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The alterations of bile acids in rats with high-fat diet/streptozotocin-induced type 2 diabetes and their negative effects on glucose metabolism

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ABSTRACT

Purpose: Bile acids (BAs) as a kind of endogenous and signaling molecules altered under the circumstance of T2DM, which could impact on the relevant pathways to further affect the glucose metabolism and insulin secretion and might be associated with the T2DM development and restoration. However, the potential mechanisms still need more various and multifaceted studies. Here, we explored the alterations of BAs features and their mechanisms, and discussed the potential effects of the altered BAs on the glucose metabolic disorder via the relevant signaling pathways.

Main methods: The high-fat diet (HFD) feeding combining with injection of low-dose streptozotocin (STZ) was employed for inducing the T2DM rat model. Based on that, we investigated the alterations of the concentrations and compositions of BAs and their mechanisms, and explored the effects of the altered BAs on the glucose metabolic disorder via farnesoid X receptor (Fxr) and G protein-coupled bile acid receptor (Tgr5)-mediated pathways.

Key findings: In rats with T2DM, the BAs in rats with T2DM exhibited characteristic alterations, especially the increased ratio of 12α-OH to non-12α-OH BAs in serum, which could be ascribed to the up-regulated Cyp8b1 mRNA expression ratio in the liver. Moreover, Additionally, the altered BAs had negative effects on glucose metabolic disorder via inhibiting the Trg5/Fxr-mediated pathways in colon, liver and pancreas in rats with T2DM.

Significance: BAs in rats with T2DM exhibited the characteristic alterations, which could provide a cue for searching biomarkers of the T2DM diagnosis, and the altered BAs might aggravate the glucose metabolic disorder.

1. Introduction

Diabetes mellitus is the third prevalent disease, and up to 90% of diabetes mellitus is type 2 diabetes mellitus (T2DM). According to WHO statistics in 2018, 422 million people, one in every eleven, are living with T2DM, which is the seventh leading cause of death worldwide. Moreover, the prevalence is predicted to continue rising if the current trends prevail, and the International Diabetes Federation estimated the global number of T2DM would dramatically increase to approximately 650 million in 2040, [1,2]. Unfortunately, T2DM is a complex endocrine and metabolic disorder, of which the pathogenic and progressive mechanisms and the complications are various and unclear, resulting in the difficulty for treatment T2DM [3]. In addition, it has been reported that diabetes even the different stages of diabetes exhibit characteristic alterations of some endogenous molecules, and analyzing the metabonomic characteristics will contribute to developing the clinical prediction technologies for the diagnosis of T2DM [4–8].

Bile acids (BAs) are a notable kind of metabolome in vivo, and they are also recognized as the natural ligands of farnesoid X receptor (Fxr) and the G protein-coupled bile acid receptor (Tgr5), playing an important role in controlling insulin secretion and glucose metabolism [9–15]. It has been demonstrated that the characteristics of BAs showed remarkable alterations in the context of T2DM [9,16,17]. Notably, it is conceivable that BAs disorder resulted from T2DM would further impact on the glucose metabolic disorder and have a relationship with T2DM development [11,13,18]. However, the potential mechanisms of

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the BAs alterations and the effects of altered BAs on glucose metabolism under the condition of T2DM have not been identified clearly and need more various and multifaceted studies. There are two aims in the present study. The first one is exploring the characteristics and the mechanisms of the alterations of BAs under the condition of T2DM. The other one is elucidating the underlying effects of the altered BAs on the already generated glucose disorder via the BAs-mediated signaling pathways on glucose homeostasis during T2DM.

High-fat-diet (HFD)-induced obesity is a high-risk pathogenic factor for T2DM, and it is the major pathogenesis basis and one of the key symptoms of T2DM [19–21]. Hence, to address the questions we mentioned above practically, in the present study, we firstly employed the T2DM rat model, which is developed by intraperitoneally injecting low-dose streptozotocin (STZ) to HFD-induced obese rat, and this model has been utilized and verified by many studies [22], and chose the HFD-induced obese rats with the normal blood glucose level as the control. We investigated the alterations of BAs concentrations and compositions in vivo and the mechanisms about the BAs alterations from rats with obesity to T2DM, and explored the further effects of the altered BAs on the glucose metabolic disorder via the Fxr/Tgr5-mediated signaling pathways in liver, colon and pancreas, which have significant correlation with insulin secretion and glucose metabolism. Generally, it is expected that the findings from this study can provide one more cue for further studies on the interaction and relationship between BAs and glucose metabolism under the condition of T2DM.

2. Materials and methods

The experimental animals study for the current work was implemented according to the guidelines of The First Hospital of Lanzhou University Ethics Review Committee and approved by the Ethics Committee for Animal Experiments of The First Hospital of Lanzhou University, and the protocol number is LDYYLL2017-72. Male Wistar rats (around 80–100 g) used as the experimental animals acclimatized in a standard laboratory in a 12-h light/dark cycle with food and water continuously available for one week before initiating the experiment.

The high-fat diet (HFD) (Rodent diet D12492) was purchased from research diets Inc. (USA), which consists of 20% protein, 20% carbohydrate and 60% fat and provides 5.24 Kcal/g calories. Streptozotocin, individual BAs, H^2-chenodeoxycholic acids (H^2-CDCA) and demethylation deoxycholic acid (NDCA) were all purchased from Sigma-Aldrich Inc. (USA). The kits for concentration quantification of serum transaminase, total BAs (TBAs), total cholesterol, triglyceride and blood glucose were purchased from Nanjing Jiancheng Bioengineering Institute (China). The RNAprep Pure Tissue Kit for total RNA isolation and the FastQuant RT Kit for cDNA synthesized from total RNA were from TianGen Biotech Corporation, LTD. (China). DyNAmo ColorFlash SYBR Green qPCR Kit for real-time quantitative polymerase chain reaction (RT-qPCR) analysis and the primers using in this study were respectively purchased from Thermo Fisher Scientific Inc. (USA) and Beijing Sunbiotech Corporation, LTD. (China).

2.1. T2DM rats model induction

T2DM rats model was induced as previous studies described with slight modifications [22,23]. Briefly, rats in T2DM group were fed with HFD for 7 weeks to induce obesity, and then injected intraperitoneally with a low dose of STZ (30 mg/kg) that was dissolved in pH 4.5 citrate buffer after fasting for 12 h, and rats were continuously fed on HFD for another week before their blood, organs and fecal samples were harvested. Rats fed with HFD for 8 weeks and injected with citrate buffer was the control group. The body weight and length of rats and the average food consumption per week in control and T2DM groups were observed persistently during the model induction period.

2.2. Samples collection

Rats were anesthetized by intraperitoneal injection of urethane (500 mg/kg body weight) after being fasted overnight for 12 h, and during the fasting time, the 12-hour feces samples were collected. After the anaesthesia, the blood was collected from the abdominal aorta, and the tissue samples of liver, ileum, colon and pancreas were obtained after rats were sacrificed by overdose anaesthesia. The samples for BAs analysis and mRNA expression determination were respectively frozen in −80 °C and in liquid nitrogen.

2.3. Measurement of transaminase, lipids and glucose in serum and TBAs in vivo

The serum levels of transaminases including aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) were analyzed by Chemistry Analyzer (OLYMPUS AU400, Tokyo, Japan) in Department of Infection in The First Hospital of Lanzhou University after being handled using their corresponding commercial kits. The concentrations of total cholesterol and triglyceride, glucose and TBAs in serum and the concentrations of TBAs in liver, ileum, colon and pancreas tissue samples and in feces were determined by a Microplate Reader (Multiskan FC, Thermo Scientific, USA) after being handled respectively according to the manufacturer's instructions of the corresponding kits.

2.4. Liver histopathology evaluation

Liver tissue ($n = 5$ in each group) was fixed in 10% aldehyde for 48 h and then approximate 5 cm liver was embedded in paraffin. The paraffin-embedded tissue was cut into around 5 μm thick sections, and then hematoxylin and eosin (H&E) staining were conducted. Liver histopathological evaluation was observed using a Motic BA210-T microscope (Motic, Xiamen, China).

2.5. Individual BAs concentrations quantification

Agilent 1260 Infinity HPLC coupled to an Agilent 6460 Tripe-Quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface (Agilent Technologies, USA) was employed for the quantification analysis of individual BAs concentrations including cholic acid (CA), β-muricholic acid (β-MCA) and chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), hyodesoxycholic acid (HDCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA), and their corresponding taurine (T) and glycine (G) conjugated BAs in serum and liver, ileum, colon and pancreas tissue ($n = 5$ per group). H^2-CDCA and NDCA were selected as the internal standards for HPLC/MS/MS analysis.

