Dissociation of intestinal and hepatic activities of FXR and LXRα supports metabolic effects of terminal ileum interposition in rodents

Running title:
FXR, LXRα and bariatric surgery

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The Farnesoid-X-receptor (FXR) and the Liver-x-receptors (LXRs) are bile acids activated receptors highly expressed in the enterohepatic tissues. The mechanisms that support the beneficial effects of bariatric surgery are only partially defined. Here we have investigated the effects of ileal interposition (IT), a surgical relocation of distal ileum into the proximal jejunum, on FXR and LXRs in rats. Seven months after surgery, blood concentrations of total bile acids, tauro cholic acid (TCA), a FXR ligand, and tauro-hyocholic acid (THCA), a LXRα ligand, were significantly increased by IT (P<0.05). In contrast, liver and intestinal concentrations of conjugated and non-conjugated bile acids were decreased (P<0.05). These changes associated with a robust induction of FXR and FXR regulated genes in the intestine, including Fgf15 a negative regulator of bile acid synthesis. IT repressed the liver expression of glucose-6-phosphatase (G6Pc) and phosphoenolpyruvate carboxykinase (Pepck) two gluconeogenic genes, along with the expression of LXRα and its target genes sterol-regulatory element-binding protein (Srepb) 1c and fatty acid synthase (Fas) in the liver. Treating IT rats with CDCA ameliorated insulin signalling in the liver. Whether confirmed in human settings, these results support the association of pharmacological therapies to bariatric surgeries to exploit the selective activation of intestinal nuclear receptors.
The Farnesoid receptor (FXR) and Liver X receptors (LXRs) are members of the nuclear receptor superfamily of transcription factors activated by bile acids and oxysterols (1,2). FXR, the master regulator of bile acid metabolism, is expressed mainly in the liver, intestine, kidney and adrenal glands (1,2). In the liver FXR engages a feedback loop that inhibits bile acid synthesis via induction of SHP, small heterodimers partner. SHP interacts with liver receptor homologue 1 (LRH1) to form a heterodimer, resulting in the repression of CYP7A1, the rate-limiting enzyme in the conversion of cholesterol to bile acids (3,4). In the intestine, FXR inhibits the absorption of bile salts via modulation of several transport proteins. FXR-dependent downregulation of the apical sodium-dependent bile salt transporter (IBAT) is thought to be mediated via SHP-dependent inhibition of LRH1 (5). In addition, FXR promotes transport of bile salts from the apical to the basolateral membrane of enterocytes via the upregulation of ileal bile acid-binding protein (IBABP) (6). Bile acids are then released into the portal circulation for return to the liver via FXR-induced expression of the organic solute transporters OSTα and OSTβ (7). Finally, FXR is a negative modulator of the sodium taurocholate cotransporting polypeptide (NTCP) which mediates absorption of bile acids from the portal circulation thus limiting hepatic bile salt levels (8). Another important function of FXR is the reduction of lipogenic pathways though downregulation of sterol-regulatory element-binding protein 1C (SREBP1C) and fatty acid synthase (FAS) genes (8). In addition, FXR exerts a substantial role in regulating hepatic carbohydrate metabolism (9). Indeed, FXR activation exerts differential effects on the regulation of hepatic gluconeogenesis during the transition from the fast to the fed state in mice. Thus, while the pharmacological activation of FXR in fed conditions negatively regulates gluconeogenic genes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6PC), in the liver, its activation in fasting does the opposite (9,10); partially explaining why FXR deficiency in rodent models of obesity seems to be beneficial on both body weight and glucose homeostasis (11). In contrast to the liver, activation of intestinal FXR
exerts beneficial effects on glucose homeostasis. Thus, the activation of intestinal FXR in the murine distal ileum induces the release of fibroblast growth factor-15 (\(Fgf15\)) (FGF19 is the human ortholog), a hormone that once secreted in the portal circulation reaches the liver to bind to the fibroblast growth factor receptor (FGFR) 4 repressing bile acid synthesis and the expression of gluconeogenic genes (12-14). This mechanism constitutes a crosstalk between the intestine and liver for the regulation of hepatic glucose production and is an appealing pharmacological target.

LXRs are ligand-activated nuclear receptors that act as cholesterol sensors. LXR\(\alpha\) is expressed in tissues with a high metabolic activity, including liver, adipose and macrophages, whereas LXR\(\beta\) is ubiquitously expressed (1). Both LXRs are activated by cholesterol derivatives, including oxysterols and 24(S),25-epoxycholesterol. Moreover, LXRs are activated by bile acids such as hyocholic acid (HCA) and hyodeoxycholic acid (HDCA) (1,2). One of the best-characterized effects of LXR\(\alpha\) is to promote reverse cholesterol transport (RCT), the process of cholesterol delivery from the periphery to the liver for excretion. The first step in RCT is the transfer of cholesterol to lipid-poor molecules in the plasma such as apolipoprotein A-I and pre-\(\beta\) high-density lipoprotein (HDL) via ATP binding cassette transporter A1 (ABCA1). LXR\(\alpha\) agonists induce ABCA1 expression in an LXR-dependent manner in macrophages and intestine (15). Another major function of LXR\(\alpha\) in the intestine is the induction of the transporters ABCG5 and ABCG8, which heterodimerize into a complex that mediates the apical efflux of cholesterol from enterocytes (16). In rodents, but not in humans, LXR\(\alpha\) activation enhances hepatic cholesterol catabolism partly through increased expression of cytochrome P450 7A1 (CYP7A1), the rate-limiting enzyme in the classical conversion of cholesterol to bile acids [17]. Others LXR target genes are SREBP1C and FAS (18-20), which promote de novo lipogenesis, and CD36, a membrane receptor capable of uptaking modified forms of low-density lipoproteins (LDL) and fatty acids from circulation (21).
Bariatric surgery is attracting increasing consideration for its role in the treatment of morbid obesity and type II diabetes (22). Despite several treatment modalities have been developed, the mechanisms that support the beneficial effects of different surgical approaches are only partially defined. In the present study we provide evidence that ileal interposition leads to a selective activation of the intestinal nuclear receptors FXR and LXR\(\alpha\) while it represses the expression/activity of these ligand-activated transcription factors in rat liver. Because in ileal transposed animals hepatic FXR and LXR\(\alpha\) become resistant to pharmacological activation, our findings provide a molecular explanation to the metabolic effects of bariatric surgery on glucose and lipid homeostasis and ground the basis for pharmacological exploitation of intestinal FXR and LXR\(\alpha\) in the treatment of type II diabetes and obesity in patients undergoing bariatric surgery.
RESEARCH DESIGN AND METHODS

