Gastric Bypass Surgery Reverses Diabetic Phenotypes in Bdnf-Deficient Mice

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Accepted for publication April 11, 2016.

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Duodenum-jejunum gastric bypass (DJB) has been used to treat morbid diabetic patients. However, neither the suitability among patients nor the mechanisms of this surgical treatment is clear. Previously, we reported a new mouse strain named Timo as type 2 diabetes model caused by brain-derived neurotrophic factor (Bdnf) deficiency. In this study, we found that DJB on Timo mice reversed their metabolic abnormalities without altering the expression of Bdnf. Glucose tolerance and insulin sensitivity were improved greatly, along with reduction of fat accumulation in liver and white adipose tissue. The gut flora population was altered by DJB with increased proportion of Firmicutes and decreased Actinobacteria and Proteobacteria in the ileum after surgery. Systemic inflammation in Timo mice was greatly suppressed with less macrophage infiltration and lower tumor necrosis factor-α levels in liver and white adipose tissue after surgery. Interestingly, the alteration of gut microbiota abundance and improved metabolism preceded the inflammation alleviation after DJB surgery. These results suggested that DJB can reverse Bdnf deficiency—associated metabolic abnormality. In addition, the reduced inflammation may not be the initial cause for the DJB-associated metabolic and microbiota alterations. The increased BDNF protein levels in hypothalamus and hippocampus may result from microbiota change after DJB surgery. (Am J Pathol 2016, 186: 2117–2128; http://dx.doi.org/10.1016/j.ajpath.2016.04.009)

Surgical treatment, especially bariatric surgery, was proposed as an effective method to maintain long-term weight loss and remission of type 2 diabetes mellitus (T2DM), in addition to lifestyle and pharmacologic interventions.1–3 Diabetes remission appears before weight reduction in patients who undergo Roux-en-Y gastric bypass surgery. Therefore, it is unlikely that weight loss is the factor that improves insulin sensitivity.4 Other hypotheses of this effect of duodenum-jejunum gastric bypass (DJB) require more direct evidence. For example, Dirksen et al5 suggested that an accelerated transit of concentrated nutrients (particularly glucose) to the distal intestine results in the increased production of insulinotropic and appetite-controlling substances. However, there are conflicting reports on the changes in gut hormone levels after bariatric surgery.5–8 Low-grade inflammation in adipose tissue and liver is one of the primary causes for low insulin sensitivity in obesity and T2DM.9–12 Several recent studies found that decreased adiposity and increased insulin sensibility after surgery was associated with gut microbiota alterations.1,5,13,14 However, how rapid changes in gut microbiota result in glycemic improvements and alterations in metabolites are not clear.

Suppression of systemic inflammation in mice and humans is associated with an increased abundance of potentially proinflammatory Proteobacteria, Bacteroides, and Ruminococcus gnavus in gut.15 Decreased Lachnospiraceae and...
Bifidobacterium abundance also correlates with reduced butyrate content in obese humans.\textsuperscript{15,16} Populations of Enterobacteriales and Verrucomicrobiales were increased in mice after Roux-en-Y gastric bypass surgery compared with sham mice.\textsuperscript{14} Increased Proteobacteria and decreased Firmicutes and Bacteroidetes were observed in Roux-en-Y gastric bypass rats.\textsuperscript{17} Previous studies of gut microflora before and after gastric bypass revealed reciprocal changes in Bacteroidetes and Firmicutes, but alterations in the Bacteroidetes/Firmicutes ratio varied in different reports.\textsuperscript{3,18} Some studies demonstrated that bacteria directly promoted gut inflammation and insulin resistance in mice fed a high-fat diet (HFD).\textsuperscript{19,20}

Timo mutant strain was generated in our laboratory as a T2DM animal model induced by Bdnf deficiency, in which a conserved genomic locus of Bdnf is disrupted by a trans gene insertion that decreases brain-derived neurotrophic factor (BDNF) levels to 30% of wild-type mice. Timo mice exhibit insulin resistance, hyperlipidemia, and metabolic inflammation before the onset of obesity and diabetes.\textsuperscript{21} However, whether the DJB surgery can reverse the metabolic phenotypes of Timo mutants is not known. In addition, Timo mice may also serve as a good model to investigate how inflammation and microbiota regulate insulin sensitivity and glucose metabolism after DJB surgery. In this study, we demonstrated that DJB surgery improved glycemic homeostasis and insulin sensitivity in Timo mice. We also observed alterations in gut microbiota before reduced inflammation and increased BDNF in Timo mice.