2.6. mRNA expressions analysis

The total RNA in liver, ileum, colon and pancreas tissue ($n = 5$ per group) was isolated according to the instructions of commercial kit respectively. cDNA was synthesized from total RNA using the Kit and following the manufacturer's instructions. The resulting cDNA with specific primers was mixed with the reagents in DyNAmo ColorFlash SYBR Green qPCR Kit in accordance with the instruction, and then was put into a 480-qPCR Light Cycler (Roche, Basel, Switzerland) for RT-qPCR, analyzing mRNA expressions of the relevant genes. Gapdh as the housekeeping gene ran for each sample to normalize expression. Specific primers sequences were exhibited in Table 1.

2.7. Statistical analysis

All data were reported as the mean ± standard deviations (S.D.). Two-tailed Student's *t*-test was employed for statistical analysis of the

Table 1
Primer sequences of rat used for RT-qPCR analysis.

Protein	Forward primer	Reverse primer
Ntcp	CACAACGTATCAGCCCCTTT	ATGCTAAGCGCCTTGTCTGT
Oatp2	CCTAGGCATAGGCATTTGGA	TCAACCAAAGCACAAAGCAG
Bsep	CCACCAGAACATGACAAACG	CCCAGTGATGACCCATAACC
Mrp2	CTTGTGGGCTTTGTTCTGTCC	GAGGCAACATCTATCCCATCA
Asbt	ATCTTCGTGGGCTTCCTCTGTCAG	TTCCAAGGCAACTGTTCGGC
Ostα	CCCTCATACTTACCAGGAAGAAGCTAC	CCATCAGGAATGAGAAACAGGC
Ostβ	TATTCCATCCTGGTTCTGGCAGT	CGTTGTCTTGTGGCTGCTTCTT
Cyp7a1	CTGCAGCGAGCTTTATCCAC	CCTGGGTTGCTAAGGGACTC
Cyp8b1	CCCCTATCTCTCAGTACACATGG	GACCATAAGGAGGACAAAGGTCT
Cyp27a1	CACACAAGGACAGCAGTGGT	CCAAGGCAAGGTGGTAGAGA
Cyp3a2	AGTAGTGACGATTCCAACATAT	TCAGAGGTATCTGTGTTTCCT
Fxr	CGAGATGCCTGTGACAAAGA	GCAGACCACACACAGCTCAT
Pxr	GATCAAGAGGAAGAAGAGGG	ATCTGGTCCTCGATAGGCAG
Car	TGGAAGATGCGGTCCATGTAG	CATACAGAAACCTGCGGCCT
Trg5	AAAGGTGGCTACAAGTGCTTC	TTCAAGTCCAAGTCAGTGCTG
Shp	CCTGGAGCAGCCCTCGT	AACACTGTATGCAAACCGAGGA
Fgf15	AGGGCCAGAAACCTTCAAAC	GATCCATGCTGTCGCTCTC
Glp-1	CCGGGTCATCTGCATCGT	AGTCTGCATTTGATGTCGGTCTT
Gapdh	ATGACTCTCCCACGGCAAG	TACTCAGCACCAGCATCACC

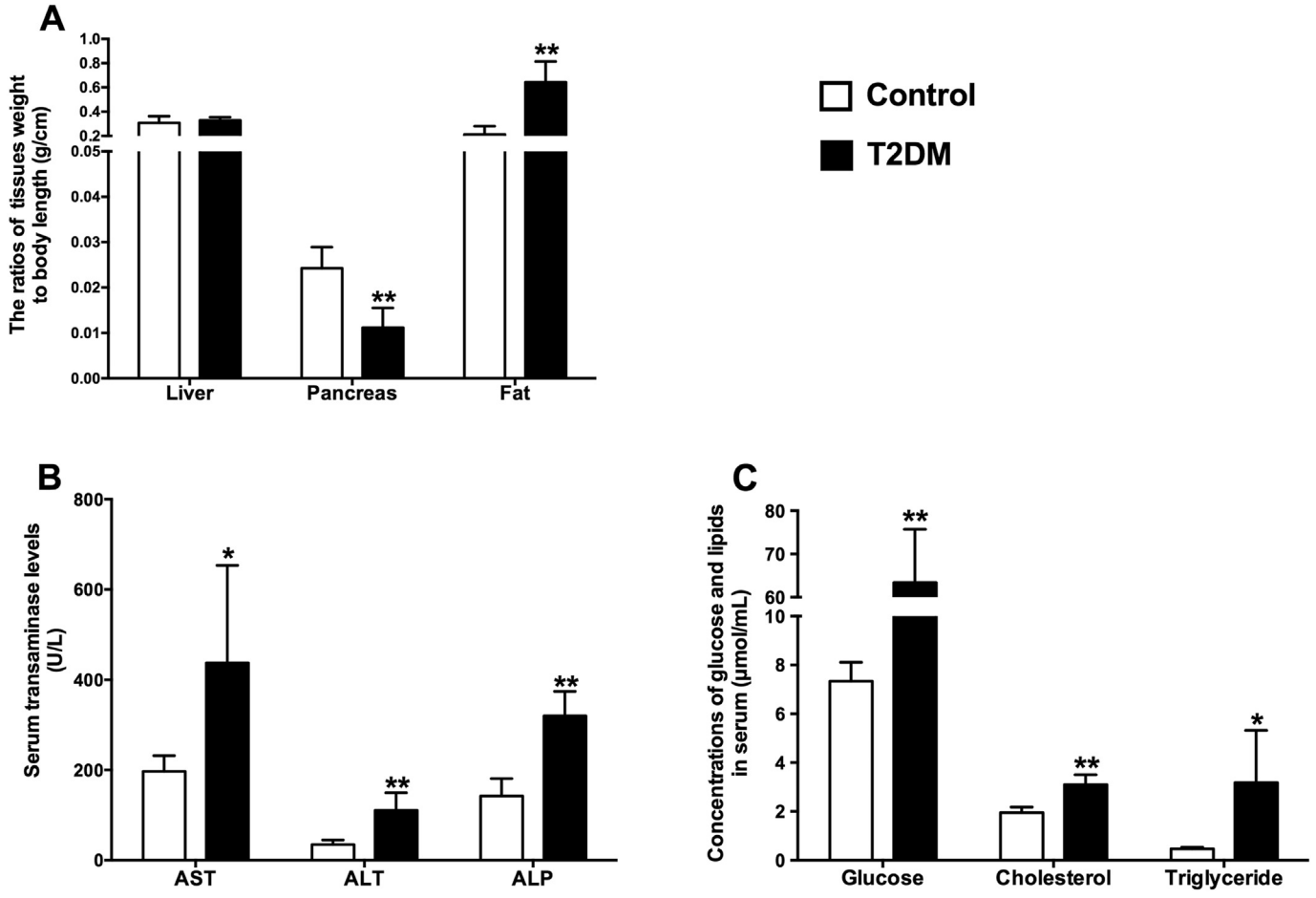

Fig. 1. Tissue weight ratios and levels of transaminase, glucose and lipids in serum. (A) The ratios of liver, pancreas and fat to body length. (B) Serum transaminase levels. (C) The concentrations of glucose, cholesterol and triglyceride in serum. All data was expressed as mean ± S.D. ($n = 5$ per group). $^{*}P < 0.05$, $^{**}P < 0.01$ indicated statistically significant difference between the T2DM group and the control group.

differences in the unpaired samples between two groups. If the P values < 0.05 or 0.01, then the difference is considered to reach statistical significance.