Animal models. Male Wistar rats, (300-325 grams), were purchased from Harlan Italy (San Pietro Natisone, Italy). Rats were housed under controlled temperature (22°C) and photoperiods (12:12-hour light/dark cycle), allowed unrestricted access to standard rat chow and tap water. All animal experimental procedures were approved by the Ethics Committee of the University of Perugia and by the Italian Health Ministry. Ileal interposition (IT) surgery involves the removal and repositioning of a distal ileum segment to the proximal jejunum in a properistaltic direction, taking care to maintain the continuity of the gastrointestinal tract and to preserve neurovascular connections. Rats undergoing surgery were fasted overnight, anesthetized with gaseous anesthesia (2% Isoflurane) and placed on a warm platform. A small midline laparotomy was performed and the cecum was exteriorized to identify the terminal ileum. Briefly, the distal margin of the loop to transpose was selected as close as possible to the ileocecal valve and the proximal margin was identified at approximately 10 cm from the distal margin. The bowel was then divided leaving its mesenteric blood supply intact. The jejunum was interrupted just 10 cm distally from the ligament of Treitz, and the ileal loop was interposed and anastomosed isoperistaltically with the jejunal stumps (IT group; N=10). The sham operation (control group; N=10) consisted of 3 enterotomies in the same locations as occurred in the ileal interposition groups. The bowel was immediately reanastomosed after transection. Both operations lasted approximately 55 minutes per animal. When necessary the sham operation was prolonged to produce a similar degree of operative stress. Post-operatively, rats received analgesia for 2 days and had access to a liquid diet for 5 days. Then, they consumed a standard diet for 7 months. Body weight and food intake were recorded monthly after 2 months of surgical procedure. At the end of 7 months rats were sacrificed and blood was collected for subsequent biochemical assays. Serum content of total cholesterol, HDL, triglycerides and aspartate aminotransferase (AST) was
measured by enzymatic assays (Wako Chemicals; Osaka, Japan). Hepatic and intestinal samples were snap frozen for RNA and protein isolation or fixed in formalin for histology. For histologic analysis colon samples were fixed in buffered formalin and routinely prepared 5-µm sections were stained with hematoxylin and eosin (H&E) and alcian blue as previously described (23).

Animal protocols: treatments with CDCA and oleanoic acid

Seven months after surgical procedure of ileal transposition, rats experimental groups (control and IT) were randomized in two subgroup (N=5) that received orally vehicle (methylcellulose 1%) or the FXR agonist CDCA (20 mg/kg), for seven days. Rats were left unfed for sixteen hours and administered again with the FXR agonist. Three hour after last administration of CDCA, blood glucose was measured in triplicate before and 20, 30, 40, 60, 80, 100 and 120 minutes after the administration of D-glucose solution (2 g/kg body weight) by oral gavage. Blood was obtained by the tail vein, and glucose was assessed using a glucometer. Blood samples were collected and insulin concentration was determined using a rat insulin ELISA Kit (Mercodia). The animals were sacrificed and liver sample were excised and immediately snap frozen for RNA and protein isolation. In another experimental settings, in order to investigate oleanolic acid effect on glucose and GLP-1 release, rats of two experimental settings were sedated with Penthotal (50 mg/kg) and blood glucose was measured before and 15, 30, 45 and 60 minutes after the administration of oleanolic acid solution (10 g/kg body weight) by oral gavage. Insulin and GLP-1 levels were measured in plasma. Insulin and GLP-1 determination were performed as described in the main text.

Western Blotting. Frozen liver samples of ≈ 100 mg, obtained from sham operated rats (untreated or treated with CDCA) and from ileal transposed rats (untreated or treated with CDCA), were homogenized in 500 µl of T-PER (Pierce) supplemented with protease and phosphatase inhibitors. The homogenates were centrifuged at 10000 g for 10 minutes and the supernatants were used as
whole cell lysates. Protein levels in tissue extract were quantified with Bradford reagent and 20 µg of proteins were used in each SDS-PAGE run, that were subsequently transferred to nitrocellulose membranes (Bio-Rad) and probed with primary antibodies pERK 1/2-Thr202/Tyr204 (#9101S, Cell Signaling), ERK 1/2 (#9102, Cell Signaling), pAKT-Ser473 (#9271, Cell Signaling), AKT (#9272, Cell Signaling), pGSK3β-Ser9 (#9336, Cell Signaling) and GSK3β (#9315P, Cell Signaling). The anti-immunoglobulin G Rabbit (Bio-Rad) was used as a secondary antibody, and specific protein bands were visualized by chemoluminescence using Supersignal West Dura reagent (Pierce). Quantification of the blots was performed by using ImageJ software. For each sample, integrated density of phospho-antibody band was divided by that of total antibody band.