Materials and Methods

Animals and Duodenum-Jejunum Gastric Bypass Surgery

Timo mice were generated in our laboratory as described previously,\textsuperscript{22} and male C57BL/6J mice were housed in a specific pathogen-free facility under 12-hour light-dark cycle and hydrated with acidified water at the Model Animal Research Center of Nanjing University. Timo mice were fed a standard chow diet ad libitum. Seven-week-old male wild-type C57BL/6J mice were fed a HFD for 17 weeks before surgery or a normal chow diet as control. The Institutional Animal Care and Use Committee of the Model Animal Research Center of Nanjing University approved the experimental protocols.

Ten-week-old Timo mice and HFD-induced C57BL/6J mice underwent DJB surgery as previously described.\textsuperscript{22} Mice were deprived of food overnight before surgery and anesthetized with intraperitoneal 1.25% Avertin (T48402; Sigma-Aldrich, St. Louis, MO). All mice received 100 μL 0.5% carprofen subcutaneously to alleviate discomfort after surgery.

Insulin and Glucose Tolerance Test

Timo mice were deprived of food 16 hours before the oral glucose [D (+)-glucose; Sigma-Aldrich] tolerance test (2 g/kg body weight). Blood glucose concentrations were tested at 0, 15, 30, 60, 90, and 120 minutes after glucose administration. Mice were injected with 0.5 U of insulin for 1 kg body weight after a 6-hour food deprivation for the insulin tolerance test (Novo Nordisk Pharmaceutical Industries, Malov, Denmark), and blood glucose concentrations were measured at 0, 15, 30, 60, 90, and 120 minutes after insulin injection. Blood concentrations were tested using a Breeze 2 Blood Glucose Meter (Bayer HealthCare LLC, Mishawaka, IN). Body weight was measured weekly at the same time point.

Metabolic Measurements

Blood was collected from Timo mice eye socket veins after overnight food deprivation in heparinized tubes, and samples were stored at room temperature for 30 minutes to separate serum from whole blood. Serum was collected after centrifugation at 3000 × g for 15 minutes. Plasma (10 μL) was used to measure insulin concentrations using a mouse insulin enzyme-linked immunosorbent assay kit (Millipore, Billerica, MA; catalog no. EZRMI-13K), according to the manufacturer’s instruction. Plasma concentrations of leptin were determined using a mouse leptin enzyme-linked immunosorbent assay kit (Millipore; catalog no. EZRMI-82K). Plasma BDNF levels were detected using a BDNF enzyme-linked immunosorbent assay kit (Boatman Tech, Shanghai, China).

Blood Chemistry for Lipid Metabolism

Lipid metabolites and hepatitis concentrations, including total cholesterol, triglycerides, high-density lipoprotein, very-low-density lipoprotein, alanine aminotransferase, and aspartate aminotransferase, in plasma of Timo mice after DJB surgery were quantified using colorimetric assays in a 7020 automatic analyzer (Hatachi High Technology, Tokyo, Japan). Food intake and energy expenditure were measured using a complementary laboratory animal metabolic system.

Histologic Analyses

Liver and adipose tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (5 mm) were generated using a Leica (Wetzlar, Germany) microtome (RM2155) and stained with hematoxylin and eosin according to standard procedures. Liver frozen sections (10 mm) were stained with Oil Red O to analyze lipid accumulation.