3. Results

3.1. Tissue weight ratio and levels of transaminases, glucose and lipids in serum

Concerning the ratio of tissue to body length of rats in the T2DM

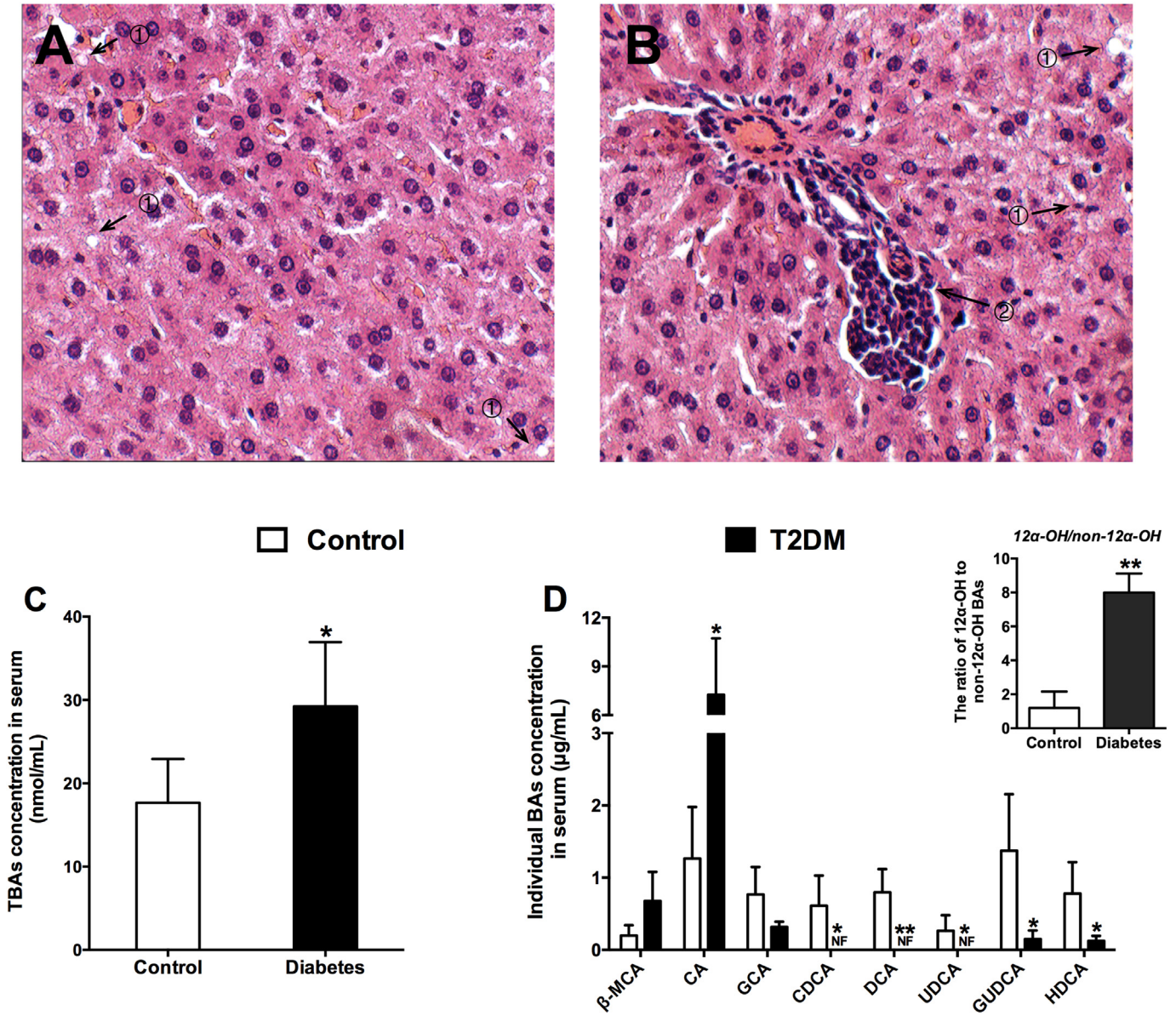

Fig. 2. Liver histologically and BAs concentrations in serum. (A and B) H & E images of liver morphology in control (A) and T2DM (B) rats, and the fat vacuoles (①) and necrosis (②) were marked with arrows; Images are shown at 20 × magnification. (C) TBAs concentration in serum. (D) The individual BAs concentrations and the ratio of 12α-hydroxylated to non-12α-hydroxylated in serum. The concentrations of individual BAs < the limits of quantitation of HPLC/MS/MS was not found. Results were shown as mean ± S.D. (n = 5 per group). $^{*}P < 0.05$, $^{**}P < 0.01$ indicated statistically significant difference between the T2DM group and the control group.

group, the liver ratio exhibited no significant difference with the control group, but the pancreas ratio and abdominal fat ratio decreased and increased profoundly respectively, compared with the control group (Fig. 1 A). In addition, the average food consumption during the model inducement period in both control and T2DM groups did not exhibit a statistically significant difference. The levels of serum transaminases including AST, ALT and ALP that reflect the liver function all increased remarkably in rats with T2DM when compared to the control (Fig. 1 B). Importantly, in comparison to the control group, the fasting blood-glucose concentration profoundly increased approximately by 8 folds, and the concentrations of lipids including cholesterol and triglyceride in serum respectively increased by 0.6 and 5.8 folds in the T2DM group (Fig. 1 C).

3.2. Histological changes of liver and BAs concentrations in serum

Histological assessments of the liver in the control obesity rats exhibited apparently fat vacuole and steatosis, as Fig. 2 A described. In comparison to the control, except for the histological changes mentioned above, noticeable inflammatory cell infiltration and local hepatocytes necrosis were observed (Fig. 2 B).

In serum, the TBAs concentration significantly increased by 65% in rats with T2DM versus the control (Fig. 2 C), which mainly exhibited the CA concentration remarkably increased from 1.27 μg/mL to 7.25 μg/mL (Fig. 2 D). In addition, the concentrations of CDCA, DCA, UDCA, GUDCA and HDCA decreased significantly as compared with the control group, and the concentrations of β-MCA and GCA had no significant difference between two groups (Fig. 2 D). Furthermore, it was found that the ratio of 12α-hydroxylated (12α-OH) (CA/GCA/DCA) to non-12α-hydroxylated (non-12α-OH) (CDCA/β-MCA/UDCA/GUDCA/

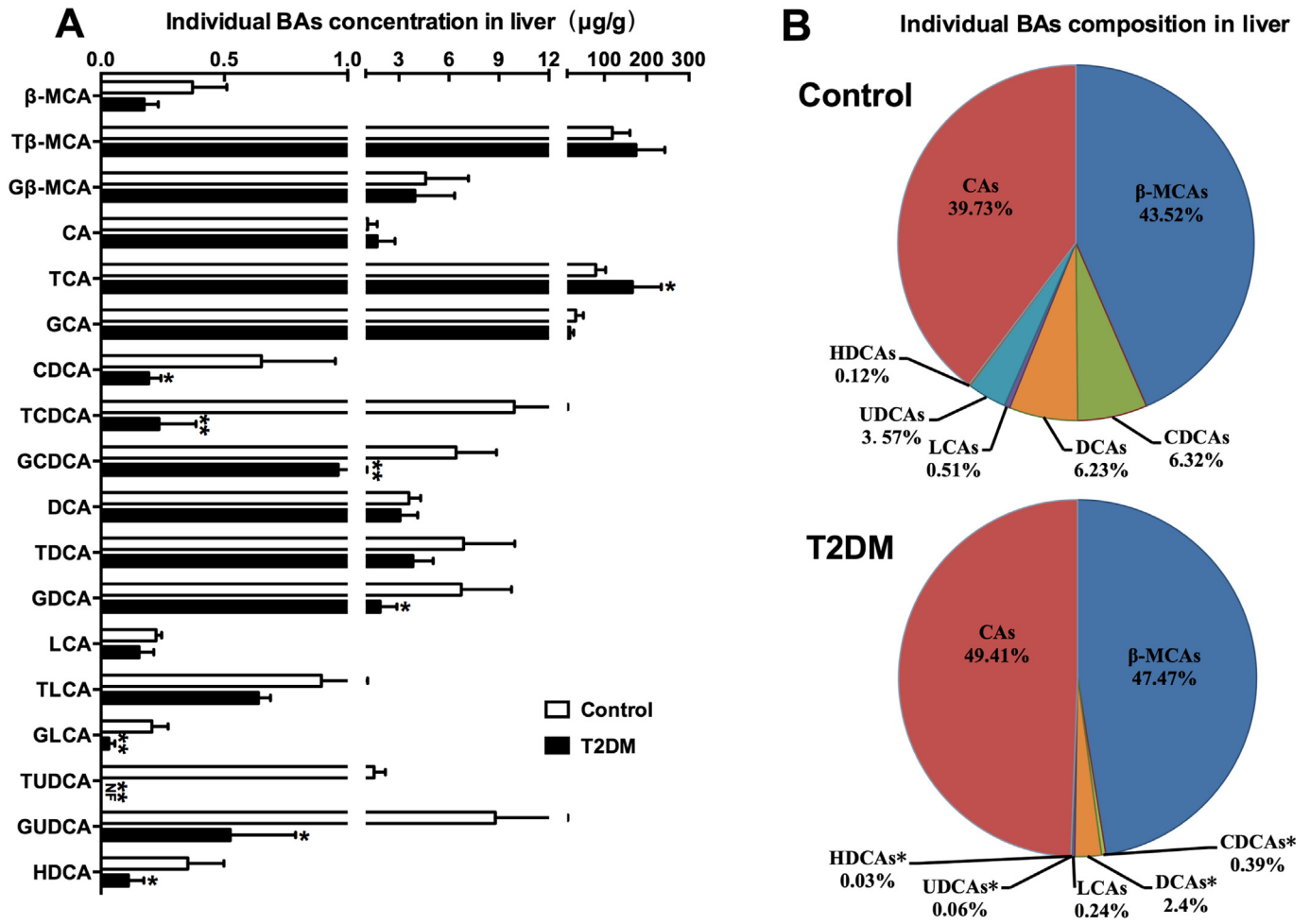

Fig. 3. The concentrations (A) and compositions (B) of individual BAs in liver tissue. The concentrations of individual BAs < the limits of quantitation of HPLC/MS/MS was not found. Results were shown as mean ± S.D. (n = 5 per group). $^{*}P < 0.05$, $^{**}P < 0.01$ indicated statistically significant difference between the T2DM group and the control group.

