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ORIGINAL CONTRIBUTIONS

Changes in Enterohepatic Circulation after Duodenal–Jejunal Bypass and Reabsorption of Bile Acids in the Bilio-Pancreatic Limb

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Abstract

Background and Aims Duodenal–jejunal bypass (DJB) shows great effects on weight loss and diabetes improvement. Previously, we reported that the bilio-pancreatic (BP) limb plays an important role in glycemic improvement and in serum bile acid (BA) level increase as reported by Miyachi et al. (Surgery 159(5):1360–71, 2016). This study aimed to investigate the mechanism of BA elevation after DJB and the relationship between these effects and BP-limb length.

Methods Otsuka Long-Evans Tokushima Fatty rats with diabetes were randomly assigned into four groups: one sham group and three DJB groups. Three DJB groups were defined according to the BP-limb length: 0 cm, 15 cm, and 30 cm. The lengths of the alimentary limb and common channel were set equally in each DJB groups. Body weight, glucose tolerance, and BA levels in the liver, bile juice, portal vein, and intestinal contents were assessed postoperatively. Changes in enterohepatic circulation of BAs were assessed using labeled BA.

Results BA elevation after DJB was higher with longer BP-limb. In the 30-cm group, the serum total BA level and BA levels in the portal vein, liver, and bile juice were greater than those in other groups. The enterohepatic circulation was shortened in the 15-cm and 30-cm groups.

Conclusions Shortening of the "enterohepatic circulation" by early reabsorption of BAs in the BP-limb, not by the early influx of bile juice into the ileum, was the main cause of BA elevation after DJB. Thus, glycemic improvement and elevation of BA concentration after DJB depend on the BP-limb length.

Keywords Bile acids · Bilio-pancreatic limb · Duodenal–jejunal bypass · Enterohepatic circulation · Diabetes mellitus

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Introduction

Metabolic surgery is the most effective treatment for morbid obesity worldwide at present [1]. In studies comparing surgical and medical treatment, long-term weight reduction effect, improvement of diseases associated with obesity, and long-term prognosis were better after metabolic surgery than after medical treatment [2, 3].

Surgical procedures can be divided into two groups: restrictive surgery such as laparoscopic sleeve gastrectomy (LSG) and combination with malabsorptive procedure such as laparoscopic Roux-en-Y gastric bypass (RYGB). Duodenal–jejunal bypass (DJB), which is the bypass surgery without stomach size reduction, is an experimental procedure that was developed to investigate the underlying mechanism responsible for the metabolic benefits after RYGB [4]. In this DJB model, we can focus only on the mechanism of intestinal bypass excluding the restrictive effect. The combination procedure of LSG and DJB (LSG+DJB) is the modification of BPD/DS and is gaining popularity mostly in Asia. Several reports indicated that adding DJB to LSG is more effective for diabetes improvement compared with LSG alone for severe diabetes cases [5–7]. The small intestine in DJB consists of three parts: alimentary limb (A-limb) through which only food passes, bilio-pancreatic (BP) limb through which only digestive juices such as bile and pancreatic juice pass, and common channel (CC) in which digestive juices and food are mixed together (Fig. 1a).

After the bypass procedure, the change in the secretion of intestinal hormones [8, 9], intestinal flora, and elevation of serum bile acid (BA) concentration [10–12] are involved in postoperative metabolic improvement effects. Among them, researchers in the field of metabolic disorder treatment have increasingly been interested in the role of BAs as a metabolic modulator [13].

The physiological function of BAs has been regarded as related to the promotion of lipid absorption so far. Recently, BAs are recognized as an endogenous mediator, because they function as signal molecules which influence energy consumption and glycolipid metabolism. BAs act as agonists of farnesoid X receptor (FXR) [13, 14] and G protein–coupled BA receptor (TGR5) [15, 16]. FXR takes control of glycolipid metabolism by suppressing the expression of phosphoenolpyruvate carboxykinase (PEPCK) and sterol regulatory element-binding protein-1c (SREBP-1c) [17, 18], BA synthesis [19, 20], and expression of BA transporters [21]. On the contrary, TGR5 enhances glucagon-like peptide-1 secretion and energy expenditure [22–24].

Most BAs are reabsorbed by the transporters in the terminal ileum and returned to the liver via the portal vein (PV). This circulation is called "enterohepatic circulation." The most supported hypothesis of increase in serum BAs after metabolic surgery is that enterohepatic circulation is short-circuited by the diversion of bile flow to the ileum [25–27]. Conversely, we reported that long BP-limb contributes to the metabolic improvement and elevation of serum BA concentration. Furthermore, these effects were canceled after removing the BP-limb [28]. Thus, we hypothesized that concentrated bile juice which is not mixed with food would be reabsorbed earlier in the BP-limb instead of the ileum.

Therefore, the aim of our study is to investigate the mechanism of BA elevation after DJB and the relationship between metabolic improvement and length of BP-limb, which may contribute to clarifying the mechanism of metabolic improvement after bypass surgery.