Quantitative RT-PCR Analyses

High-quality total RNA from liver, white adipose tissue (WAT), brown adipocyte tissue (BAT), and muscle was isolated by extraction with RNAiso plus (9108; TaKaRa, Tokyo, Japan). cDNA was synthesized using a PrimeScript RT reagent Kit with a gDNA Eraser kit (RR047A; TaKaRa). The primers used for F4/80, Cd11b, Cd11c,
tumor necrosis factor-α (Tnfa), IL-6 (Il6), IL-1 β (Il1b), and monocyte chemoattractant protein 1 (Mcp1), and 36B4 were as follows: F4/80, 5'-AGTACGATGTTGG-GCTTTTG-3' (forward) and 5'-CCCCATCTGTACATCT-CACCT-3' (reverse); Cd11b, 5'-CAGTCCAGGGC-TCTCA-3' (forward) and 5'-GGAGCCATCACTGAA-GAG-3' (reverse); Cd11c, 5'-ATGGGACCTCAAGA-CAGGAC-3' (forward) and 5'-GGATCTGGGGATCT-GAAATC-3' (reverse); Mcp-1, 5'-CATCCACGTTG-GCTCA-3' (forward) and 5'-GATCATCTTGGCTGT-GAATGAGT-3' (reverse); Tnf-α, 5'-CTCTCTATTCT-CTGCTTTGGA-3' (forward) and 5'-GGTTCTGGGCGC-TAGAACTGA-3' (reverse); II-1β, 5'-AACCTGCTGTGG-GTGTAACGTTC-3' (forward) and 5'-AGACAGAGGCT-TTTTGTGTGT-3' (reverse); II-6, 5'-CGCATGAA-GTTCCTCCTCTGC-3' (forward) and 5'-CCTCTTGTA-AGTCTCCTTCTCC-3' (reverse). Quantitative reverse transcription-PCR for each gene was performed using SYBR Premix Ex Taq (RR420A; TaKaRa) in an ABI 7700 sequence detector (Applied Biosystems, Foster City, CA). The relative abundance of target gene transcripts was normalized to 36B4 expression.

Western Blot Analysis

Total protein extracts from WAT, liver, hypothalamus, and hippocampus were homogenized in RIPA lysis buffer that contained a 1% protease inhibitor cocktail (Sigma-Aldrich) and a 1% tyrosine phosphatase inhibitor cocktail (Sigma-Aldrich). Immunoblotting was performed as previously described.23 Total TNF-α was immunoblotted using a rat anti-TNF-α antibody (BD Biosciences, San Jose, CA; catalog no. 559064). Total BDNF was immunoblotted using a rabbit anti-BDNF antibody (Santa Cruz Biotechnology, Santa Cruz, CA; sc-546).

TNF-α Injection and Measurement

Plasma TNF-α levels were determined using a mouse TNF-α enzyme-linked immunosorbsent assay kit (MTA00B; R&D Systems, Minneapolis, MN), according to the manufacturer’s protocol. Mouse recombinant TNF-α (10 μg/kg) (GenScript, Nanjing, China; Z02918-100) was continuously injected intraperitoneally into Timo mice for 7 days after DJB surgery.

Bacterial DNA Extraction

Fecal samples and different parts of the biliopancreatic limb, the Roux limb, ileum, cecum, colon, and rectum were collected from DJB and sham mice and stored at -80°C for further use. Bacterial genomic DNA was extracted using the QiaAmp DNA stool Mini DNA-Isolation Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Concentrations were measured using the Qubit 2.0 instrument and the Qubit dsDNA HS Assay (Life Technologies, Invitrogen Division, Darmstadt, Germany).

16S rRNA Gene Primer and Amplicon Library Construction

Primers were used to amplify the 16S rRNA hyper variable region V6 according to the report by Huber et al24; all primers sequences are as follows: 967F-PP, 5'-CNACGG-GAAAGACCTTAN-C-3'; 967F-UC1, 5'-CAACGCCAA-AAACCTTACC-3'; 967F-UC2, 5'-CAACGCCGGAACACTTACC-3'; 967F-UC3, 5'-ATACGGGARGAACACTTACC-3'; 1046R, 5'-CGACAGGCTAGCANCACC-3'; 1046R-AQ1, 5'-CGACGGCATGCANCACC-3'; and 1046R-AQ2, 5'-CGACGACCAGCCANCACC-3'. The generated amplicon libraries were used in the Ion Plus Fragment Library Kit (Life Technologies, Invitrogen Division; catalog no. 4471252) according to the manufacturer’s instructions. Library concentrations were measured using the Qubit 2.0 instrument and the Qubit dsDNA HS Assay (Life Technologies, Invitrogen Division).