HDCA) BAs in serum increased profoundly from 1.2 to 8.0 (Fig. 2 D).

3.3. BAs concentrations and compositions in liver tissue

As data shown in Fig. 3 A, in comparison to the individual BAs concentrations in liver tissue of the control group, only TCA concentration increased profoundly, the concentrations of CDCA, TCDCA, GCDCA, GDCA, GLCA, TUDCA, GUDCA and HDCA significantly decreased, and the concentrations of other BAs including β-MCA, Tβ-MCA, Gβ-MCA, CA, GCA, DCA, TDCA, LCA and TLCA had no statistical changes. For the individual BAs compositions in liver tissue (Fig. 3 B), as compared with the control group, the compositions of CDCAs, DCAs, UDCAs and HDCAs decreased remarkably, and the CAs composition increased from approximately 40% to around 50% (not reach significantly statistical difference), and the β-MCAs composition exhibited no great change, in the T2DM group. Additionally, in liver tissue of rats with T2DM, the composition of the primary BAs (β-MCAs, CAs and CDCAs) increased, but that of the secondary BAs (DCAs, LCAs, UDCAs and HDCAs) was reduced from 10.4% to 2.7%.

3.4. BAs concentrations and compositions in ileum tissue

The alterations of individual BAs concentrations and compositions in ileum tissue of rats in T2DM group compared to the controls (Fig. 4), suggested that the concentrations of β-MCA, CA, CDCA, DCA, LCA, UDCA, GUDCA and HDCA was reduced remarkably, the concentrations of Tβ-MCA, TCA, TDCA and TUDCA elevated significantly. The UDCAs composition decreased significantly, which decreased from 4.55% to 0.82%, and the alterations of other individual BAs concentrations and compositions did not reach the apparently statistical difference.

3.5. BAs concentrations and compositions in colon tissue and BAs fecal excretion

In colon tissue, most of individual BAs concentrations decreased under the condition of T2DM in rats as compared with the control rats, especially the concentrations of β-MCA, CDCA, DCA, LCA, UDCA, GUDCA and HDCA (Fig. 5 A). Additionally, the 12-h fecal excretion amount of TBAs was dramatically reduced by about 80% in T2DM rats versus the control rats. Moreover, as Fig. 5 B described, compared with the control group, the compositions of CAs and LCAs elevated by about 6-fold and 2-fold, the compositions of UDCAs and HDCAs decreased remarkably, and the UDCAs compositions even decreased to the level that could not be detected. The DCAs composition decreased from 55% to 41% but its difference did not reach the statistical significance, and the compositions of β-MCAs and CDCAs had no significant difference between the two groups. Additionally, the secondary BAs composition reduced from 86% to 63%.

3.6. BAs concentrations and compositions in pancreas tissue

As results exhibited in Fig. 6 A, in pancreas tissue, the

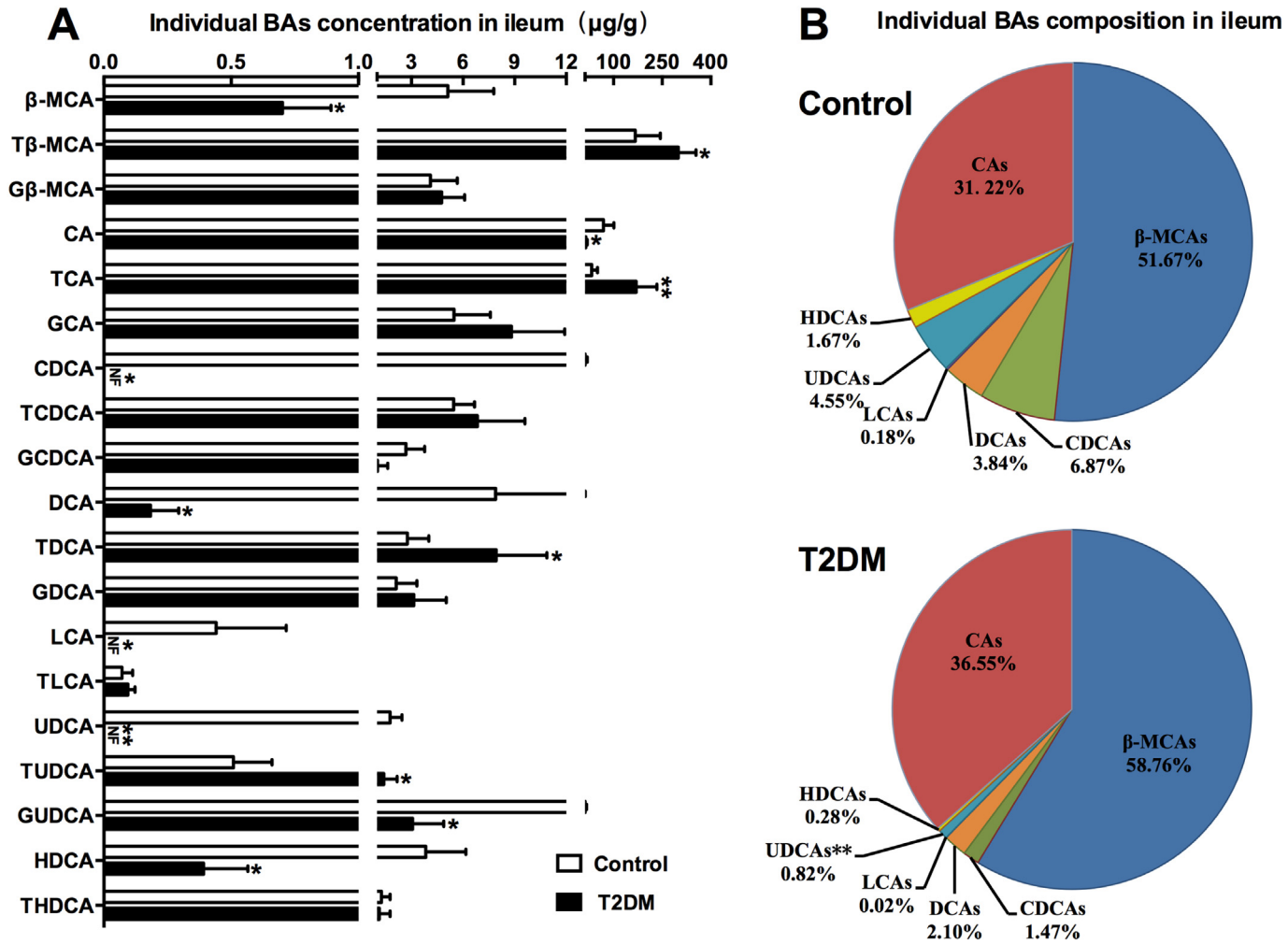

Fig. 4. The concentrations (A) and compositions (B) of individual BAs in ileum tissue. The concentrations of individual BAs < the limits of quantitation of HPLC/MS/MS was not found. Results were shown as mean ± S.D. (n = 5 per group). $^{*}P < 0.05$, $^{**}P < 0.01$ indicated statistically significant difference between the T2DM group and the control group.

concentrations of TCDCA, TLCA, and GUDCA decreased profoundly, but that of UDCA and HDCA increased significantly, and other BAs concentrations exhibited insignificant difference. For BAs compositions in pancreas tissue (Fig. 6 B), the compositions of CDCAs and LCAs decreased profoundly to around 11% and 21%, and the compositions of UDCAs and HDCAs increased remarkably, which respectively increased from 11.75% to 41.18% and from not be detected to 11.17%. The differences of others BAs (CAs and DCAs) compositions did not reach the statistical significance.

3.7. mRNA expressions of functional genes in the liver

In the liver of T2DM rats, in comparison to the control, the mRNA expressions quantity of most of the functional genes decreased (Fig. 7). Especially, the mRNA expressions of nuclear receptor-small heterodimer partner (Shp), BAs biosynthesis enzymes including cholesterol 7α-hydroxylase (Cyp7a1) and sterol 27-hydroxylase (Cyp27a1), BAs metabolism enzyme Cyp3a2 (homologous as human CYP3A4) and transporters including Na^{+}-taurocholate *co*-transporting polypeptide (Ntcp), organic anion transporting polypeptide 2 (Oatp2), bile salt export pump (Bsep) and multidrug resistance-associated protein 2 (Mrp2), all significantly down-regulated in the context with T2DM. The mRNA expression of fibroblast growth factor 15 (Fgf15) in the liver of T2DM rats up-regulated by 36% as compared with the control, but the difference did not reach the statistical significance (Fig. 7 A). Other functional genes expressions including Fxr and sterol 12α-hydroxylase (Cyp8b1) did not change remarkably. Importantly, the mRNA expression ratio between Cyp8b1 and Cyp7a1 elevated from 5:1 in control group to 400:1 in T2DM group, and the ratio between Cyp8b1 and Cyp27a1 elevated from 6:1 to 16:1.