Material and Methods

Animals

In this study, Otsuka Long-Evans Tokushima Fatty (OLETF) rats were used in the experiment. These rats lack cholecystokinin-A receptors and develop obesity and hyperglycemia with

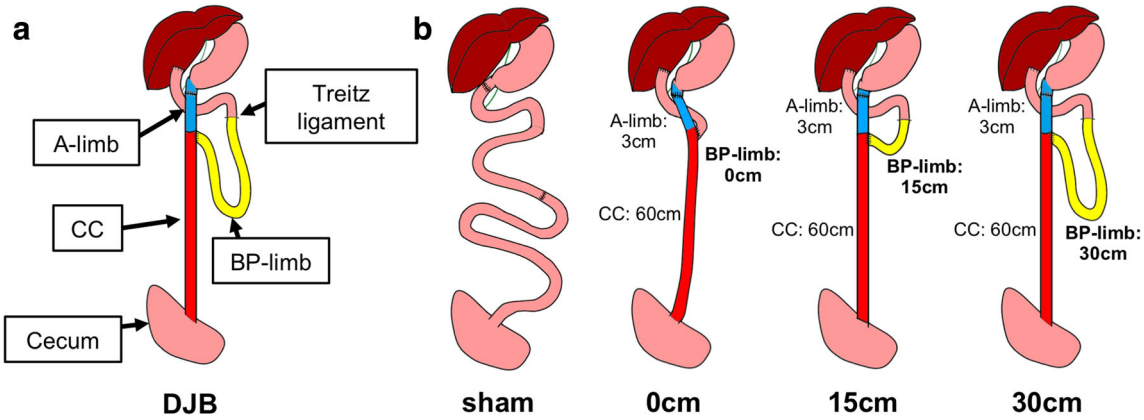

Fig. 1 Illustration of operations and intestinal segments. **a** Schematic illustration of duodenal–jejunal bypass (DJB), alimentary limb (A-limb), bilio-pancreatic limb (BP-limb), common channel (CC). **b** In this study, the length of each limb was fellows. Zero-centimeter group (A-limb 3 cm, BP-limb 0 cm, CC 60 cm), 15-cm group (A-limb 3 cm, BP-limb 15 cm, CC 60 cm), 30-cm group (A-limb 3 cm, BP-limb 30 cm, CC 60 cm)

hyperinsulinemia after overeating [29–33]. They have been used as a model of type 2 diabetes based on obesity. We obtained 5-week-old male OLETF rats from Japan SLC, Inc. (Shizuoka, Japan). All rats were single-housed under specific pathogen-free conditions and fed normal chow (Labo MR stock; Nosan Corporation, Yokohama, Japan, composed of 15.2 kcal% fat, 32.6 kcal% protein, and 52.3 kcal% carbohydrates (CHO)) ad libitum. All experiments were approved by the animal care and utilization committee of our institute.

Operative Procedure

Operation was performed on rats aged 20 weeks after overnight fasting. Surgical procedures were performed according to our previous reports [28, 34, 35]. To compare the effect of improving the postoperative metabolism due to the difference in lengths of the BP-limbs, OLETF rats were divided into four groups ($n = 8$ in each group, Fig. 1b): one sham group and three DJB groups (0-, 15-, and 30-cm BP-limb length group). In the sham group, the duodenum close to the pylorus and the jejunum 30 cm distal from the ligament of Treitz were transected and reanastomosed. In the 30-cm group, the duodenum was divided immediately distal to the pylorus, and the distal end was closed. The jejunum was transected 30 cm distal to the ligament of Treitz, and the distal end of the jejunum was anastomosed end-to-end to the proximal end of the duodenum. The proximal end of the jejunum was anastomosed in an end-to-side fashion to the jejunum (Y-anastomosis) 3 cm distal to the duodenal–jejunal anastomosis. In the 0-cm group, 30 cm of the BPL was resected and Y-anastomosis was created in the same manner. In the 15-cm group, 15 cm of the BPL was resected and Y-anastomosis was created in the same manner. Since the total length of small intestine is around 90 cm, the A-limb and CC were equally set as about 3 cm and about 60 cm, respectively, in all DJB groups. Water was provided ad libitum from the day of operation, and normal chow was provided ad libitum from the first operation day until sacrificed.

Body Weight and Food Intake

Body weight was monitored weekly. Daily food intake was monitored for three consecutive days for 12 weeks after surgery.

MTT and ITT

All tests were performed after overnight fasting. Meal tolerance test (MTT) was performed 8 weeks after the operation. Rats were administered a liquid mixed meal, Ensure® H (1.5 kcal/ml, 28 kcal% fat, 15 kcal% protein, and 57 kcal% CHO) (Abbott Japan Co., Chiba, Japan) by oral gavage at a dose of 1.2 g CHO/5.8 ml/8.76 kcal/kg body weight. Blood samples were drawn from the tail vein at baseline and at 15, 30, 60, 90, and 120 min after administration. Blood glucose levels were measured with Ascensia Breeze 2 (Bayer, Osaka, Japan). Insulin tolerance test (ITT) was performed 11 weeks after the operation. Insulin was injected subcutaneously (0.5 U/kg). The blood glucose levels were measured at baseline and at 15, 30, 45, 60, 90, and 120 min after injection.