Microbiota 16S rRNA Gene Sequencing and Analysis

The Ion Torrent Personal Genome Machine Template OT2 200 kit (Life Technologies; catalog no. 4480974) was applied to perform emulsion PCR using the Ion OneTouch 2 system as described in the User Guidelines (Part No. 4469004 Rev. B 07/2011). Amplicon libraries were sequenced on the Ion Torrent Personal Genome Machine system using the Ion Torrent Personal Genome Machine Sequencing 200 Kit v2 (catalog no. 4482006; Life Technologies) according to the manufacturer’s protocol. The sphere was loaded to a 316 chip (Life Technologies) according to the method of Sebastian.25 ShangHai Biotechnology Corporation (Shanghai, China) analyzed 16S rRNA gene sequences. The relative abundance of bacterial taxonomic groups was compared in all sequences, and a principal coordinate’s analysis plot of weighted UniFrac distances was also performed in different groups.

Denaturing Gradient Gel Electrophoresis

Fecal bacteria DNA (100 ng) was amplified by 16rRNA v6-v8 variation region primers (968F primer contains GC clamp: 5'-CAGCCGCGGCGCGCCGCCGGCGGCGGCGGAGGGAACGCGGAAGACCTTACC-3'; 1401R primer: CGTGTGTAACAGACC) using a 2X Taqplus Master Polymerase mix (Vanzyme; P212-01/02/03), 433-bp amplicon was generated. Eight percent acrylamide gel (acylamide:bisacrylamide, 37.5:1) with a 35% to 55% (w/v) urea denaturant gradient was run at 80V for 12 hours. The gels were stained by 2 mg/mL silver in Cairns' fixation solution, and 1.5% NaOH that contained 0.4% formaldehyde as developer. The special band was extracted, transformed into Escherichia coli.

Statistical Analysis

Statistically significant differences between Timo mice and wild-type littermates are presented as means ± SEM. All data were analyzed using one-way analysis of variance (when three groups are compared) or repeated measurement analysis of variance (for time course data) and unpaired Student’s t-test. \( P < 0.05 \) was considered significant.

Results

Obesity, Glucose Intolerance, and Insulin Insensitivity in Timo Mice Are Reversed after DJB Surgery

Timo mice exhibited obesity, diabetes, and inflammation at 4 weeks of age (Supplemental Figure S1). To investigate whether DJB surgery reversed obesity in Timo mice, we performed DJB surgery on 10-week-old male Timo mice according to Woods et al \(^ {22} \) (Supplemental Figure S2). We found that DJB surgery caused a decrease in body weight from 34.99 ± 4 g to 26.59 ± 3.14 g after 1 week in Timo mice (Figure 1, A–C). HFD-induced obese mice, as a positive control, also exhibited body weight loss from 40.88 ± 0.18 g to 31.55 ± 0.79 g 1 week after surgery, and

![Image](https://example.com/image1.png)

**Figure 1**  Body weight and metabolism phenotypes of male Timo mice decrease after DJB surgery. **A:** Body weight of 10-week-old male severely obese Timo mice decreases 8 weeks after surgery. **B:** Weekly body weights for each of the sham and DJB mice. **C:** Relative body weight changes in the sham group and DJB group after DJB surgery. **D** and **E:** Fat and lean mass of Timo mice 8 weeks after DJB surgery by dual-energy X-ray absorptiometry analysis. **F:** Food intake of Timo mice 6 weeks after DJB surgery by complementary laboratory animal metabolic system. **G:** \( \text{VO}_2 \) of Timo mice 6 weeks after DJB surgery by complementary laboratory animal metabolic system. **H:** \( \text{VCO}_2 \) per kilogram of lean body weight of Timo mice 6 weeks after DJB surgery by complementary laboratory animal metabolic system. **I:** RER of Timo mice 6 weeks after DJB surgery by complementary laboratory animal metabolic system. **J:** Heat production of Timo mice 6 weeks after DJB surgery by complementary laboratory animal metabolic system. Data are expressed as means ± SEM. \( n = 8 \) to 13 (A); \( n = 5 \) (J). *\( P < 0.05 \), **\( P < 0.005 \), and ***\( P < 0.001 \). DJB, duodenum-jejunum gastric bypass; RER, respiratory exchange ratio (\( \text{VCO}_2/\text{VO}_2 \)); \( \text{VCO}_2 \), carbon dioxide release; \( \text{VO}_2 \), oxygen consumption.
exhibited better control of glucose (Supplemental Figure S3). Decreased body weight in Timo mice directly correlated with reductions in fat mass (Figure 1, D and E). Timo mice consumed fewer calories but exhibited no differences in physical activity from that of sham mice 8 weeks after DJB surgery (Figure 1, F-J, and Supplemental Figure S4). Our data confirm that DJB significantly reduced body weights in Timo mice and reduced food intake.