3.8. mRNA expressions of functional genes in ileum, colon and pancreas

In ileum, the position that plays a pivotal role in BAs reabsorption, only the mRNA expression of apical sodium-dependent bile acids transporter (Asbt), the chief BAs transporter at apical membrane mediating > 95% BAs reabsorption from intestinal lumen, down-regulated profoundly under the circumstance of T2DM in rats as compared with the control (Fig. 8 B). The mRNA expressions of others genes, including nuclear receptors (Fxr, Shp and Fgf15) and BAs reabsorption transporters expressing at the basolateral membrane of enterocyte named heterodimeric organic solute transporters α and β (Ostα/β), had no significant difference between T2DM and control groups (Fig. 8 A and B).

In the colon, as compared with the control group, the mRNA expressions of nuclear receptor Tgr5 and glucagon-like peptide-1 (Glp-1), which develop the Tgr5-Glp-1 pathway to play an important role in regulating glucose homeostasis and insulin secretion, down-regulated significantly by 59.4% and 55.1%, respectively (Fig. 8 C).

Pancreas expressing Fxr and Tgr5 is the key organ that secretes

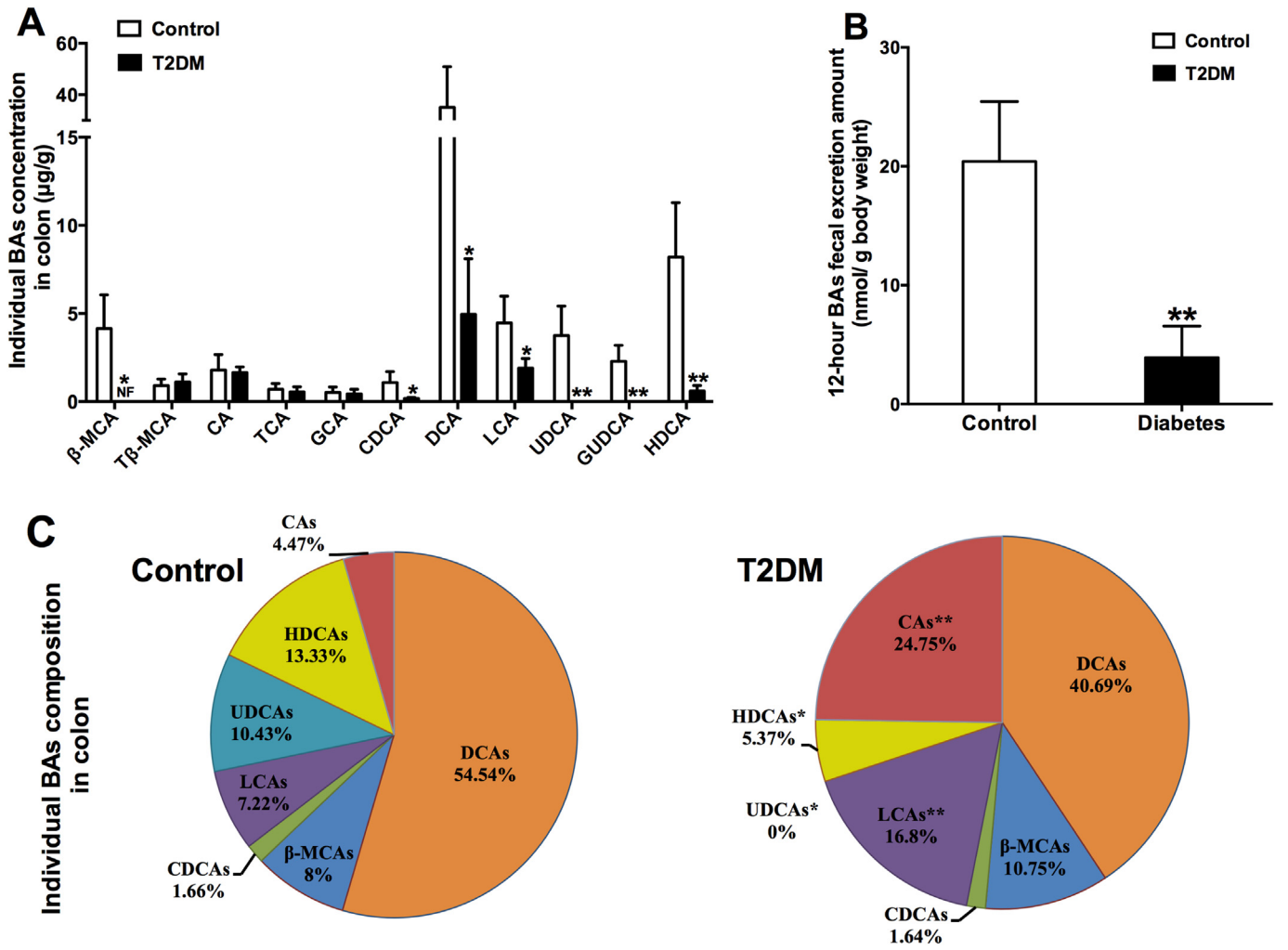

Fig. 5. The concentrations (A) and compositions (B) of individual BAs in colon tissue. The concentrations of individual BAs < the limits of quantitation of HPLC/MS/MS was not found. Results were shown as mean ± S.D. (n = 5 per group). $^{*}P < 0.05$, $^{**}P < 0.01$ indicated statistically significant difference between the T2DM group and the control group.

insulin and glucagon to determine glucose homeostasis in vivo. As the results shown in the Fig. 8 D, in T2DM group, the mRNA expressions of Fxr, Tgr5 and Glp-1 in pancreas down-regulated by 86.1%, 53.0% and 66.4% respectively as compared with the control group, but the difference of Tgr5 mRNA expression between two groups did not exhibit statically significance.

4. Discussion

T2DM is a prevalent and complex endocrine and metabolic disease [3,24,25]. In the recent years, it has been demonstrated that, BAs as a kind of endogenous and signaling molecules altered under the circumstance of T2DM, which could impact on the relevant pathways to further affect the glucose metabolism and insulin secretion and might be associated with the T2DM development and restoration. Moreover, the characteristics of BAs alterations analysis could contribute to the T2DM diagnosis [11,12,14,15,18,26]. Here, in the present study, following by employing the HDF-induced obesity associated with low dose STZ to develop T2DM rats model, we explored the alterations of BAs features and their underlying mechanisms, and discussed the potential effects of the altered BAs on the glucose metabolic disorder via the relevant signaling pathways.

For the characteristics of BAs alterations under the condition of T2DM in rats, in liver tissue, the most of individual BAs concentrations decreased, only TCA concentration increased profoundly (Fig. 3). Our findings suggested that this BAs feature in the liver could be ascribed to the down-regulation of most of the genes explored for BA's synthesis and metabolism in the liver in T2DM rats (Fig. 7), and the up-regulated ratio of the mRNA expressions between Cyp8b1 and Cyp7a1 in liver associated with the increased TCA concentration. More importantly, in the current study, an increased ratio of 12α-OH to non-12α-OH BAs in plasma was observed in rats with T2DM (data was shown in Fig. 2 D), and this finding was dovetailed with other studies that were exerted in patients and rodents with insulin resistant or T2DM [16,27]. This major characteristic of blood BAs alteration would potentially assist diagnosing diabetes [26,28,29]. It is, however, that, the mechanism about the increased ratio of 12α-OH BAs has not been identified. The present data raised the possibility that the significantly elevated ratios of the mRNA expressions between Cyp8b1 and Cyp7a1 and between Cyp8b1 and Cyp27a1 in the liver of rats with T2DM were associated with the increased ratio of 12α-OH/non-12α-OH BAs (Fig. 7). Liver expressing abundant enzymes is the pivotal organ for BAs biosynthesis and determining BAs characteristics. In the liver, Cyp7a1 is the rate-limiting enzyme for primary BAs biosynthesis in classical pathway, and Cyp27a1 is the essential enzyme for the hepatic direct generation of CDCAs and indirect generation of β-MCAs, which are the major non-12α-OH BAs in vivo. Cyp8b1 is the pivotal enzyme determining the hepatic biosynthesis of 12α-OH BAs that is mainly CAs [30,31]. Consequently, the significantly elevated ratios of Cyp8b1 to Cyp7a1 and to Cyp27a1 increased the ratio of 12α-OH/non-12α-OH BAs in rats with