Blood, Tissue, and Intestinal Content Collection

Twelve weeks after the operation, all rats were administered Ensure® H by oral gavage at the same dose as MTT after an overnight fasting. All rats were euthanized 1 h after meal loading, and we collected tissue, blood, bile, and intestinal contents. Blood samples were withdrawn from the aorta (Ao), PV, and inferior vena cava (IVC) and collected in ice-chilled tubes containing ethylenediaminetetraacetic acid. Plasma samples were obtained by centrifuging blood (1200 rpm for 10 min at 4 C) and stored at − 80 °C. Liver, bile, and intestine were stored at − 80 °C. Intestine was fixed in buffered formalin (10%). The collected intestinal contents (Fig. 4a) were stored at − 80 °C.

BA Analysis

The Total BA level in Ao, PV, and IVC were examined using the total BA test kit (Wako, Osaka, Japan). Each BA concentration (29 species; Supplementary Table 1) was analyzed using liquid chromatography–mass spectrometry (LC-MS/MS) system.

Plasma and bile were diluted with methanol to prepare a sample solution. Liver or intestinal contents were diluted after homogenization and used as a sample solution. Then, 50 μL of the internal standard (IS) solution, 50 μL of ethanol, and 850 μL of water were added to 50 μL of the sample solution, followed by the pretreatment using an OASIS WAX cartridge (Waters Co., Milford, MA, USA). Nexera liquid chromatograph (Shimadzu Co., Ltd., Kyoto, Japan) and InertSustain C18 column (GL Sciences, Co., Tokyo, Japan) were connected to QTRAP 6500 tandem mass spectrometer (SCIEX, Framingham, MA, USA), and purified sample solutions were analyzed by LC-MS/MS.

Measurement of the Enterohepatic Circulation

The experiment for measurement of the enterohepatic circulation was performed 12 weeks after surgery. All rats were administered Ensure® H by oral gavage at the same dose of MTT after an overnight fasting. We cannulated the catheter (Intramedic polyethylene tubing PE50; Becton Dickinson, Franklin Lakes, NJ, USA) into the common bile duct and place the Surflo (Terumo, Tokyo, Japan) into the duodenum. TCA-d4 (taurocholic acid-d4; Cayman, Ann Arbor, MI, USA)

at 0.01 mg was administered into the duodenum 1 h after meal loading. Then, saline was administered at 3 ml/h continuously. We collected the bile from the catheter placed in the bile duct every 15 min for 3 h after administration of TCA-d4 (Fig. 5a). After deproteinization of 50 μL of bile, it was analyzed under conditions similar to BA analysis.

PCR

The expression of Na taurocholate cotransporting polypeptide (*ntcp*), bile salt export pump (*bsep*), and cytochrome P450 7A1 (*cyp7a1*) in the liver and apical sodium-dependent bile salt transporter (*asbt*), fatty acid binding protein 6 (*fabp6*), and organic solute transporter (*ost*) α/β in the small intestine was analyzed by real-time polymerase chain reaction (PCR). Total RNA from liver and intestine samples (30–60 mg) was extracted using the RNeasy Plus Universal Mini Kit (QIAGEN, Hilden, Germany). RNA integrity was determined using NanoDrop (Thermo Scientific, Waltham, MA, USA). cDNA was prepared using SuperScript™ VILO™ Master Mix (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Quantitative PCR was performed in the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq II, ROX plus (Takara Bio, Shiga, Japan). The following primer pairs are shown in Supplementary Table 2. Relative quantification of mRNA within the samples was examined using the comparative threshold cycle method, and results were averaged and normalized to the expression of rat glyceraldehyde 3-phosphate dehydrogenase gene in each sample.

Histopathological Analysis

The small intestine fixed in buffered formalin (10%) was embedded in paraffin. Serial, 3-μm thick sections were stained with hematoxylin and eosin. Histopathological analyses were performed by an expert pathologist. The villous height was measured using BZ-9000 and BZ-II image analysis application (KEYENCE, Osaka, Japan). The mean value of the 20 villous height which was randomly measured in × 4 field was the value of each position.

Data Analysis

All data were presented as mean ± standard error of the mean (SEM). All statistical analyses were performed using the JMP 13.2.0 statistical software package (SAS International Inc., Cary, NC, USA). In all analyses, $P < 0.05$ was taken to indicate significance. To determine significant differences between the sham and other groups, Dunnett's test or Steel test was used after Levene test.