**Analysis of blood glucose levels, insulin, GLP-1 and oral glucose tolerance test (OGTT).** OGTT was performed after 12–14 hours of fasting (7 months after surgical procedure). To reduce the stress during blood collection, rats were sedated with Penthotal (50 mg/kg). Blood glucose was measured in triplicate before (baseline) and 20, 30, 40, 60, 80, 100 and 120 minutes after the administration of D-glucose solution (2 g/kg body weight) by oral gavage. Blood was obtained by the tail vein and glucose was assessed using a glucometer (One Touch Ultra, Lifescan; Johnson & Johnson, Milpitas, Calif). Blood samples were placed in centrifuge tubes containing 50-mmol/L EDTA and 100-µmol/L DPP-4 inhibitor that was added to avoid GLP-1 degradation. After centrifugation (3000 rpm for 10 minutes at 4°C), plasma was collected and stored at -80°C until assay. Insulin concentration was determined using a rat insulin ELISA Kit (Mercodia) and plasma levels of GLP-1 were measured using an EIA kit (Phoenix Pharmaceuticals).

**Quantitative real time(RT)-PCR.** Immediately after sacrifice, liver and ileum were excised from animals of the control group and IT group. Total RNA was isolated from these samples using the TRIzol reagent (Invitrogen) accordingly with manufacturer protocol; 1 µg of purified RNA was treated with DNase-I and reverse transcribed to cDNA with Superscript-II (Invitrogen) in a 20 µl
reaction volume using random primers. For quantitative RT-PCR, 25 ng of template was dissolved in a 25 μl solution containing 200 nM of each primer and 12.5 μl of SYBR GreenER qPCR SuperMix (Invitrogen) for iCycler iQ instrument (Bio-Rad). All reactions were performed in triplicate, and the thermal cycling conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, followed by 50 cycles of 95 °C for 15 s, 58°C for 30 s and 72°C for 30 s. The relative mRNA expression was calculated accordingly with the \( C_t \) method. All PCR primers were designed using the software PRIMER3 (http://frodo.wi.mit.edu/primer3/) using published sequence data obtained from the NCBI database (Supplementary Table I).

**Bile acids determination and sample preparation.** The stock solutions of the individual tauro-conjugated and un-conjugated bile acids were prepared separately in methanol at a concentration of 1 mg/mL. All stock solutions were stored at −20 °C. Calibration standards were prepared by combining appropriate volumes of each bile acid stock solution and methanol. The calibration range was from 10 nM to 100 μM of each bile acid in the final solution. Rat serum sample aliquots of 100 μL were deproteinized with 1 mL of cold acetonitrile with 5% of NH₄OH vortexing for 1 min (23). After centrifugation at 16000 g for 10 min, the clear supernatant was transferred to a new vial, snap frozen and lyophilized. The sample was then re-dissolved in methanol–water (2:1, v/v) for tauro-conjugated bile acids determination and in methanol-ammonium acetate 10 mM with 0.005% formic acid (3:2, v/v) for unconjugated bile acids determination. A bile acids extraction yield of 95% has been measured using bile acids standards addition in plasma sample before and after deproteinization procedure. For colon and liver samples, 100 mg of lyophilized tissue was homogenized for 20 min in 10 mL H₂O. After 45 min of sedimentation, 500 μL of liver homogenate were added to 2mL of ice-cold alkaline ACN. Samples were vortexed and shaked continuously for 30 min, and then centrifuged at 16,000×g for 10 min. The supernatant was aspirated and the pellet was extracted with another 1ml of ice-cold alkaline ACN. Supernatants from the 2
extraction steps were pooled, lyophilized and reconstituted in 100 μl of methanol–water (2:1, v/v) for tauro-conjugated bile acids determination and in 100 μl of methanol-ammonium acetate 10 mM with 0.005% formic acid (3:2, v/v) for unconjugated bile acids determination (24).

**Liquid chromatography and mass spectrometry.** For LC–MS/MS analysis, chromatographic separation was carried out on the HPLC–MS system LTQ XL ThermoScientific equipped with Accelera 600 Pump and Accelera AutoSampler system. The mixture was separated on a Jupiter 5μ C18 column from Phenomenex (150X2.00 mm). Tauro-conjugated bile acids were separated at a flow rate of 200 μl/min using a methanol–aqueous ammonium acetate (NH₄OAc) gradient (25). Mobile phase A was 5% methanol in water containing 2mM ammonium acetate at pH 7, mobile phase B was methanol, containing ammonium acetate at 2mM. The gradient started at 30 % B and increased to 100% B in 20 min, kept at 100% B for 5 min then decreased to 30% B in 1 min and kept at 30% B for 10 min. ESI was performed in negative ion mode, the ion source temperature was set at 280 °C. The tune page parameters were automatically optimized injecting taurocholic acid at 1 μM as standard. The MS/MS detection was operated in MRM mode using a collision energy of 20 (arbitrary units), the observed transitions were: Tauromuricholic acid (t-MCA) at 13.5 min MRM of 514.28 Th→514.28 Th, taurohyocholic acid (t-HCA) at 15.6 min MRM of 498.29 Th→498.29 Th, taurocholic acid (t-CA) at 16.6 min MRM of 514.28 Th→514.28 Th, taurochenodeoxycholic acid (t-CDCA) at 18.5 min MRM of 498.29 Th→498.29 Th, and taurodeoxycholic acid (t-DCA) at 18.9 min MRM of 498.29 Th→498.29 Th. Unconjugated bile acids were separated at a flow rate of 200 μl/min using 10 mM Ammonium Acetate in water at 0.005% formic acid as the mobile phase A 10 mM Ammonium Acetate in methanol at 0.005% formic acid as mobile phase B (26). The gradient program started at 60% B and increased to 95% B in 25 min, kept at 95% B for 9 min then decreased to 60% B in 1 min and kept at 60% B for 10 min. ESI was performed in negative ion mode, the ion source temperature was set at 280 °C (27). The tune
page parameters were automatically optimized injecting CA at 1 μM as standard. The MS/MS detection was operated in MRM mode using a collision energy of 15 (arbitrary units). The observed transitions were: hyocholic acid (HCA) at 8.9 min MRM of 391.29 Th→391.29 Th, cholic acid (CA) at 10.2 min MRM of 407.28 Th→407.28 Th, chenodeoxycholic acid (CDCA) at 13.8 min MRM of 391.29 Th→391.29 Th, deoxycholic acid (DCA) at 14.4 min MRM of 391.29 Th→391.29 Th and lithocholic acid (LCA) at 17.5 min MRM of 375.28 Th→375.28 Th.