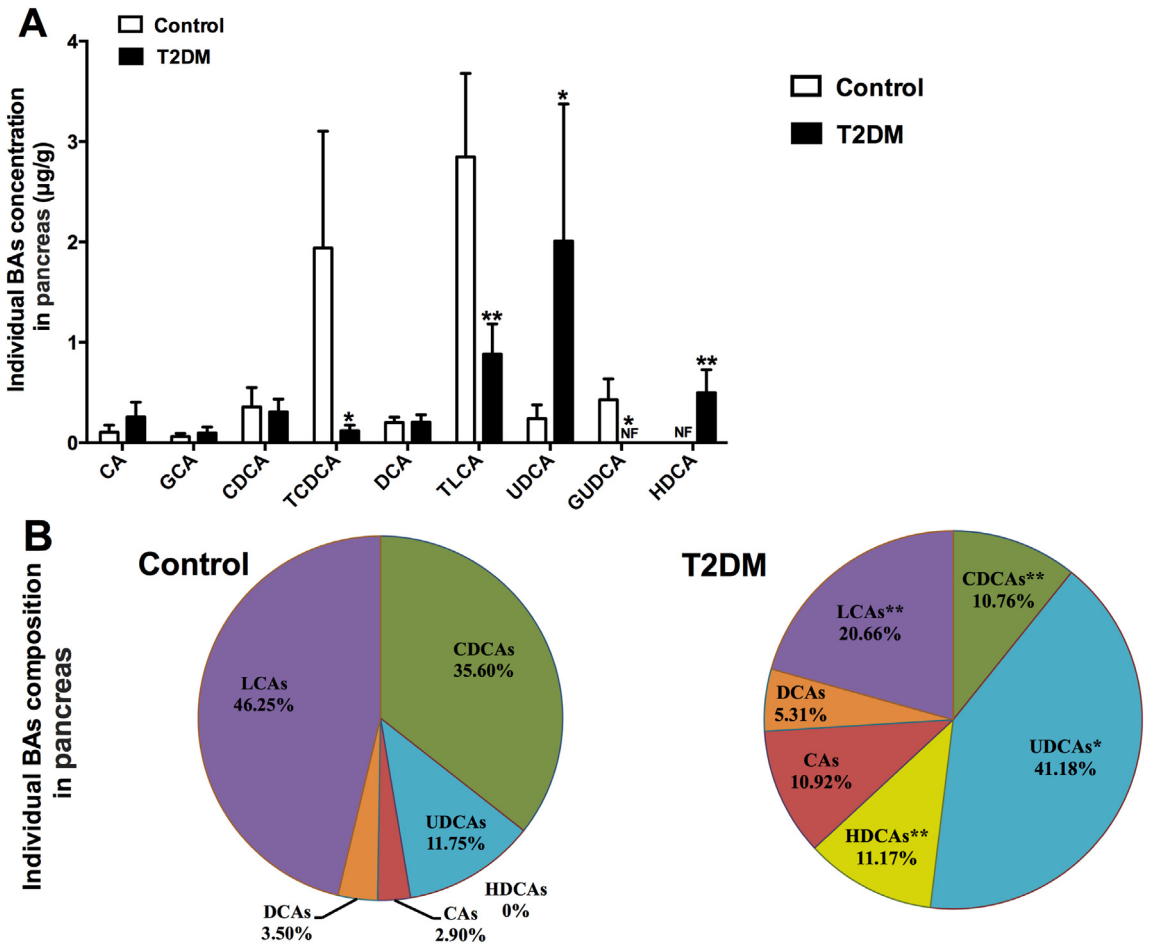

Fig. 6. The concentrations (A) and compositions (B) of individual BAs in pancreas tissue. The concentrations of individual BAs < the limits of quantitation of HPLC/MS/MS was not found. Results were shown as mean ± S.D. (n = 5 per group). $^{*}P < 0.05$, $^{**}P < 0.01$ indicated statistically significant difference between the T2DM group and the control group.

HFD/STZ-induced T2DM.

Which could be the underlying mechanisms about the adaptive alterations of Cyp8b1, Cyp7a1 and Cyp27a1 expression at transcriptional level? Except for the Cyp8b1, the mRNA expressions of most of the hepatic functional genes including Cyp7a1, Cyp27a1, Cyp3a2 and transporters involved in BAs transportation (Ntcp, Bsep, Oatp2 and Mrp2) down-regulated significantly (Fig. 7), which could be ascribed to the liver injury that had been identified by the increased serum transaminase and liver histological assessment in T2DM rats (Figs. 1 and 2). T2DM is a complex and multifactorial metabolic syndrome, which is often followed by multiple organ dysfunction and the relevant diseases. Thereinto, liver function disorders including hepatic steatosis, fibrosis even necrosis, and liver diseases, such as non-alcoholic fatty liver disease and steatohepatitis, are the common complications of T2DM [32–35]. Based on the results from our current and previous studies, compared to the normal rats without HFD, only moderate hepatic steatosis was observed in the HFD-induced obesity rats (the control group), and the serum transaminases levels that reflect the liver function were normal in obesity rats (Data was not shown), which indicated that the obesity did not result in the liver damage. Furthermore, we found the STZ was also not the factor resulting in liver injury (data was not shown). Altogether, liver injury was the occurrence accompanying with T2DM, and it resulted in the down-regulation of the most genes in the liver.

In addition, for the potential mechanism of the unaltered mRNA expression of Cyp8b1, it could be ascribed to the decreased concentration and composition of CDCAs including CDCA, TCDCA and GCDCA derepressing the signaling pathway of Fxr-Shp-Cyp8b1 in the liver of T2DM rats (Figs. 3 and 7). CDCAs are the potent agonists of Fxr, and it has been demonstrated that Cyp8b1 gene transcription is tightly repressed by Shp whose gene expression is promoted by Fxr activation in the liver, and this is so-called hepatic Fxr-Shp-Cyp8b1 signaling pathway [36–39]. In rats with T2DM [40–42], the concentration and composition of CDCAs in liver tissue decreased significantly (Fig. 3). Meanwhile, the hepatic mRNA expression of Shp directly promoted by Fxr activation and reflecting the Fxr activation [43], down-regulated remarkably (Fig. 7). Therefore, the results indicate that the hepatic Fxr-Shp pathway was inactivated under the condition of T2DM in rats, which relatively up-regulated the hepatic Cyp8b1 gene expression and improved the Cyp8b1 mRNA expression maintaining at the normal range under the condition of liver injury in rats with T2DM. Furthermore, it has been reported that improved Cyp8b1 expression is an adverse therapy for T2DM [24].

In the current study, it was found that the levels of secondary BAs (DCAs, LCAs, UDCA and HDCAs) in liver, ileum, colon and pancreas tissues decreased apparently in T2DM rats. Secondary BAs are formed by modifying the primary BAs in the large intestinal lumen by the gut microbiota [30,44,45]. It has been revealed that gut microbial dysbiosis contributes to the metabolic syndrome occurrence and development, and it was observed in the context of HFD and diabetes [46–49]. Consequently, it seemed plausible that the decreased secondary BAs in BAs pool could be ascribed to the alteration of gut microbiota under the circumstance of HFD/STZ-induced T2DM in rats. Regrettably, for the reason that the gut microbiota is complex, we could not explore the