Bariatric surgery is one of the new clinical treatments for severe T2DM.26,27 Insulin sensitivity was normalized to the level of wild-type littermates 2 weeks after DJB surgery (Figure 2A). The effect of DJB surgery on insulin sensitivity was maintained for at least 8 weeks (Figure 2, B and C). Plasma insulin concentrations were reduced to normal concentrations in DJB Timo mice compared with sham wild-type littermates 9 weeks after DJB surgery (Figure 2D). The correction of glucose intolerance in Timo mice was maintained for at least 8 weeks after surgery (Figure 2, E and F).

Lipid Accumulation Is Reduced in Timo Mice after DJB Surgery

Obesity and T2DM are accompanied by leptin resistance because lipid accumulation damages leptin signaling.28,29 Lipid accumulation in liver and adipose tissue (WAT and BAT) and enlarged adipocyte size occurred in 8-week-old male Timo mice (Figure 3A). Timo mice also exhibited a tendency for leptin resistance with increased concentrations at 6 weeks (Supplemental Figure S5A).

Histologic analyses and Oil Red O staining revealed that fat content decreased significantly in the livers of Timo mice 8 weeks after surgery compared with that of the sham Timo mice (Figure 3B). Hepatitis (alanine aminotransferase) was

![Figure 2](image-url) Insulin sensitivity increases after DJB surgery in male Timo mice. A–C: Insulin tolerance improves 2 (A), 4 (B), and 8 (C) weeks after DJB surgery by insulin tolerance test. D: Plasma insulin concentration decreases to normal after DJB surgery for 9 weeks by enzyme-linked immunosorbent assay. E and F: Oral glucose tolerance increases 6 and 8 weeks after DJB surgery by glucose tolerance test. Data are expressed as means ± SEM. n = 3 to 7 (E and F). *P < 0.05, **P < 0.005, ***P < 0.001, and ****P < 0.0005. AUC, area under the curve; DJB, duodenum-jejunum gastric bypass.)
alleviated 9 weeks after DJB surgery, which contributed to the improvement in lipid metabolism (Figure 3D). We measured serum concentrations of total cholesterol, triglycerides, high-density lipoprotein, and very-low-density lipoprotein to investigate whether the decreased weight of Timo mice 9 weeks after DJB surgery reflected changes in lipid lysis. Total cholesterol and very-low-density lipoprotein decreased postoperatively in male and female Timo mice after DJB surgery (Figure 3C and Supplemental Figure S5B). Leptin concentrations in serum also decreased to normal concentrations in Timo mice 8 weeks after DJB surgery (Figure 3E).

Microbiota Alterations and Inflammation Suppression Results from DJB Surgery

Accumulating evidence indicates that bariatric surgery affects the diversity of gut microbiota.13,14,17 We analyzed fecal bacterial composition 1 week and 2 weeks after DJB surgery using denaturing gradient gel electrophoresis analysis to examine immediate changes in gut microbiota. Fecal bacteria exhibited different dominant populations with lower diversity in DJB mice than in wild-type littermates. Bacterial communities were unstable, which was indicated by large variations in different samples (Figure 4A). Desulfovibrionaceae abundance was much higher in the sham group of Timo mice (Table 1 and Supplemental Figure S6A) 1 week after surgery, Clostridiales abundance decreased (Table 2, Supplemental Figure S6A), and Verrucomicrobiaceae abundance increased (Supplemental Figure S6A and Table 2) in Timo DJB mice 2 weeks after surgery. The predominant gut bacteria community became consistent within the same experimental group 2 weeks after DJB surgery. The increase in Verrucomicrobiaceae abundance was more significant in Timo DJB mice (Table 2, Supplemental Figure S6B).