Tissue triglyceride and cholesterol determination

For hepatic and ileal determination of triglycerides and cholesterol, fragments of ≈ 100 mg of liver and ileum were homogenized with 1 ml of T-PER (Pierce). The homogenates were used for protein concentration analysis (Bradford assay-Biorad) and 100 μl of tissue extracts added to 1.6 ml chloroform:methanol (2:1) for 16 hours at +4°C, after which 200 μl of 0.6% NaCl was added and the solution was centrifuged at 2000 g for 20 minutes. The organic layer was removed and air-dried in chemical hood. The resulting pellet was dissolved in 400 μl Phosphate Saline Buffer containing 1% Triton X-100 (Sigma-Aldrich). Triglycerides and cholesterol were measured by specific enzymatic reagents (Wako Chemicals; Osaka, Japan).

Statistical Analysis. All values are expressed as means ± SE of n observations/group. Comparisons of more than 2 groups were made with a one way ANOVA with post hoc Tukey’s test. Comparison of 2 groups was made using Student’s t test for unpaired data when appropriate. Differences were considered statistically significant at values of P < 0.05.
RESULTS

Effects of ileal interposition surgery on systemic and biochemical parameters. Over the 7 months post-surgery all rats (sham and IT) consumed a similar amount of diet and there was no significance difference in body weight among the two groups (Table 1). While similar plasmatic levels of total cholesterol, HDL, AST and glucose were observed, a strong reduction of plasmatic levels of triglycerides was documented in IT rats (Table 1) (28-30). Glucose tolerance was significantly improved at 15 and 30 min in IT rats (Fig. 1A). Moreover, glucagon like peptide-1 (GLP-1) and insulin levels were significantly higher in IT rats in comparison to sham operated animals (Fig. 1B and C). Since it is recognized that IT surgery increases the secretion of GLP-1, a well defined target gene for the bile acid-activated plasma membrane receptor GPBAR-1, we have next examined the mRNA levels of \textit{Gpbar-1} and \textit{pro-glucagon-1} (a GLP-1 precursor) in the ileum. Results from these experiments demonstrate that both \textit{Gpbar-1} and \textit{pro-glucagon-1} genes were induced in the ileum of IT rats (Fig. 1D). The histopathological analysis of ileum sections demonstrated an increase in length and number of villi in the interposed ileal segment compared with the ileum of sham operated rats (Fig. 1E). The role of GPBAR-1 was further investigated by challenging sham and IT rats with oleanolic acid, a GPBAR-1 ligand. These functional studies demonstrated that treating rats with oleanolic acid activated intestinal \textit{Gpbar-1} and induced a rapid and robust increase of GLP-1 and insulin, thus improving glucose profile in response to OGTT (Supplementary Fig. 1).

Serum bile acids. We have next investigated the bile acids composition in the plasma, liver and small intestine in IT rats. Consistent with previous works (28, 31, 32) we found that IT increases the total plasmatic levels of bile acids (Fig. 2A). The plasmatic concentrations of non conjugated bile acids, chenodeoxycholic acid (CDCA), cholic acid (CA) and hydroxylated CA (HCA) were significantly increased in IT rats (Fig. 2B). Moreover, IT surgery significantly increased plasma...
concentration of tauro-β-muricholic acid (TβMCA) and tauro-CDCA (TCDCA), while reduced that of tauro-deoxycholic acid (TDCA) (Fig. 2C). When these changes were expressed as a percentage of total bile acids we found that IT rats had a robust increase in the percentage of primary bile acids CDCA, CA and HCA (4%, 17% and 42% respectively) compared to sham operated rats (1%, 6% and 24%). Moreover, as reported in Figures 2D and E, a reduction in the percentage of tauro-conjugated bile acids TCA, TDCA and THCA was found in the IT group in comparison to sham operated rats (14%, 2% and 12% respectively versus 22%, 7% and 29%).

**Hepatic bile acids.** In the contrast to what observed in the serum, hepatic levels of total bile acids were lower in IT rats compared to sham operated animals (Fig. 3 A). The analysis of quantitative distribution of bile acids in the liver highlighted that the concentration of primary bile acids CA and HCA increased in IT rats (Fig. 3 B). Furthermore, the concentration of tauro-conjugated bile acids TCDCA and TDCA enhanced while that TCA was strongly reduced after bariatric surgery (Fig. 3 C). Noteworthy, the compositional analysis of plasmatic bile acids expressed as a percentage showed that ~ 95 % of bile acids in the liver were tauro-conjugated in both experimental groups and confirmed that IT surgery enhanced the hepatic content of THCA and reduced hepatic levels of TCA (THCA: IT 23% vs sham 4%; TCA: IT 49% vs sham 84%).