Results

Weight Gain, Food Intake, Glucose Metabolism

The weight gain after the operation was significantly suppressed in the 30-cm group compared with the sham group (Fig. 2a). The blood glucose levels and its area under the curve (AUC) were significantly lower throughout the MTT in the 30-cm group. However, in the 15-cm group, the blood glucose level was significantly lower only at 15-min point (Fig. 2b, c). Throughout the ITT, the blood glucose levels and its AUC were also significantly lower in the 15-cm and 30-cm groups (Fig. 2d, e). The weight gain and blood glucose level in the 0-cm group did not differ from those in the sham group. From the above, the glycemic improvement effect of DJB was higher with longer BP-limb. No intergroup differences were observed in food intake (Supplementary Fig. 1).

Serum Total BA Level

The total BA levels in the PV and Ao were significantly higher in the 30-cm group. The total BA level in the IVC tended to be higher in the 30-cm group. As the BP-limb was longer, the serum total BA level was higher (Fig. 3a).

BA Composition in the PV

In the PV, most BA fraction concentrations were significantly elevated in the 30-cm group compared with the sham group (Supplementary Fig. 2A). Primary and secondary BA concentrations were significantly higher in the 15-cm and 30-cm groups compared with the sham group. There was no remarkable change in the ratio of primary BAs to secondary BAs (Supplementary Fig. 2B). Conjugated BA concentration in the 15-cm and 30-cm groups and unconjugated BA concentration in the 30-cm group were significantly higher compared with the sham group. Furthermore, the ratio of conjugated BAs to unconjugated BAs was significantly higher in the 30-cm group compared with the sham group (Fig. 3b).

BA Compositions in the Liver and Bile Juice

The BA concentrations in the liver and bile juice in the 30-cm group were significantly higher compared with the sham group. Most of the BAs in the liver and bile juice were consisted of conjugated BA in all groups (Fig. 3c, d) (Supplementary Figs. 3A, B and 4A, B).

Conjugated BA Concentration in the Intestine

The small intestine was divided into seven segments as shown in Fig. 1c. The conjugated BA concentration in segments A–E were significantly higher in the 30-cm group, and those in segment D and E were significantly higher in the 0-cm group compared with