We also sequenced the gut bacterial 16S rRNA gene variation region from the intestines of DJB Timo mice, sham Timo mice, and sham wild-type littermates 9 weeks after DJB surgery (Figure 4 and Supplemental Figure S7). A total
of 105 samples from 15 mice were analyzed (average 92,376 16S rRNA gene sequences per sample) (Supplemental Table S1). Principal coordinate analysis plots of weighted UniFrac distances demonstrated that DJB surgery exhibited stronger effects on gut microbiota in DJB Timo mice than in the sham mice (Figure 3, A and D), especially in distal small intestine (Figure 3, A and E, and Supplemental Figure S7, A and B), large intestine, and feces (Figure 4, B and F, and Supplemental Figure S7, C–E). We found a higher abundance of anti-inflammatory Firmicutes and lower proinflammatory Proteobacteria in ileum in Timo mice 9 weeks after surgery (Figure 4E), but Verrucomicrobiae abundance increased significantly in the ileum and feces of Timo DJB mice (Figure 4, E and F). A decrease in the abundance of Bacteroides was observed in different parts of the large intestine (Figure 4F and Supplemental Figure S7, C–E). These data support that pathogenic bacteria decreased and beneficial microflora increased in Timo DJB mice, which may be associated with the improvements in the metabolism of Timo mice after DJB surgery.

Figure 4  Average relative abundance of ileum and fecal bacterial orders in DJB and sham Timo mice. A and B: Ileum and fecal microbial composition of individual mice from sham control (SI/SF, WSI/WSF) and DJB-operated groups (BI/BF) 9 weeks after DJB surgery by microbiota 16S rRNA gene sequencing and analysis. C and D: Principal coordinates analysis plot of weighted UniFrac distances. Each dot represents ileum or fecal community in the sham group and DJB group, indicated by color. E and F: Relative abundance of different microbiota in ileum and fecal sample in DJB group and sham group. Data are expressed as means ± SEM. n = 5. *P < 0.05, **P < 0.005. BI/BF, DJB-operated group; DJB, duodenum-jejunum gastric bypass; SI/SF, Timo sham group; WSI/WSF, wild-type sham group; WT, wild-type.
Timo Mice and Sham Control Mice after Surgery

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DGGE, denaturing gradient gel electrophoresis.

DJB Surgery Suppressed Systemic Inflammation in Timo Mice

Systemic inflammation was reported in obese humans and many rodent models.11,15,20,30–32 Inflammation was observed in WAT, liver, BAT, and muscle tissue of adult Timo mice, as indicated by the up-regulation of macrophage markers and inflammatory cytokines (Figure 5, A and B, and Supplemental Figure S5, D and E). The up-regulation of active macrophage markers and proinflammatory cytokines in WAT, BAT, and skeletal muscle occurred at 4 weeks of age, which occurred before the significant increase in body weight at 6 weeks in male Timo mice (Figure 5A and Supplemental Figure S5, D and E), and liver inflammation occurred at a later stage (8 weeks) (Figure 5B). The inflammation was aggravated with progression of obesity and diabetes in Timo mice, as indicated by the significant increase in the adipokines Tnf-α and Mcp-1 at 8 weeks of age (Figure 5A and B).

DJB surgery significantly ameliorated inflammation in the liver and WAT of Timo mice. The expression of macrophage markers (F4/80, Cd11b, and Cd11c) and proinflammatory cytokines (Tnf-α and Mcp-1) were significantly down-regulated 8 weeks after DJB surgery in the WAT and liver of Timo mice (Figure 5, F and G). Serum levels of TNF-α were also significantly lower than before DJB surgery (Figure 5, C–E). The number of macrophages in livers of Timo mice also decreased (Figure 5, H–K). To investigate whether inflammation alleviation resulted in insulin sensitivity improved in Timo

Table 2 DGGE Band Analysis from the Feces of Timo Mice and Sham Control Mice after Surgery for 2 Weeks

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DGGE, denaturing gradient gel electrophoresis.
mice, we studied the inflammation of WAT and liver of Timo mice 1 week after surgery. We found that the expression of macrophage markers (F4/80, Cd11b, and Cd11c) and proinflammatory cytokines (Tnf-α and Mcp-1) were not down-regulated, suggesting inflammation had not been suppressed (Figure 5, L and M). Furthermore, TNF-α expression was still elevated after DJB operation for 2 weeks (Figure 5N). This result supported the fact that the prominent long-time not short-time effects of DJB surgery on glycemic control result in the alleviation of inflammation postoperatively.