**Ileal bile acids.** IT reduces the total bile acids content in the intestinal wall, a measure of bile acid absorption by the intestine (Fig. 4 A). Thus, while the intestinal content of CDCA, CA and LCA was higher in the IT group in comparison to sham operated rats (Fig. 4 B), the intestinal concentration of conjugated bile acids TCA, TDCA and THCA was significantly reduced in IT rats compared to sham operated animals (Fig. 4 C). As in the liver, the analysis of intestinal bile acids showed that ~ 95 % were tauro-conjugated in both experimental groups. In particular, the percentage of TCA and THCA (FXR and LXRα agonists respectively) did not change between the two experimental groups while that of TβMCA (a TGR5 ligand) and CA enhances after IT (Fig. 4 D and E).
**Ileal interposition dissociates intestinal and liver FXR activities.** Having shown that surgical interposition of the distal ileum into the proximal jejunum strongly decreases hepatic content of TCA (a well recognized FXR agonist in rodents) but does not change its intestinal content, we have further investigated whether this shift in relative bile acid composition modulates the expression of FXR and its target genes in both the ileum and liver of IT rats. Results from Real-Time PCR data analysis demonstrated that the IT procedure upregulates the expression of Fxr and its target genes Ostα and Fgf15 in the ileum (Fig. 5A). Because Fgf15 inhibits hepatic gluconeogenesis through a pathway involving the inhibition of PGC-1α, a coactivator of the nuclear receptor glucocorticoid receptor (GR), the master regulator of gluconeogenic genes G6PC and PEPCK (9, 33, 34), we have then investigated the FXR/FGF15 pathway in sham operated and IT rats. For this purpose we performed an OGTT test in IT rats treated for 7 days with the natural FXR agonist CDCA. On the seventh day rats were fasted overnight and treated with CDCA 3 hour before the OGTT. Results from these experiments demonstrated that administering IT rats with CDCA improves the glucose tolerance at all time points, with the greatest differences observed between 60 and 120 minutes (Supplementary Fig. 2A). Furthermore, in comparison to sham operated rats, administering IT rats with CDCA resulted in a robust reduction of insulin plasma levels (Supplementary Fig. 2B). The IT surgery, per se, increased intestinal expression of Fgf15 (Fig. 5A) and exposure to CDCA resulted in a further ~ 6 folds induction of this hormone (Supplementary Fig. 2 C). These changes resulted in a robust suppression of gluconeogenic genes in the liver. Thus, IT surgery resulted in a robust reduction of Pepck, G6pc and Pgc1α mRNA expression in the liver (Fig. 5B) and exposure to CDCA further amplified this effect (Supplementary Fig. 2 D), while no changes in hepatic expression of Gr was observed (Fig. 5 B and Supplementary Fig. 2 D). Because these findings suggest that the activation of intestinal FXR/FGF15 axis regulates hepatic gluconeogenesis in IT rats, we have further dissected the FGF15 and insulin signals in the liver by investigating the phosphorylation
status of ERK, AKT and GSK3β by Western blot analysis (Supplementary Fig. 3A). Results of these experiments demonstrated that CDCA significantly reduces GSK3β phosphorylation while enhances the phosphorylation of ERK1/2 on both Thr 202 and Tyr 204 residues (Supplementary Fig. 3 B). Moreover, AKT phosphorylation was not changed (Supplementary Fig. 3 B) (34).

The investigation of Fxr gene expression in the liver also revealed that IT surgery had no effect on the transcription of this nuclear receptor as well as that of its canonical target genes Shp and Bsep, while the expression Ostα, another FXR target gene, was repressed by ~ 70% (Fig. 5 C). Similarly to Ostα, results from Real-Time PCR experiments showed that the liver expression of genes involved in bile acid uptake (i.e. Ntcp) and bile acid synthesis (Cyp7a1, Cyp8β1, Cyp27Aα1 and Cyp7β1) was reduced by 60-80% by IT surgery.

Ileal interposition dissociates intestinal and liver LXR activities. Because the IT surgery increases the concentration of THCA, a ligand for LXRα, we have then analyzed both ileal and hepatic expression of LXRα, the main regulator of reverse cholesterol transport (RCT) and lipidogenesis. As illustrated in Figure 6 A, the IT surgery resulted in a robust induction of Lxrα and its target genes Abca1, Abcg5 and Abcg8, involved in the reverse cholesterol transport pathway (15, 16). Despite it has been reported that intestinal-specific LXRα activation leads to decreased intestinal cholesterol absorption and improved lipoprotein profile (35), we did not observed any reduction of plasma cholesterol levels in IT rats (Table-I). However, IT rats had a significant reduction in the expression of the Lxrα target gene Npc1l1, a central player in intestinal cholesterol absorption (36, 37). Noteworthy, the investigation of fatty acid transport protein-4 (Fatp4), which is the principal fatty acid transporter in enterocytes (37, 38), as well as that of Cd36, another LXRα regulated gene involved in the up-take of oxydated LDL, revealed that the expression of these proteins was not changed between the two experimental animal groups, sham and IT (Fig. 6 A).
As shown in Figure 6 B, the analysis of hepatic expression of Lxrα demonstrated that this transcription factor and its target gene Cd36 were reduced in the liver of IT rats, while no change was observed in the hepatic expression of others LXRα target genes such as Abca1, Abcg5 and Abcg8. In line with these results, the hepatic mRNA expression of the LXRα target gene Srebp1c, the main regulator of fatty acid synthase (Fas) expression, was reduced following the surgical procedure (Fig. 6 B).