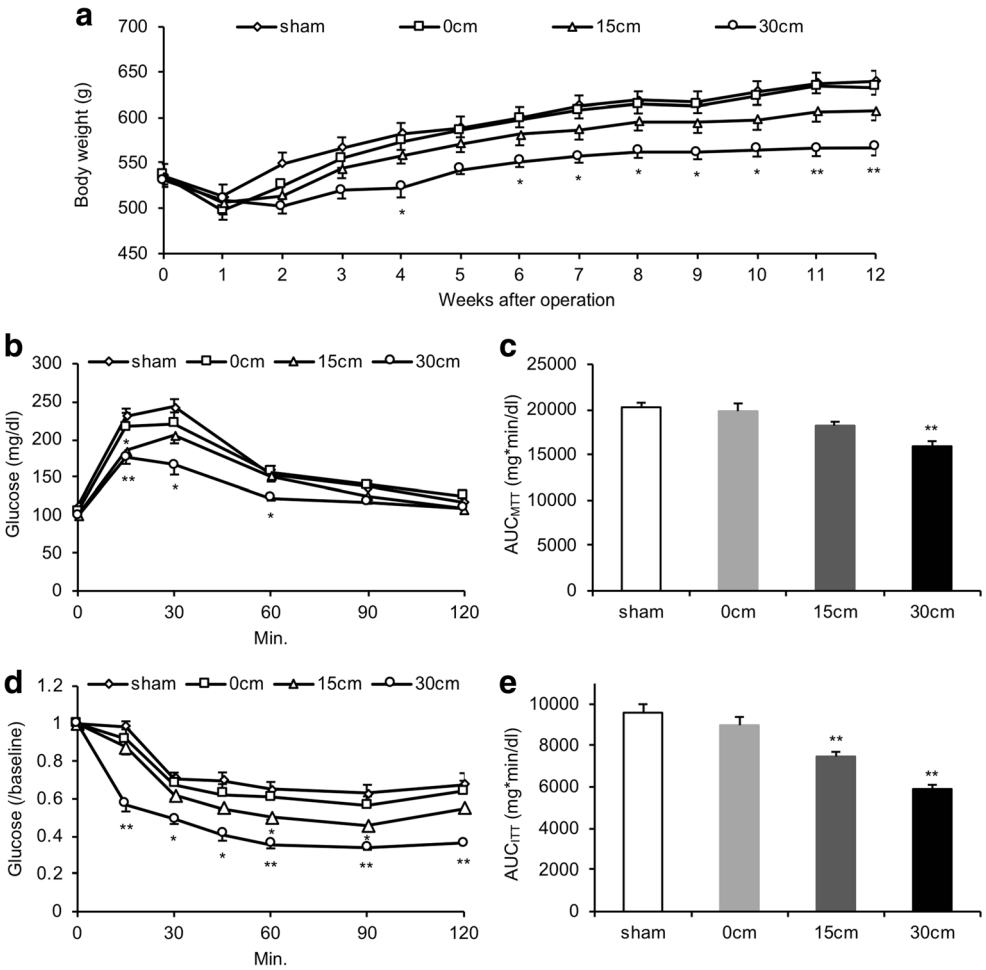

Fig. 2 Effect of duodenal–jejunal bypass. **a** Body weight after the operation. **b** Glucose excursion curve in meal tolerance test 8 weeks after the operation. **c** Area under the curve in meal tolerance test. **d** Glucose excursion curve in insulin tolerance test. **e** Area under the curve in insulin tolerance test. Data are presented as mean ± SEM; $n = 8$ rats per group; $^{*}P < 0.05$, $^{**}P < 0.01$ versus values in the sham group (Dunnett test or Steel test after Levene test)

the sham group. Those in segment G were significantly lower in the 0-, 15-, and 30-cm groups compared with the sham group. The conjugated BA concentration decreased gradually in the BP-limb (Fig. 4b).

Enterohepatic Circulation

After bolus injection of TCA-d4 into the duodenum, the labeled BA level in the common bile duct was gradually elevated and reached its peak level, and it was decreased thereafter in all groups. In the sham and 0-cm groups, the labeled BA appeared slowly in the common bile duct. In contrast, the labeled BA appeared rapidly in the 15-cm and 30-cm groups, and their peak levels were about twice as higher than the peak of 0-cm or sham group (Fig. 5b).

Gene Expression of CYP7A1 and BA Transporters in the Liver and BP-Limb

In the proximal part of the BP-limb (segment A), only the expression of *asbt* was significantly decreased in the 30-cm group compared with the sham group, and there was no significant difference in the expression of *fabp6* and *ostα/β* (Fig. 6a). In the distal part of the BP-limb (segment B), only the expression of *ostα/β* was significantly increased in the 30-cm group compared with the sham group, and there was no significant difference in the expression of *asbt* and *fabp6* (Fig. 6b). In the liver, the expression of *bsep* was significantly increased in the 30-cm group compared with the sham group, and there was no significant difference in the expression of *cyp7a1* and *ntcp* among all groups (Fig. 6c).

Histopathology of the BP-Limb

The histological findings showed some atrophy of the intestinal mucosa in the BP-limb (Supplementary Fig. 5A, B). However, there was no significant difference in the villous height among all groups compared with the sham group (Supplementary Fig. 5C).

Discussion

BAs are recognized as endogenous mediator influences on energy expenditure and glycolipid metabolism. BA