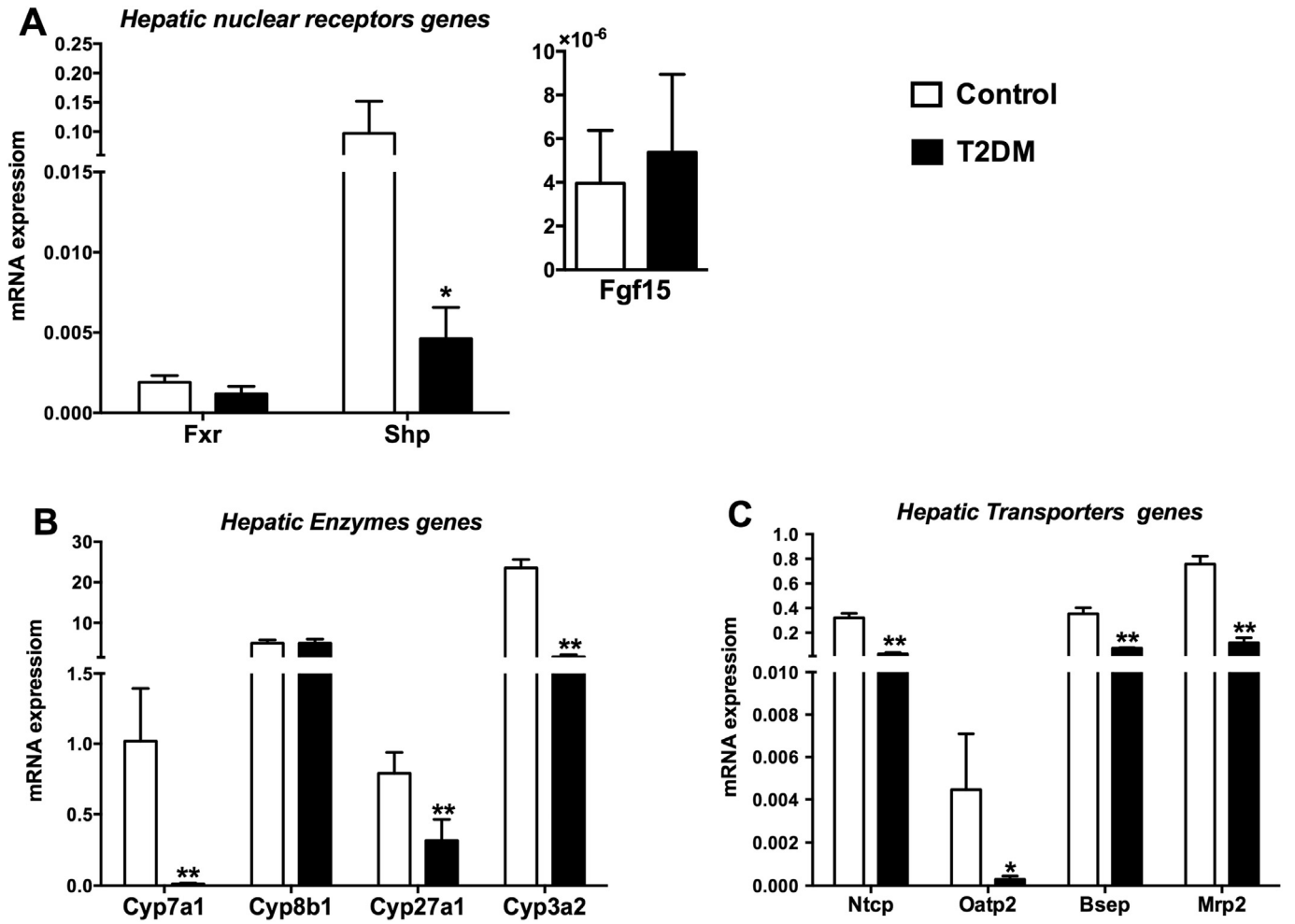

Fig. 7. The mRNA expressions of the functional genes in liver. (A) The mRNA expressions of the relevant nuclear receptors. (B) The mRNA expressions of BAs biosynthesis and metabolism enzymes. (C) The mRNA expressions of BAs transporters. All data was expressed as mean ± S.D. (n = 5 per group). $^{*}P < 0.05$, $^{**}P < 0.01$ indicated statistically significant difference between the T2DM group and the control group. Gapdh as the housekeeping genes was used to normalize expression.

alterations of gut microbiota in the current study.

BAs as the important signaling molecules in vivo can bind with two key nuclear receptors including Fxr and Tgr5 to regulate insulin secretion and glucose homeostasis (the details of the regulatory pathways were described in Fig. 9), of which the alterations should be responsible for the glucose metabolism disorders [9,50–54], but whether the altered BAs as the signaling molecules exerting positive or negative effects on glucose disorder under the circumstance of T2DM has not been clarified. As a result, another focus of this study was to further explore the effects of the altered BAs on the already generated glucose disorder in rats with T2DM. In vivo, three pivotal positions including colon, pancreas and liver that express Fxr and Tgr5 mediating signaling pathways play an important role in the regulation of glucose homeostasis and insulin secretion (Fig. 9), so the major findings of this study were discussed as follows.

(1) Tgr5 that expresses at the membrane of the colonic epithelial cells plays a crucial role in the regulation of the generation of Glp-1 which can promote insulin secretion from pancreatic islet β cells to reduce the glucose level. DCAs and LCAs as the secondary BAs highly existing in the colon are the potently endogenic agonists of Tgr5, and they can up-regulate Glp-1 gene expression at the transcriptional level via activating Tgr5, which is described in the Fig. 9 [10,11,54–56]. Nevertheless, in the HFD/STZ-induced T2DM rats, we found that the concentrations of DCA and LCA in colon tissue decreased profoundly (Fig. 8 C) and the mRNA expression of Glp-1 down-regulated significantly, which should exert a negative effect on glycemia and further induce the T2DM development in rats.

(2) Pancreas possessing islet α and β cells that respectively excrete glucagon and insulin is the chief organ determining the glucose level in vivo. Tgr5 expressed at the membrane of the islet α cell, which is activated by DCAs and LCAs, and the activation can promote Glp-1 transcription. The Glp-1 can bind with the relevant receptors at the membrane of islet β cell to induce insulin secretion [14,54,57]. In addition, in the islet β cell, there is Fxr which can be activated by its potent endogenic agonist CDCAs to promote the insulin secretion [15]. Altogether, the activations of the DCAs/LCAs-Tgr5-Glp-1 pathway and the CDCAs-Fxr pathway in the pancreas can promote insulin secretion to ameliorate glycemia. In the present study, we found that, in pancreas tissue of rats with T2DM, the concentrations of TLCA and TCDCA and the compositions of LCAs and CDCAs decreased significantly (Fig. 6), which could contribute to the remarkable down-regulation of the mRNA expressions of Glp-1 via inhibiting Tgr5 and Fxr (Fig. 8 D). These findings indicated that the decreased LCAs and CDCAs, resulting in the inactivation of Tgr5-Glp-1 and Fxr pathways in pancreas tissue of T2DM rats, had negative effects on insulin secretion and glucose metabolism during HFD/STZ-induced T2DM in rats.

(3) Glycolysis, glycogenesis and gluconeogenesis in the liver are other pivotal factors for determination of glucose level in vivo, and it has

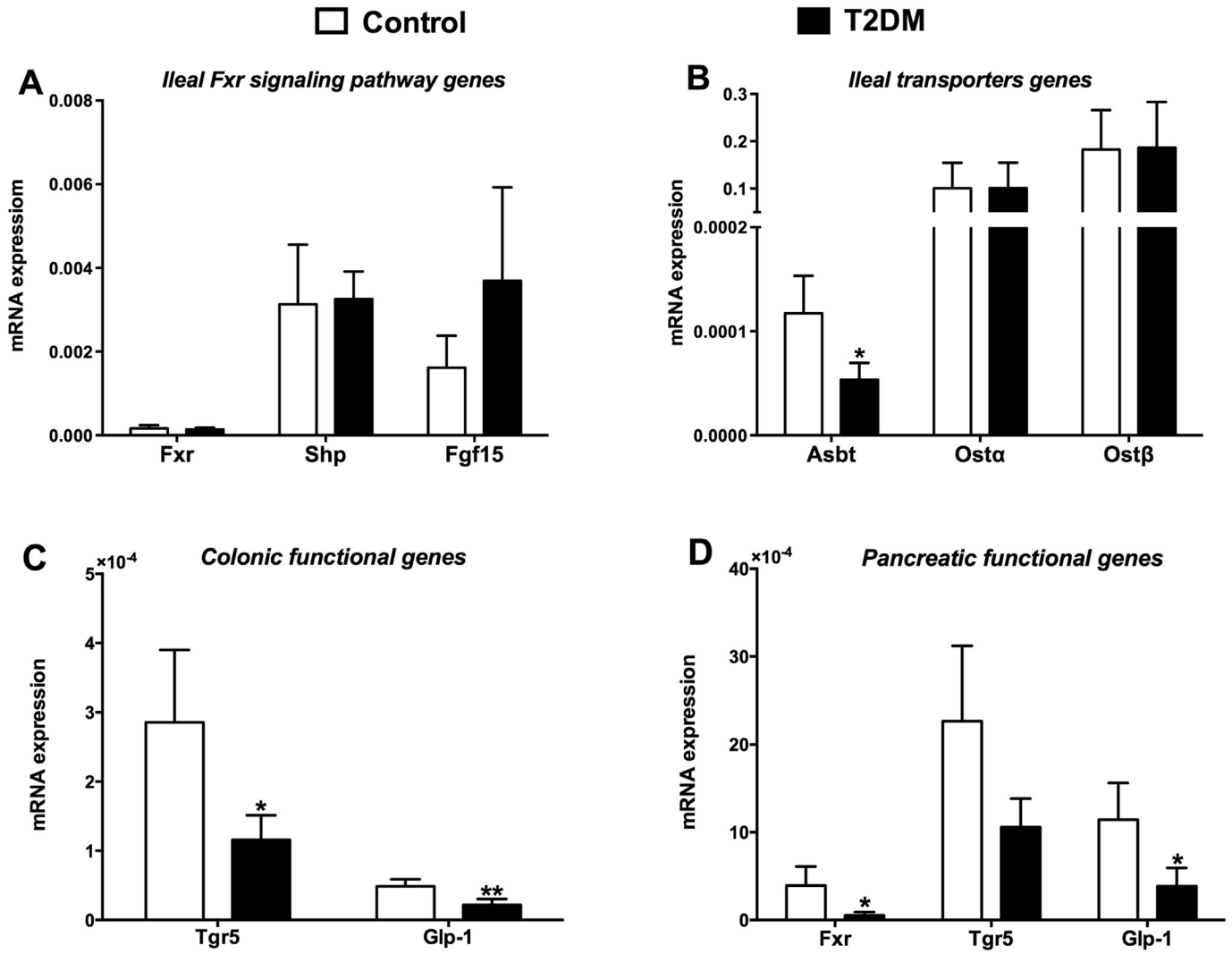

Fig. 8. The mRNA expressions of the functional genes in ileum, colon and pancreas. (A) The mRNA expressions of the relevant nuclear receptors in ileum. (B) The mRNA expressions of BAs transporters in ileum. (C) The mRNA expressions of the relevant genes in colon. (D) The mRNA expressions of the relevant genes in pancreas. All data was expressed as mean ± S.D. (n = 5 per group). $^{*}P < 0.05$, $^{**}P < 0.01$ indicated statistically significant difference between the T2DM group and the control group. Gapdh as the housekeeping genes was used to normalize expression.