Finally we found no significant changes in terms of hepatic and ileal content of cholesterol and triglycerides among the two experimental groups (Fig. 6 C, D and E). Noteworthy, ileal content of triglycerides were strongly up-regulated in IT rats compared to sham operated animals (Fig. 6 F).
DISCUSSION

Bariatric surgery, such as Roux-en-Y gastric bypass (RYGB), is currently the most effective long-term treatment for obesity (39, 40) and has been shown to markedly improve glucose homeostasis in type 2 diabetes (41-43), but the mechanisms by which this occurs remain poorly defined. The improvement of glucose homeostasis after bariatric surgery has been attributed to weight loss resulting from a reduction in gastric volume and/or reduced nutrient absorption, depending on the type of surgery. However, results from clinical studies support a role for adaptive endocrine changes. First, in patients with type 2 diabetes undergoing bariatric surgery, such as RYGB, glucose normalization often occurs before substantial weight loss (43, 44). Second, bariatric surgeries involving bypass of the proximal small intestine, such as ileal interposition and/or biliopancreatic diversion are more effective at improving obesity and reversing type 2 diabetes than bariatric surgeries involving only gastric restriction (43, 45). These observations have led to the concept that the release of insulinotropic hormones, such as GLP-1 and PYY, from L cells located in the distal ileum plays an important mechanistic role (46). Increased secretion of these hormones after bariatric surgery has been demonstrated in a number of clinical studies and may contribute to weight loss and improved glucose metabolism (45-47).

In the present study we have investigated the molecular mechanisms that underlie the metabolic benefits induced by ileal transposition, a surgical procedure in which the distal ileum is interposed in the proximal jejunum in an isoperistaltic direction. The results presented here demonstrate that IT improves glucose homeostasis and lipid metabolism by altering the physiology of bile acids. In particular, IT increases total plasma bile acids concentrations while reduces their content in intestinal wall and liver. Compositional analysis of hepatic bile acids pool revealed that the IT surgery reduces the percentage of TCA, a well recognized FXR agonist, enhances the quote of THCA, a well identified LXRα agonist and has no effect on the percentage of
\(\beta\)MCA, a recognized TGR5 agonist. By contrast, the relative amount of FXR and LXR\(\alpha\) agonists in the intestine remain constant but the amount of \(\beta\)MCA is increased in the ileum of IT rats. These findings raised the concept that IT, by resetting the compositions of bile acids in entero-hepatic tissues or by increased cycling of bile acid from the intestine to the liver (i.e. their entero-hepatic cycling), reset the expression and activity of bile acid activated receptors (i.e. Gpbar-1, Fxr and Lxr\(\alpha\)) throughout the whole intestine.

To confirm this hypothesis we examined whether IT regulates intestinal GPBAR-1 expression/activity and demonstrated that the mRNA expression of this G-protein activated receptor was increased after the surgical procedure and that the relative increase of \(\beta\)MCA, a GPBAR-1 agonist, was likely responsible for GPBAR-1 mediated secretion of GLP-1 in the intestine of IT rats.

A key observation of the present study was the demonstration that IT exerts opposite effects on FXR signaling in the intestine and liver. Results from our studies demonstrate that while ileal expression of Fxr and its target genes Ost\(\alpha\) and Fgf15 increases in response to IT, the expression Fxr and its target genes (Ost\(\alpha\), Bsep and Shp) in the liver is down-regulated or remains unchanged. Together with the fact that hepatic bile acid concentrations are dramatically reduced by IT, these data indicate that this surgical procedure activates the expression/activity of ileal FXR while its activity in the liver is repressed. This finding was further confirmed by the analysis of genes involved in liver metabolism of bile acids. Thus, the liver expression of genes involved in bile acid uptake (i.e. Ntcp) and bile acid synthesis (such as Cyp7\(\alpha\)1, Cyp8\(\beta\)1, Cyp27\(\alpha\)\(\alpha\)1 and Cyp7\(\beta\)1) were reduced by 60-80% by IT. Because these genes are directly regulated by intracellular concentrations of bile acids, the fact that bile acid synthesis is repressed, despite the robust reduction of liver bile acid concentrations caused by IT, indicates that IT induces an escape mechanism that superimpose to liver FXR. Indeed, regulation of Cyp7\(\alpha\)1 and Cyp8\(\beta\)1 by FXR is
mediated by activation of several indirect mechanisms (34, 48, 49). FXR-mediated activation of
SHP and Fgf15 (FGF19 in humans) has been shown to be responsible for this suppression. By using
novel genetically modified mice, Kong et al (49) have shown the intestinal FXR/FGF15 pathway is
critical for suppressing both Cyp7α1 and Cyp8β1 gene expression, while the liver FXR/SHP
pathway has a minor role in suppressing Cyp7α1 gene expression. Present data are fully
consistent with these findings and indicate that ileal transposition causes a selective activation of
intestinal FXR and that the FXR/FGF15 pathway mediates, in its turn, the repression of bile acid
synthesis observed in this study.

Another important observation we have made in this study was that the activation of intestinal
FXR/FGF15 axis represses hepatic gluconeogenesis. Indeed, the FXR-mediated induction of Fgf15
in the intestine resulted in a strong down-regulation of the expression of two gluconeogenic
genes, G6pc and Pepck, in the liver of IT animals. In addition, administering rats with CDCA
increased ERK1/2 phosphorylation while reduced that of GSK3β. Since a previous work reported
that FGF19 (a human ortholog of FGF15) induces glycogen synthesis by enhancing ERK1/2
phosphorylation and by repressing GSK3β (50), our results seem to suggest that the FXR mediated
induction of FGF15 could also induce glycogen synthesis. Thus, the selective inhibition of hepatic
activity of FXR achieved by the surgical strategy may be an appealing option for the therapy of
obesity and the diseases related to dysfunctions of glucose homeostasis.