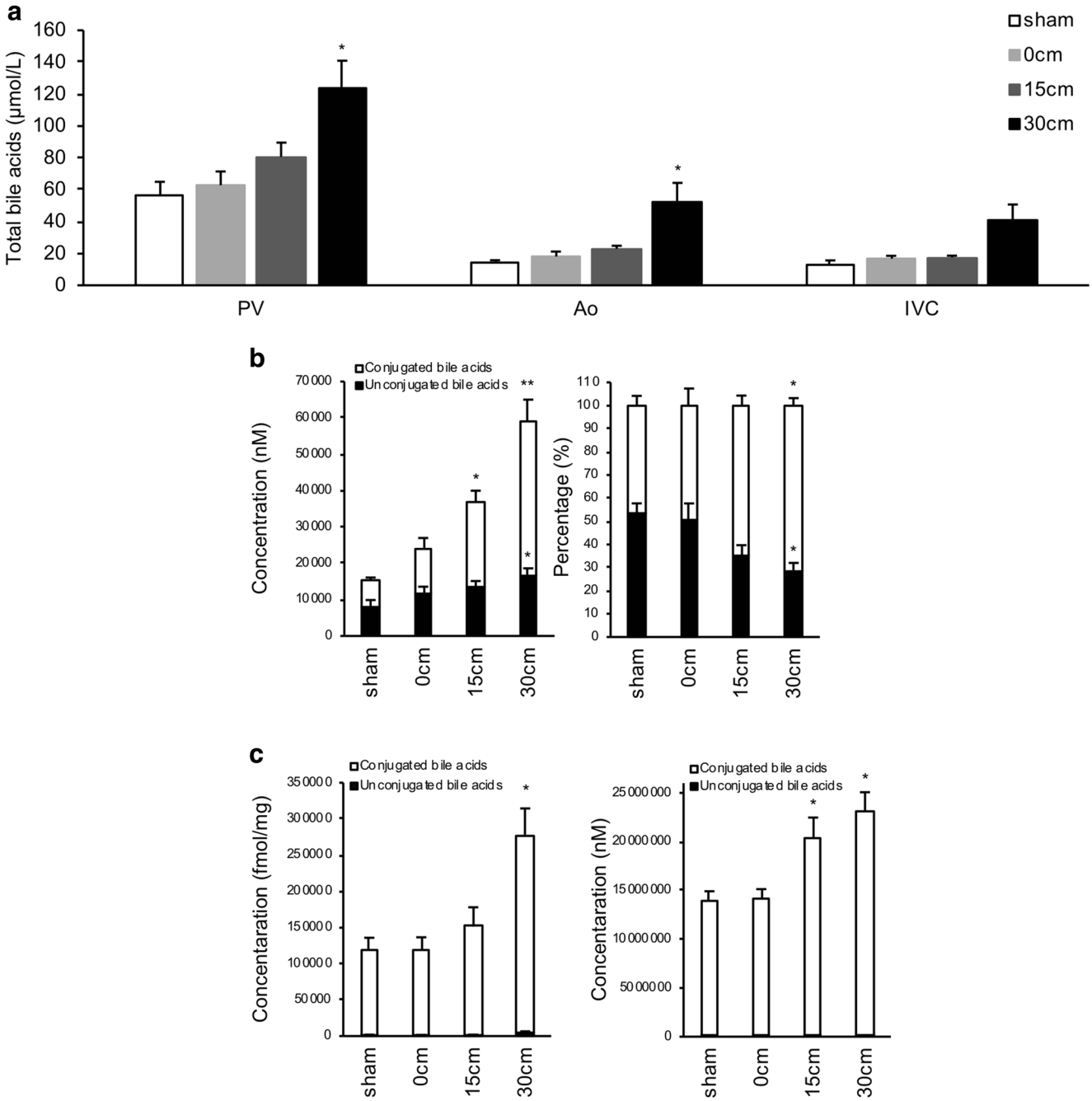

Fig. 3 Analysis of bile acids. **a** Serum total bile acid concentration in portal vein (PV), aorta (Ao), and inferior vena cava (IVC). $n = 8$ rats per group. **b** Concentration of conjugated and unconjugated bile acids and ratio of conjugated to unconjugated bile acids in PV. $n = 8$ rats per group. **c** Concentration of conjugated and unconjugated bile acids and ratio of conjugated to unconjugated bile acids in the liver. $n = 8$ rats per group. **d** Concentration of conjugated and unconjugated bile acids and ratio of conjugated to unconjugated bile acids in the bile juice. $n = 7$ rats per group. Data are presented as mean ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$ versus values in the sham group; NS not significant (Dunnett test or Steel test after Levene test)

themselves act as agonists of TGR5 and FXR [14–16], and they have physiological activity and show metabolic improvement [17, 18, 22–24]. In addition, the serum BA concentration is elevated after metabolic surgery [36, 37], and it did not work for FXR knockout mice [38]. Therefore, the elevation of serum BA levels after metabolic surgery would be the main mechanism of glycemic improvement effect.

To date, the most supported hypothesis of serum BA elevation after metabolic surgery was shortening of the enterohepatic circulation due to the diversion of the bile flow to the terminal ileum [25–27]. Our study clearly demonstrated that reabsorption of conjugated BAs in the BP-limb, which is the proximal jejunum, was promoted after DJB. That is, this is considered to be the mechanism of shortening of

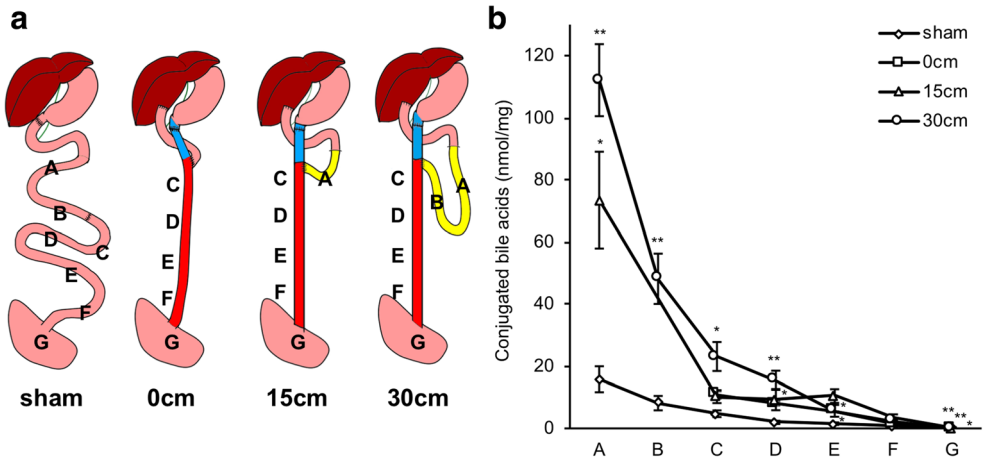

Fig. 4 Analysis of bile acids in the intestine. **a** Intestine was divided into seven segments at the time of collecting tissue and intestinal contents. **b** Concentration of conjugated bile acids in the intestine. Data are presented as mean ± SEM; $n = 8$ rats per group; $^{*}P < 0.05$, $^{**}P < 0.01$ versus values in the sham group (Dunnett test or Steel test after Levene test)