been reported that the glycolysis and glycogenesis progressions can be promoted by hepatic Fxr activation to induce glucose consumption. Furthermore, the gluconeogenesis progression that can be repressed by hepatic Fxr activation can inhibit hepatic glycogen decomposing into glucose [14,53,58]. Apparently, the Fxr activation is a benefit for improving glucose disorder in the context of T2DM. Based on the results from our experiment, the concentration and composition of CDCAs including CDCA, TCDCA and GCDCA, as the most potent agonists of Fxr, profoundly decreased in liver tissue of the rats with HFD/STZ-induced T2DM (Fig. 3). Meanwhile, the hepatic mRNA expression of Shp directly promoted by Fxr activation and reflecting the Fxr activation increased (Fig. 7A), which could demonstrate the Fxr in the liver was activated [43]. As a result, it suggested that the hepatic Fxr should be inactivated in rats with T2DM, and as the conclusion from others studies mentioned above, we speculated that the Fxr inactivation could promote T2DM aggravation in rats.

Taken together, in the light of the findings from the present study, it was pointed out that the alterations of BAs in colon, pancreas and liver tissue of rats with HFD/STZ-induced T2DM had negative effects on glucose disorder via disturbing the Tgr5 and Fxr-mediated relevant signaling pathways, which might have relationship with the T2DM development, but the concrete mechanisms about the effects of BAs alterations on glucose disorder need to be fully explored.

5. Conclusion

In conclusion, the results indicate that the BAs in rats with T2DM exhibited characteristic alterations, especially the increased ratio of 12α-OH to non-12α-OH BAs in serum, which could be ascribed to the up-regulated Cyp8b1 mRNA expression ratio in the liver, and this characteristic could provide a cue for searching biomarkers of the T2DM diagnosis. Additionally, we proposed that the altered BAs had negative effects on glucose metabolic disorder via inhibiting the Trg5/Fxr-mediated pathways in colon, liver and pancreas in T2DM rats model, which might further result in the T2DM development. However, the concrete mechanisms need to be comprehensively verified and whether these results could be translated to the human situation also need to be further studied.

Abbreviations

T2DM type 2 diabetes mellitus

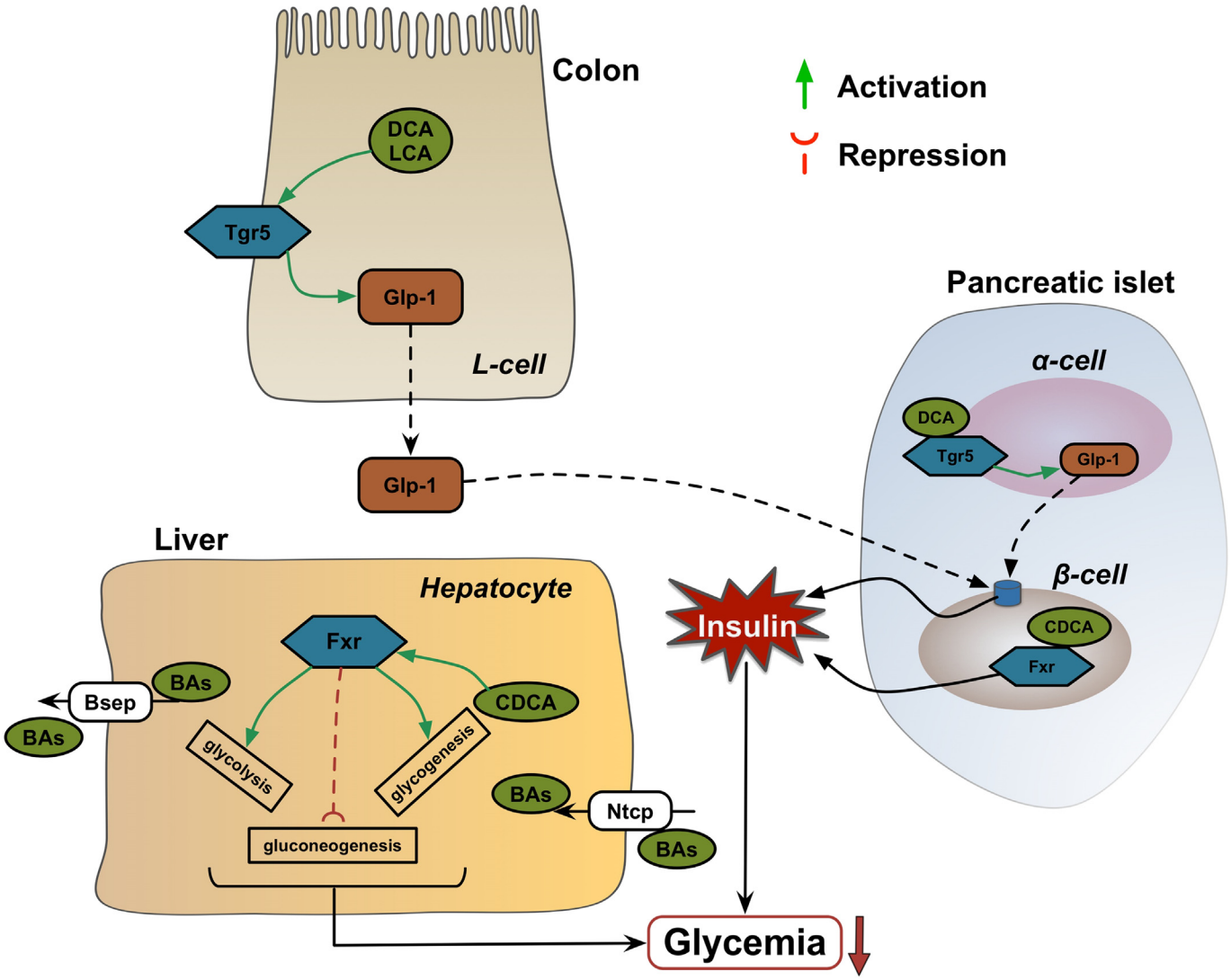

Fig. 9. Schematic overview of the regulatory signaling pathways of BAs on glucose metabolism and insulin secretion. DCA, deoxycholic acid; LCA, lithocholic acid; Tgr5, the G protein-coupled bile acid receptor; Glp-1, glucagon-like peptide-1; Fxr, farnesoid X receptor; BAs, bile acids; CDCA, chenodeoxycholic acid; Ntcp, Na^+-taurocholate *co*-transporting polypeptide; Bsep, bile salt export pump.

BAs	bile acids
TBAs	total bile acids
Fxr	farnesoid X receptor
Tgr5	the G protein-coupled bile acid receptor
STZ	streptozotocin
HFD	high-fat diet
H^2-CDCA	H^2-chenodeoxycholic acid
NDCA	demethylation deoxycholic acid
AST	aspartate transaminase
ALT	alanine transaminase
ALP	alkaline phosphatase
CA	cholic acid
β-MCA	β-muricholic acid
CDCA	chenodeoxycholic acid
DCA	deoxycholic acid
HDCA	hyodesoxycholic acid
LCA	lithocholic acid
UDCA	ursodeoxycholic acid
T-	taurine conjugated bile acid
G-	glycine conjugated bile acid
RT-qPCR	real-time quantitative polymerase chain reaction
Shp	small heterodimer partner
Cyp7a1	cholesterol 7α-hydroxylase
Cyp27a1	sterol 27-hydroxylase
Cyp3a2	cytochrome P450 3a2, homologous as human CYP3A4
Ntcp	Na^+-taurocholate *co*-transporting polypeptide
Oatp2	organic anion transporting polypeptide 2
Bsep	bile salt export pump
Mrp2	multidrug resistance-associated protein 2
Fgf15	fibroblast growth factor 15
Cyp8b1	sterol 12-alpha-hydroxylase
Asbt	apical sodium-dependent bile acids transporter
Ostα/β	heterodimeric organic solute transporters alpha and beta
Glp-1	glucagon-like peptide-1

Disclosures

No conflicts of interest, finances or otherwise are declared by the authors who have taken part in this study.

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