Ileal interposition surgery also dissociates the expression/activity LXRs. In this regard we have
observed that IT modulates Lxrα expression and activity. Moreover, we found that the increased
expression of Lxrα in the ileum associated with an up-regulation of LXRα target genes Abca1,
Abcg5 and Abcg8, and with a repression of the Npc1l1, a protein which mediates the cholesterol
absorption in the intestine. Of note, the expression of Fatp4, the main mediator of intestinal
absorption of triacylglycerols, as well as that of Cd36, which mediates the absorption of oxydated
LDL, was not changed between sham and IT rats. Accordingly with RT-PCR data, ileal interposed rats had a robust increase in the intestinal content of TG.

In conclusion this study demonstrated that IT, a bariatric surgery, produces improvements of glucose tolerance and obesity by altering the homeostasis of bile acids in rats. The altered physiology of bile acids results in extensive remodeling of expression/activity of bile acid activated receptors in entero-hepatic tissues. These data unveil potential novel strategies for the prevention and treatment of obesity and type 2 diabetes.

AKNOWLEDGMENTS

SF is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Author contributions AD and SF conceived the study; AM, CS and LG performed ileal interposition surgery; AM, CD and CS performed OGTT test; CD and AB performed in vitro experiments; SC performed histologic analysis; BR performed Real-Time PCR data analysis; MCM performed serum and intrahepatic and intestinal bile acid quantization; ED contributed to statistical analysis; BR and SF wrote the manuscript. The authors declare no competing financial interests.
REFERENCES


### Table 1. Clinical and biochemical assessments  7 months after intestinal interposition.

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<th>Parameters</th>
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<td>Daily food intake</td>
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<td>(Grams/100 g of body weight)</td>
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<td>Final Weight (grams)</td>
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<td>Glucose (mg/dL)</td>
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<td>HDL-cholesterol (mg/dL)</td>
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<tr>
<td>Triacylglycerols (mg/dL)</td>
<td>62.5 ± 5.7</td>
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<tr>
<td>AST (U/L)</td>
<td>82.5 ± 3.7</td>
<td>85.3 ± 4.7</td>
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The values are expressed as mean ± SE. (* p<0.05 IT group versus sham group N=10).
FIG. 1. Ileal interposed rats show an improvement in glucose tolerance. Oral glucose tolerance test (OGTT) performed at 7 months after surgical procedure in ileal interposed and sham operated rats after 18 hours of food withdrawal (“fasting”). (A) Blood glucose curve, (B) plasmatic levels of GLP-1 and (C) insulin. (D) Ileal gene expression of Gpbar-1 and pro-glucagon-1. Values are normalized relatively to Gapdh mRNA and are expressed relative to those of sham operated rats, which are arbitrarily set to 1. Data are mean ± SE of 5 animals per group (*p<0.05 IT compared to Sham group). (E) Representative Hematoxylin/Eosin and alcian blue staining of ileal section obtained from sham operated and ileal interposed animals.

FIG. 2. Characterization of plasmatic bile acids. (A) Total bile acids. (B) Plasmatic concentrations of non-conjugated bile acids: cholic acid (CA), chenodeoxycholic acid (CDCA), hyocholic acid (HCA), deoxycholic acid (DCA) and lithocholic acid (LCA); (C) plasmatic concentrations of conjugated bile acids: tauro(T)-βmuricholic acid (TβMCA), T-cholic acid (TCA), T-chenodeoxycholic acid (TCDCA), T-hyocholic acid (THCA) and T-deoxycholic acid (TDCA). Qualitative analysis of bile acids composition in (D) sham operated and (E) ileal interposed rats. The values are expressed as mean ± SE. (*p<0.05 IT compared to Sham group, N=5).

FIG. 3. Characterization of hepatic bile acids. (A) Total bile acids. (B) Hepatic concentrations of non-conjugated bile acids: CA, CDCA, HCA, DCA and LCA; (C) hepatic concentrations of conjugated bile acids: TβMCA, TCA, TCDCA, THCA and TDCA. Qualitative analysis of bile acids composition in (D) sham operated and (E) ileal interposed animals. The values are expressed as mean ± SE. (*p<0.05 IT compared to Sham group, N=5).

FIG. 4. Characterization of ileal bile acids. (A) Total bile acids. (B) Ileal concentrations of non-conjugated bile acids: CA, CDCA, HCA, DCA and LCA; (C) ileal concentrations of conjugated bile acids: TβMCA, TCA, TCDCA, THCA and TDCA. Qualitative analysis of bile acids composition in (D) sham operated and (E) ileal interposed animals. Data are mean ± SE of 5 animals per group (*p<0.05 IT compared to Sham group).

FIG. 5. Ileal interposition dissociates ileal and hepatic FXR expression/activity. (A) Ileal mRNA relative expression of FXR, OSTα and FGF15. (B) Hepatic mRNA relative expression of Pepck, G6pc, Pgc1α and Gr. (C) Hepatic mRNA expression of Fxr, Shp, Ntcp, Cyp7a1, Cyp7b1, Cyp8b1, Cyp27α1, Bsep and Ostα. Values are normalized relatively to Gapdh mRNA and are expressed relative to those of control rats, which are arbitrarily set to 1. Data are mean ± SE of 5 animals per group (*p<0.05 compared to Sham group).