enterohepatic circulation after bypass surgery. In addition, this phenomenon is probably the cause of elevated serum BA concentration after DJB. Previously, we reported the presence of the BP-limb; the proximal jejunum, through which food does not pass, plays an important role in elevating serum BA levels [28]. However, the detailed function of the BP-limb remains unknown so far. BAs synthesized from cholesterol in the hepatocyte are secreted into the duodenum via the bile ducts. Then, most BAs are reabsorbed in the terminal ileum by active transport, and they returned to the liver via the PV blood flow. This circulation is called enterohepatic circulation of BAs. Some amounts of reabsorbed BAs flow into the systemic circulation and are excreted in the urine [39]. In this study, the concentration of conjugated BAs in the PV and the ratio conjugated to unconjugated BAs were both significantly higher in the 30-cm group which had longer BP-limb (Fig. 3b). In this study, the concentration of conjugated BAs in the BP-limb decreased gradually while flowing to the distal end (Fig. 4b), and the labeled BA administered into the duodenum was secreted to the bile earlier in the 15-cm or 30-cm group in which the BP-limb exists (Fig. 5b). These results strongly suggest that more BAs are reabsorbed in the BP-limb than in the terminal ileum. To our knowledge, this is the first demonstration of short-circuited enterohepatic circulation by reabsorption of conjugated BAs in the BP-limb.

Regarding the mechanism of reabsorption of conjugated BAs in the BP-limb after DJB, the mechanism of BA absorption in the intestine is classified into active absorption by the transporters and passive absorption by concentration gradient. BA transporters in the intestine are mainly composed of ASBT at apical membranes, FABP6 in cell matrix, and OSTα/β at the basolateral membranes [40, 41]. ASBT expression is decreased [42] and that of FABP6 and OSTα/β are increased by FXR activation [40, 43]. Since active absorption through the BA transporters has been reported to be concentration-dependent [44–48], BA concentration is also an important factor influencing enterohepatic circulation in addition to the change in expression of BA transporters. Therefore, we assumed the following three mechanisms promoting BA absorption: the increase in the contact area between bile juice and intestinal mucosa, increased expression of BA transporters in the BP-limb, and elevation of BA concentration in the BP-limb. In this study, we could not find any changes in the histological structure of intestinal mucosa in the

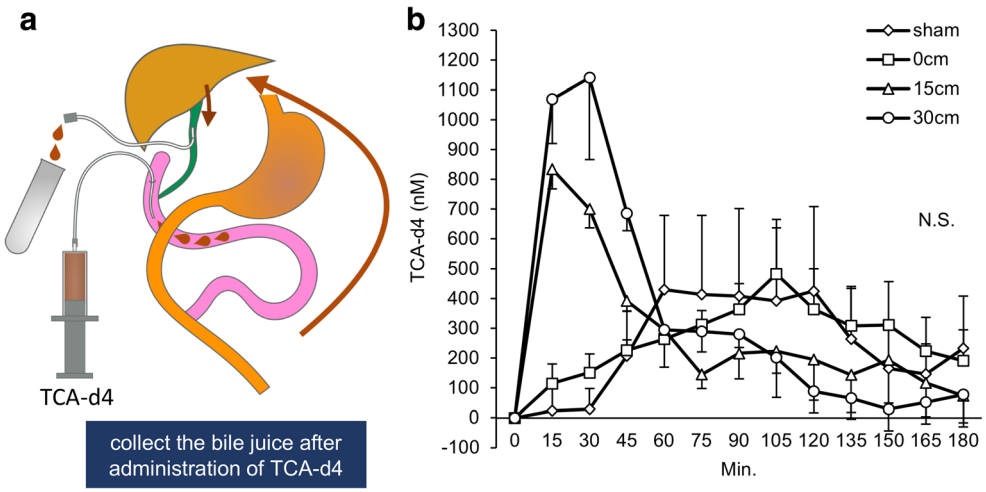

Fig. 5 Analysis of enterohepatic circulation. **a** We cannulated the catheter into the common bile duct and collected the bile every 15 min for 3 h after administration of TCA-d4 into the duodenum. **b** Concentration of TCA-d4 in bile juice after administration of TCA-d4 into the duodenum. Data are presented as mean ± SEM; $n = 5$ rats per group; NS not significant (Dunnett test or Steel test after Levene test)