DJB Surgery Did Not Affect Bdnf Transcript Level in Hypothalamus and Hippocampus

The lower expression of BDNF in Timo mice contributes to obesity and diabetes phenotypes.21 We measured mRNA levels and protein levels of Bdnf genes in the hypothalamus and hippocampus.

**Figure 5** Inflammation changes in WAT and livers of male Timo mice before and after DJB surgery. A and B: mRNA levels of macrophage marker and inflammatory factor changes in WAT and liver of 4-, 6-, and 8-week-old male Timo mice by RT-qPCR. C and D: Plasma levels of TNF-α in 4- and 6-week-old male Timo mice. E: Plasma values of TNF-α in male Timo mice 9 weeks after DJB surgery by enzyme-linked immunosorbent assay. F and G: Macrophage markers and inflammation were decreased in liver and WAT of Timo mice 9 weeks after DJB surgery by RT-qPCR. H–K: Flow cytometric analyses of macrophages in stromal vascular fraction of livers of old male Timo mice 8 weeks after surgery. L and M: Macrophage markers and inflammation were not changed change in liver and WAT of Timo mice 1 week after DJB surgery by RT-qPCR. N: TNF-α remains high in Timo mice after surgery for 2 weeks and insulin sensitivity improves by Western blot analysis. Data are expressed as means ± SEM. n = 5 (A and B); n = 5 to 7 (E, H–K); n = 6 (F and G); n = 4 (L and M); n = 3 to 7 (N). *P < 0.05, **P < 0.005, and ***P < 0.001. DJB, duodenum-jejunum gastric bypass; RT-qPCR, quantitative RT-PCR; TLR-4, Toll-like receptor 4; TNF-α, tumor necrosis factor-α; WAT, white adipose tissue.
and hippocampus of Timo mice after DJB surgery to investigate whether the reverse effects of DJB surgery were due to alterations in BDNF expression. No significant changes in Bdnf were observed in brains of Timo mice 9 weeks after DJB surgery (Figure 6, A and B), and BDNF protein levels increased in hypothalamus and hippocampus of Timo mice after DJB surgery but not in the peripheral system (Figure 6, C–E). Otherwise, no significant difference was observed in HFD-fed mice 9 weeks after surgery compared with sham control mice (Supplemental Figure S8).

To investigate whether the BDNF level change in Timo mice contributed to microbiota change, we examined the BDNF level in Timo mice after surgery for 1 week while its microbiota altered. Our results show that no significant change of BDNF occurred in Timo mice after DJB surgery for 1 week (Figure 6F). These data suggest that the BDNF change in DJB Timo mice was indirectly affected by insulin sensitivity improvement.

Discussion

More than a dozen major studies demonstrated that bariatric Roux-en-Y gastric bypass surgery is therapeutic in T2DM human patients, rat, and mice by improving insulin sensitivity.6,17,33–35 Most research on gastric bypass was performed on HFD-fed rodent models.14,17,35,36 To our knowledge, the present work confirmed for the first time that DJB surgery effectively combats systemic inflammation and metabolic abnormalities in Timo mice, which is a Bdnf deficiency-induced T2DM model. In addition, we found the DJB surgery did not alter the expression of Bdnf, indicating a compensatory pathway for the DJB effects.

Accumulating evidence indicates that insulin resistance and lipid metabolism disorder are the hallmarks of obesity and contribute to the development of T2DM, cardiovascular disorders, and nonalcoholic fatty liver disease.2,9,37 Inflammation, especially in WAT and liver, is a key feature of obesity and T2DM.11,20 Individuals with increased levels of TNF-α and IL-6 exhibit insulin resistance.9,38 We also observed that systemic inflammation appeared before weight gain and insulin resistance in Timo mice. We found that DJB surgery significantly suppressed inflammation and TNF-α levels in Timo mice. This result is consistent with reports that barbassic surgery alleviated inflammation in mice with high sucrose—induced nonalcoholic steatohepatitis.32 However, inflammation alleviation was not the direct cause for improvements in the insulin sensitivity that