FIG. 6. Ileal interposition dissociates ileal and hepatic LXR expression/activity. (A) Real-time PCR analysis of ileal expression of Lxrα, Fatp4, Npc1l1, Cd36, Abca1, Abcg5 and Abcg8. (B) Real-time PCR analysis of hepatic expression of Lxrα, Srebp1c, Fas, Cd36, Abca1, Abcg5 and Abcg8. Values are normalized relatively to Gapdh mRNA and are expressed relative to those of control rats, which are arbitrarily set to 1. Cholesterol and triglycerides determinations in the liver (C-D) and intestine (E-F) of sham operated and ileal interposed animals. Data are mean ± SE of 5 animals per group (*p<0.05 compared to Sham group).
Figure 1

Panel A: Glucose (mg/dL) response over time (mins) for Sham and IT groups.

Panel B: GLP-1 (ng/ml) response over time (mins) for Sham and IT groups.

Panel C: Insulin (µg/l) response over time (mins) for Sham and IT groups.

Panel D: Relative mRNA expression of Gpbar-1 and Pro-glucagon 1 for Sham and IT groups.

Panel E: Histological images comparing Sham and IT groups.
Figure 2

Diabetes

PLASMA

A. Total bile acids (µmol/L)

B. Non-conjugated bile acids (nmol/L)

C. Conjugated bile acids (nmol/L)

D. Pie chart of non-conjugated bile acids in Sham group

E. Pie chart of conjugated bile acids in Sham group

F. Pie chart of non-conjugated bile acids in IT group

G. Pie chart of conjugated bile acids in IT group

Legend:
- CA
- CDCA
- HCA
- DCA
- LCA
- TβMCA
- TCA
- TCDCA
- THCA
- TDCA

Significance:
- * p < 0.05

Sham vs. IT comparison:
- Significant differences (*) indicate statistical significance between the two groups.
Figure 3
Figure 4
**A. Intestine**

- **Fxr**: 
- **Ostα**: 
- **Fgf15**: 

**Relative RNA expression**

**B. Liver**

- **Gr**: 
- **G6Pc**: 
- **Pepck**: 
- **Pgc1α**: 

**Relative RNA expression**

**C. Liver**

- **Ntcp**: 
- **Cyp7β1**: 
- **Cyp27α1**: 
- **Cyp8β1**: 
- **Cyp7α1**: 
- **Fxr**: 
- **OstTα**: 
- **Bsep**: 
- **Shp**: 

**Relative RNA expression**

*Figure 5*
A. Intestine

- **Lxrα**
- **Fatp4**
- **Npc1l1**
- **Cd36**
- **Abca1**
- **Abcg5**
- **Abcg8**

**Relative mRNA expression**

B. Liver

- **Lxrα**
- **Srebp1c**
- **Fas**
- **Cd36**
- **Abca1**
- **Abcg5**
- **Abcg8**

**Relative mRNA expression**

C. Hepatic cholesterol (μg/mg protein)

- **Sham**
- **IT**

D. Ileal cholesterol (μg/mg protein)

E. Hepatic triglycerides (μg/mg protein)

F. Ileal triglycerides (μg/mg protein)

Figure 6
Supplementary figure legends

**Supplementary FIG. 1.** Oral administration of oleanolic acid induces a reduction of glycemia (A), a rapidly increase of GLP-1 plasmatic levels (B) and an increase of insulin plasmatic levels (C) after 60 min from GP-BAR1 agonist administration.

**Supplementary FIG. 2.** Administration of chenodeoxycolic acid inhibits hepatic gluconeogenesis via FGF15. Panels A-B. Oral glucose tolerance test (A) and insulinemia (B) were performed at 7 months after surgical procedure in ileal interposed and sham operated rats treated for 7 days with CDCA, unfed overnight, and treated with CDCA for a further 3-hour period. Data are mean ± SE of 5 animals per group. * P<0.05 vs control group. Panel C. Ileal mRNA relative expression of FGF15. Panel D. Hepatic mRNA relative expression of PEPCK, G6Pase, PGC1α and GR. Values are normalized relatively to GAPDH mRNA and are expressed relative to those of control rats, which are arbitrarily set to 1. Data are mean ± SE of 5 animals per group. * P<0.05 vs control group.

**Supplementary FIG. 3.** FXR activation after ileal interposition induces ERK1/2 phosphorylation. (A) Schematic representation of FGF15 and insulin signaling pathways involved in the regulation of glycogen synthesis. (B) Western blotting analysis of phospho-ERK1/2 (Thr 202 -Tyr 204), total ERK1/2, phospho Akt (Ser 473), total Akt, phosphor-GSK3β (Ser 9) and total GSK3β on liver homogenates. From left to right: Lanes 1-3, liver samples from control rats; Lanes 4-6, liver samples from control rats administered CDCA; Lanes 7-9, liver samples from IT rats; Lanes 10-12 liver samples from IT rats administered CDCA. (C) Image J densitometry of western blotting experiments. The scheme presented in panel 3A is partially derived by Kir et al. (ref. 50).
Supplementary Figure 1
Supplementary Figure 3

A. FGF15 → FGFR4 → ERK → RSK → GSK3β → GS

Glycogen synthesis

B. Controls and treatment effects on phosphorylation levels:

- pERK 1/2
- ERK 1/2
- pAKT
- AKT
- pGSK3β
- GSK3β

CTRL, CTRL+ CDCA, IT, IT+ CDCA

C. Graphs for pERK/ERK, pAKT/AKT, and pGSK3β/GSK3β levels:

- CTRL vs. IT with or without CDCA
Supplementary Table I. List of forward and reverse primers used for RT-PCR.

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