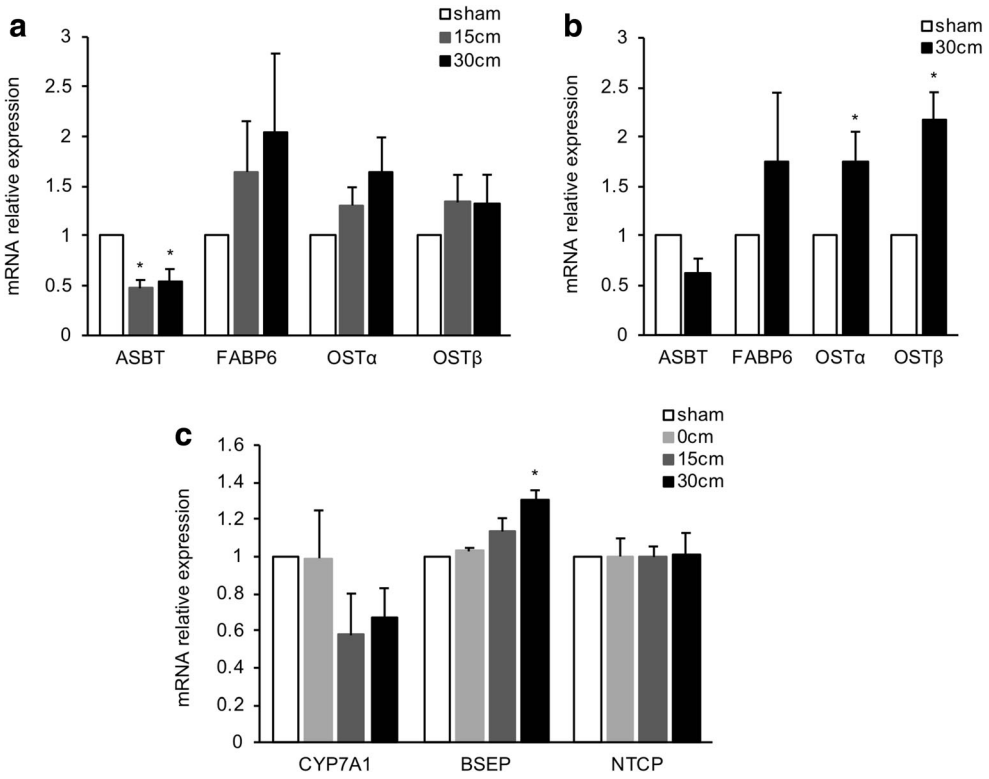

Fig. 6 The expression of cyp7a1 and bile acid transporters. **a** mRNA relative expression of bile acid transporters (*asbt*, *fabp6*, *ostα/β*) in the proximal BP-limb. **b** mRNA relative expression of bile acid transporters (*asbt*, *fabp6*, *ostα/β*) in the distal BP-limb. **c** mRNA relative expression of *cyp7a1* and bile acid transporters (*bsep*, *ntcp*) in the liver. Data are presented as mean ± SEM; $n = 6$ rats per group; $^*P < 0.05$ versus values in the sham group (Dunnett test or Steel test after Levene test)

BP-limb (Supplementary Fig. 5A, B, C), and the expression of *asbt* was significantly decreased in the BP-limb (Fig. 6a). On the contrary, the conjugated BA concentration in the BP-limb was significantly higher than that of the sham group (Fig. 4b). Therefore, the increase in conjugated BA absorption in the BP-limb is considered a result of elevation of conjugated BA concentration in the BP-limb, and we think it is caused by the influx of high-concentrated bile juice, which is not diluted by food.

Moreover, regarding the relationship between the length of BP-limb and postoperative metabolic improvement, we made four groups with different lengths of the BP-limb to compare the effect of postoperative metabolic improvement and change in BA kinetics. A-limb and CC were set to 3 cm and 60 cm, respectively, in each group to equalize the length of the small intestine involved in absorption of nutrient (Fig. 1b). As a result, BA concentrations in the liver and blood and the improvement effect after surgery depended on the length of the BP-limb, and the metabolic improvement effect was canceled in the 0-cm group in which the BP-limb was resected. These findings support that postoperative metabolic improvement effect is not attributed to shortening of the small intestine involved in the absorption of nutrients, but depends on the length of the BP-limb. Thus, the BP-limb itself contributes to the improvement of metabolism through elevation of BA concentration. This result might be the key to clarify the mechanism of metabolic surgery and become the supporting evidence to set the long BPL in clinical practice.

Limitation

Our study has some limitations and leaves some questions unanswered. In this study, we used rats as models, although BA fractions varied greatly among the animal species. In humans, the bile secreted from the liver is stored in the gall bladder and is concentrated; on the contrary, rats do not have gallbladder. Therefore, it is unknown whether the change in BA kinetics after surgery in rats applies in humans. Further studies are needed to know the exact mechanism of BA reabsorption in the BP-limb in human clinical setting.

Conclusion

In conclusion, we confirmed the shortening of the enterohepatic circulation by early reabsorption of conjugated BAs in the BP-limb, not by the early influx of bile juice into the ileum, after DJB in the type 2 diabetes rat model. This shortening of enterohepatic circulation was caused by the high concentration of BAs in the BP-limb, and we speculate that it is caused by the influx of high-concentrated bile juice which is not diluted by food into the BP-limb. It was enhanced as BP-limb lengthened. Therefore, the metabolic improvement effect and elevation of BA concentration after DJB both depend on the length of the BP-limb. We hope that this study will contribute to the elucidation of the metabolic improvement effect of bypass surgery.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Statement of Animal Rights All procedures in this study were approved by ethics committee for animal research of our institute.

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