Figure 6  Bdnf level in different tissues of male Timo mice 9 weeks after surgery. Bdnf transcripts were determined using quantitative real-time RT-PCR. Bdnf transcripts I/I and IV/IV were investigated. A and B: Levels of different Bdnf transcripts in hypothalamus and hippocampus of DJB Timo mice and sham control mice by quantitative RT-PCR. All data are from 9 weeks after surgery in Timo mice. C and D: Plasma levels of BDNF in male Timo mice 2 and 9 weeks after DJB surgery by enzyme-linked immunosorbent assay. E: Protein levels of BDNF increase in hypothalamus and hippocampus of Timo mice 1 week after surgery by Western blot analysis. F: Protein levels of BDNF increase in hypothalamus and hippocampus of Timo mice 1 week after surgery by Western blot analysis. Data are expressed as means ± SEM, n = 6 per group (A and B). *P < 0.05, **P < 0.05, and ***P < 0.001. BDNF, brain-derived neurotrophic factor; DJB, duodenum-jejunum gastric bypass.
resulted from DJB surgery because enhanced insulin sensitivity preceded the TNF-α down-regulation. Inflammation reduction has been related to long-time intermittent deprivation of food both in mice and rat. However, all Timo mice after DJB surgery were only deprived of food one time (16 hours or 6 hours) for glucose tolerance test or insulin tolerance test and re-fed at least 2 weeks before inflammation measurement, we believed that food deprivation and re-feeding did not affect the conclusion about DJB effects on inflammation after DJB surgery. The lower inflammatory status may be beneficial for the maintenance of normal metabolic homeostasis because TNF-α administration partially impaired insulin sensitivity after DJB surgery in Timo mice (Supplemental Figure S5C).

An increasing abundance of Proteobacteria was involved in inflammation-associated dysbiosis. Studies suggested that the decreased abundance of Bacteroides and increase in Verrucomicrobia correlated with the enhancement of insulin sensitivity after bariatric surgery. Our data also demonstrated an increase in Proteobacteria in the ileum and feces of Timo mice (Figure 3, A, B and G), which is consistent with the upregulated inflammation in Timo mice (Figure 4D). We observed an increase in the abundance of Verrucomicrobia (genus: Akkermansia) and decrease in Bacteroidetes in Timo mice after DJB surgery, and a significant change in Verrucomicrobia appeared before the insulin sensitivity improvement after DJB surgery for 1 week (Figure 3A and Figure 1K). TNF-α levels remained high in WAT and liver of DJB Timo mice at this time (Figure 4L). The higher abundance of Firmicutes in ileum demonstrated lipid metabolism improvements after DJB surgery. The time course of different physiologic events after surgery may indicate that microbiota abundance alterations, but not inflammation, directly suppressed the effects on obesity and diabetes.

One of the possibilities for metabolic improvements may be the direct or indirect influence of DJB on brain BDNF expression. We examined BDNF expression after DJB surgery to exclude this possibility. We did not observe significant changes in the hypothalamus or hippocampus of DJB Timo mice before its metabolism homeostasis improved (Figure 6F). Bercik et al. found that the intestinal microbiota affects BDNF and behavior in BALB/c mice. Another group confirms that the gut microbiota affects hypothalamic and brainstem body fat–regulating circuits in C57Bl/6J male mice involved in reduction of leptin sensitivity and the expression of the obesity-suppressing neuropeptides proglucagon and Bdnf. Therefore, current data suggest that the changes in insulin sensitivity, inflammation, and gut microbiota are independent of BDNF changes (Figures 5 and 6).

Our results revealed the role of inflammation and microbiota in the effects of DJB surgery, and these data indicated that suppressed inflammation is the result, not the cause, of diabetes reversal in Timo mice after DJB surgery. Gut microbiota alterations may be the key reason for diabetes remission after DJB surgery, but more mechanistic studies are needed to explain how different families of microbiota regulate nutrient metabolism in the host. Given the potential role of microbiota in mediating inflammation, gut microbiota transplantation may become an effective treatment of enteritis and related bowel diseases.

**Acknowledgments**

S.J., Q.W., Z.H., A.S., Y.P., S.H., S.G., W.Z., and S.Y. performed experiments; S.J., Z.L., and X.G. designed the experiments and wrote the manuscript.

**Supplemental Data**

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2016.04.009.

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