependent manner (26), whereas the nuclear receptor, SHP, is a target of FXR (27). In the present study, the mRNA and protein expression levels of PGC-1α (Fig. 4) were significantly higher in the liver tissues of the T2DM rats, compared with those in the control rats, and these expression levels were significantly decreased following treatment with CDCA (Fig. 4). Notably, the mRNA and protein expression levels of SHP in the liver tissues of the T2DM rats were significantly lower, compared with those in the control rats (Fig. 5), and treatment with CDCA partly reversed this decreased expression (Fig. 5).

Discussion

The present study examined the effects of FXR activation by CDCA in a rat diabetic model. The results confirmed that FXR activation lowered body weight, and levels of blood glucose and insulin in the rats. In addition, FXR activation partially inhibited the downregulation in the mRNA and protein expression of FXR in T2DM rats. Furthermore, the upregulated expression levels of PEPCK, G6Pase and PGC-1α, as well as the downregulated expression of SHP in the liver tissues of the T2DM rats were partly reversed by FXR activation. These results supported the hypothesis that the activation of FXR is beneficial in diabetes treatment.

T2DM is one of the most prevalent endocrine disorders in developing and developed countries. Despite advances in understanding of the pathophysiology of T2DM, and substantial progress in efforts to control T2DM, the global burden of this disease remains high (28). Several novel therapeutic strategies have been developed. Novel therapeutic targets include glucagon-like peptide-1, dipeptidyl peptidase-4 and sodium-glucose co-transporter 2 inhibitors (29). However, there remains a requirement to identify novel molecules, which

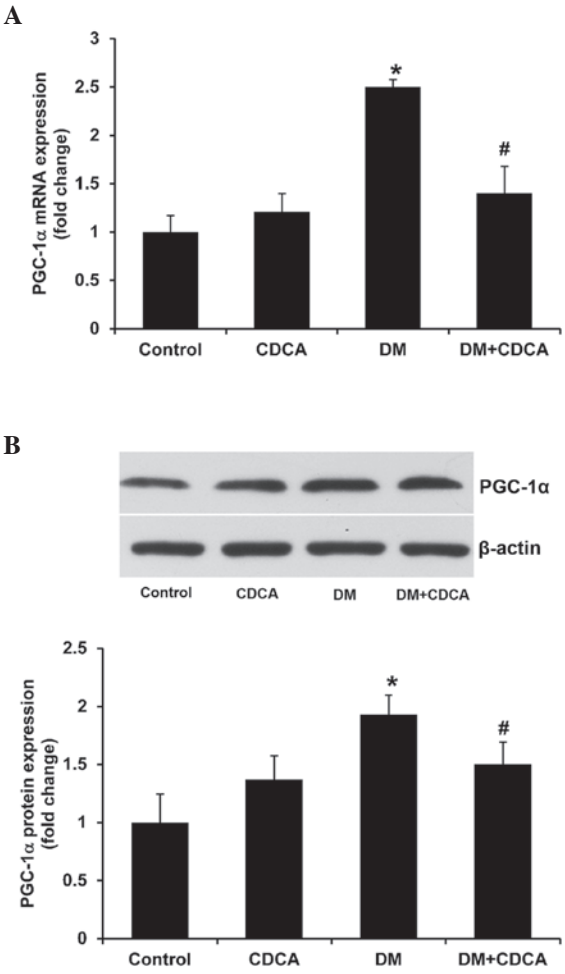

Figure 4. mRNA and protein expression levels of PGC-1 in rat liver tissues. (A) mRNA expression levels of PGC-1α were determined using reverse transcription-quantitative polymerase chain reaction. (B) Protein expression levels were determined using western blotting. Results are expressed as the mean ± standard error of the mean (n=10). *P<0.05, vs. control; #P<0.05, vs. DM. PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1; CDCA, chenodeoxycholic acid; DM, diabetes mellitus.

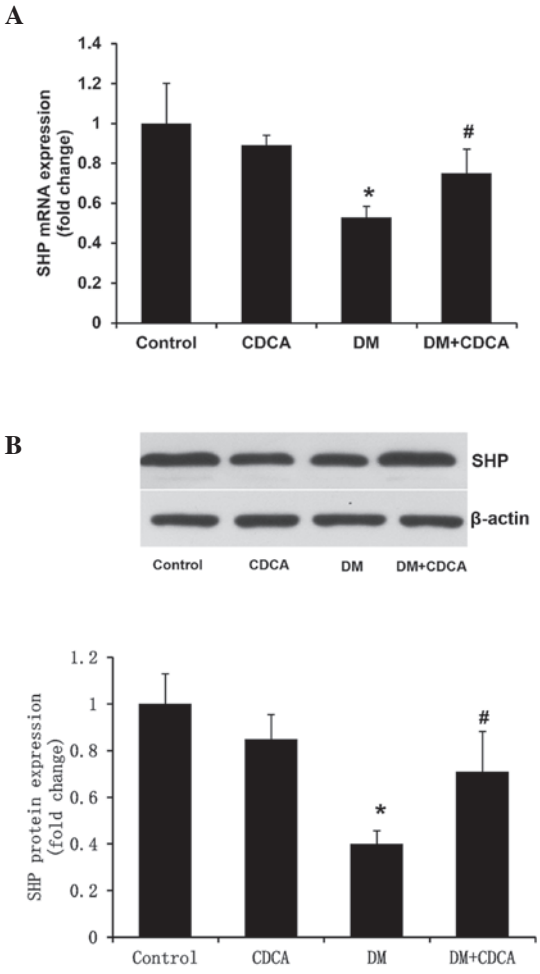

Figure 5. mRNA and protein expression levels of SHP in rat liver tissues. (A) mRNA expression levels of SHP were determined using reverse transcription-quantitative polymerase chain reactio. (B) Protein expression levels were determined using western blotting. Results are expressed as the mean ± standard error of the mean (n=10). *P<0.05, vs. control; #P<0.05, vs. DM. SHP, short heterodimer partner; CDCA, chenodeoxycholic acid; DM, diabetes mellitus.

are more specific and effective against T2DM. The primary aim of the present study was to determine whether CDCA, a synthetic FXR-specific agonist, is able to reduce hyperglycemia and hyperinsulinemia in rats with T2DM, which was induced by a HFD for 8 weeks and a low-dose STZ injection. Administration of CDCA significantly decreased levels of fasting and post-prandial plasma glucose, TG concentrations, and insulin levels in the rats with T2DM. RT-qPCR and western blotting demonstrated that CDCA treatment partly reversed the abnormal mRNA and protein expression levels of FXR, G6Pase, SHP and PGC-1α in the T2DM rats.

The beneficial effects of FXR have been reported in numerous studies. Activation of FXR by GW4064 or hepatic overexpression of constitutively active FXR significantly lowered blood glucose levels in obese *db*/*db* and wild-type mice (14). Treatment with GW4064 also improves whole body insulin resistance in obese *ob*/*ob* mice *in vivo* (16). The activation of FXR also promotes insulin sensitivity in the liver and skeletal muscles (30,31). However, other studies did not report concordant results. Prawitt *et al* (17) demonstrated that the deletion of FXR improved adipose tissue, but not hepatic insulin sensitivity, in *ob*/*ob* mice, suggesting that FXR deficiency, not activation, beneficially affects body weight and glucose homeostasis in obesity. Furthermore, the previous study demonstrated that total, but not liver-specific, FXR deficiency protects from diet-induced obesity and insulin resistance (17), indicating that liver FXR may not be important for glucose and lipid metabolism in obesity. Another investigation also reported that FXR knockout mice gained less body weight in an *ob*/*ob* mice background (18), indicating that loss of FXR prevents diet-induced or genetic obesity, and accelerates liver carcinogenesis under diabetic conditions. Furthermore, in a vertical sleeve gastrectomy model, Ryan *et al* (32) reported that the preoperative weight of wild-type mice was already markedly higher, compared with that of FXR-deficient mice, and that sham-operated wild-type mice gained an additional 10 g during the experiment, whereas sham-operated FXR-deficient mice remained at preoperative weight. This suggested that FXR exerts a detrimental effect on the control of body weight in diabetes. In the present study, the results demonstrated that FXR activation led to reduced body weight gain, lower blood glucose/insulin levels and improved metabolism in a T2DM

rat model established by a HFD and a single injection of STZ. These data support the beneficial effect of FXR activation in diabetes.

Previous studies have indicated that PGC-1α, G6Pase, SHP and PEPCK may be involved in the beneficial effects of FXR agonist in different animal models (33,34). PGC-1α may be one of the most critical metabolic switches in diabetes (35), obesity (36) and exercise-inducing effects (37,38). Wang *et al* (39) reported that the orphan nuclear receptor, SHP, represses the promoter activity of PGC-1α. and PGC-1α has also been demonstrated to be closely associated with the gluconeogenic gene, PEPCK, and G6Pase (34,40). Therefore, a reduction in the levels of FXR in T2DM may inhibit the expression of SHP and PGC-1a, resulting in an increase in PEPCK and G6Pase activities. However, the direct effect of FXR activation on expression levels of PGC-1α, G6Pase, SHP and PEPCK in the liver has not been reported. The results of the RT-qPCR and western blotting in the present study demonstrated that FXR activation by CDCA downregulated the expression levels of PGC-1α, G6Pase and PEPCK, and upregulated the expression of SHP in liver tissues. Deficits of PGC-1α, G6Pase and PEPCK may be an important underlying cause of insulin resistance (41). In addition, the overexpression of SHP recovered impaired glucose-stimulated insulin secretion (42). Therefore, the reversal of changes to the expression levels of PGC-1α, G6Pase, PEPCK and SHP by FXR activation suggested the presence of improved insulin sensitivity in the rat T2DM model. These results were concordant with a putative role for these proteins in the pathogenesis and treatment of T2DM (43), suggesting that the FXR agonist is able to attenuate the development of insulin resistance and T2DM in the rat model.

In conclusion, the results of the present study demonstrated that the FXR agonist reduced blood glucose/insulin levels and partly inhibited the changes in the expression of metabolism-associated genes in the liver tissue s of T2DM rats. These results may provide additional evidence to support the current hypothesis that FXR agonists may be useful in the treatment of T2DM and hypertriglyceridemia.

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