Roux-en-Y gastric bypass surgery reduces bone mineral density and induces metabolic acidosis in rats

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Roux-en-Y gastric bypass surgery reduces bone mineral density and induces metabolic acidosis in rats. Am J Physiol Regul Integr Comp Physiol 305: R999–R1009, 2013. First published September 11, 2013; doi:10.1152/ajpregu.00038.2013.—Roux-en-Y gastric bypass (RYGB) surgery leads to bone loss in humans, which may be caused by vitamin D and calcium malabsorption and subsequent secondary hyperparathyroidism. However, because these conditions occur frequently in obese people, it is unclear whether they are the primary causes of bone loss after RYGB. To determine the contribution of calcium and vitamin D malabsorption to bone loss in a rat RYGB model, adult male Wistar rats were randomized for RYGB surgery, sham-operation–ad libitum fed, or sham-operation–body weight-matched. Bone mineral density, calcium and phosphorus balance, acid-base status, and markers of bone turnover were assessed at different time points for 14 wk after surgery. Bone mineral density decreased for several weeks after RYGB. Intestinal calcium absorption was reduced early after surgery, but plasma calcium and parathyroid hormone levels were normal. 25-hydroxyvitamin D levels decreased, while levels of active 1,25-dihydroxyvitamin D increased after surgery. RYGB rats displayed metabolic acidosis due to increased plasma lactate levels and increased urinary calcium loss throughout the study. These results suggest that initial calcium malabsorption may play a key role in bone loss early after RYGB rats, but other factors, including chronic metabolic acidosis, contribute to insufficient bone restoration after normalization of intestinal calcium absorption. Secondary hyperparathyroidism is not involved in postoperative bone loss. Uregulated vitamin D activation may compensate for any vitamin D malabsorption.

Roux-en-Y gastric bypass; vitamin D; metabolic acidosis; secondary hyperparathyroidism

BARIATRIC SURGERY IS CURRENTLY the only effective long-term treatment for morbid obesity and its comorbidities. Roux-en-Y gastric bypass (RYGB) is the most commonly performed procedure and can be considered the gold standard for weight loss surgery. Recently, however, there has been an increasing focus on potential negative side effects of weight loss surgery, in particular, its effects on changes in bone metabolism (46, 49). Several prospective studies have reported significant decreases in bone mineral density (BMD) of the lumbar spine, hip, and femoral neck in men and women during the first year after RYGB surgery (13, 19, 39). The scarce data on long-term effects of RYGB surgery on bone mass suggest that reduced BMD may persist beyond the first postsurgical year (26, 50).

The anatomical rearrangement of the gastrointestinal tract after RYGB surgery leads to malabsorption of several micronutrients, including vitamin D (1, 19), and vitamin D deficiency is a common finding in patients after RYGB surgery (6). This may reduce intestinal calcium absorption (41), cause secondary hyperparathyroidism (2° HPT), and ultimately cause bone loss (19). Therefore, it has been proposed that postsurgical vitamin D levels should be regularly monitored and supplemented when necessary (43). However, several studies have reported a decrease in BMD after RYGB surgery despite vitamin D supplementation, indicating that vitamin D deficiency may not be the only cause of RYGB-associated changes in bone metabolism (12, 13, 39, 44). In addition, obesity per se is generally associated with low vitamin D levels due to vitamin D sequestration in adipose tissue and insufficient sunlight exposure (16). Therefore, presurgical evaluation of vitamin D and parathyroid hormone (PTH) status in patients undergoing RYGB often reveal substantial vitamin D deficiency and 2° HPT (21, 44). This complicates the assessment of postsurgical RYGB-induced changes and the interpretation of their specific contribution to bone loss. Finally, most studies report only values for 25-hydroxyvitamin D [25(OH)D] and not for the biologically active form 1,25-dihydroxyvitamin D [1,25(OH)2D]. To investigate the functional consequences of vitamin D malabsorption after RYGB surgery, measurement of 1,25(OH)2D is indispensable. Interestingly, while presurgical levels of 1,25(OH)2D have been reported to be decreased in RYGB patients (21), some studies report unaltered (1, 2) or even increased (44) postsurgical 1,25(OH)2D levels. Overall, these conflicting results suggest that the role of vitamin D malabsorption as a single cause for postsurgical bone loss after RYGB seems unlikely and that more in-depth studies are required.

The aim of this longitudinal study was to characterize the time course of dynamic changes in bone metabolism after RYGB surgery in rats under standardized conditions without dietary calcium or vitamin D supplementation. We hypothesized that factors other than vitamin D deficiency may contribute to increased bone resorption and dysregulation of calcium homeostasis after RYGB.

MATERIALS AND METHODS

Animals and housing. Thirty-two adult male Wistar rats weighing 450–500 g were allocated to either RYGB surgery (n = 15), sham...
operation with no dietary manipulation \( n = 9 \), ad libitum fed sham rats (AL)), or sham operation with food restriction to match the postoperative body weight of RYGB rats \( n = 8 \), body weight-matched sham rats (BWm)). RYGB and AL rats were housed in groups of four and three animals per cage, respectively; BWm rats were housed individually to allow food restriction. Animals were maintained under an artificial 12:12-h light-dark cycle at room temperature \( (24^\circ C) \) and had ad libitum access to normal chow (Provimi Kliba, Kaiseraugst, Switzerland) and tap water unless otherwise stated. All experiments were approved by the Veterinary Office of the Canton Zurich, Switzerland.

Surgery. RYGB surgery was performed as previously described \( (10) \). Briefly, the small bowel was transected \( \pm 20 \) cm distal to the pylorus of the stomach, creating a proximal and distal end of small bowel. The proximal end constituted the bilipancreatic limb and was anastomosed to the ileum \( (24^\circ C) \) and had ad libitum access to normal chow (Provimi Kliba, Kaiseraugst, Switzerland) and tap water unless otherwise stated. All experiments were approved by the Veterinary Office of the Canton Zurich, Switzerland.

Sample collection. Rats were allowed to recover from anesthesia and CT scans for 1 day before being single-housed in metabolic cages (Tecniplast, Buguggiate, Italy) for urine and feces collection. Food consumption, water consumption, and body weight were recorded daily. After a 3-day adaptation period, urine and feces were collected for 24 h. Urine samples were collected under mineral oil to prevent evaporative losses. Rats were then anesthetized by inhalation of isoflurane/air, and venous blood was collected from the sublingual vein. Heparinized blood was immediately analyzed for pH, blood gases, and electrolytes on an ABL 800 Flex blood gas analyzer (Radiometer, Copenhagen, Denmark). Serum was stored at \( -20^\circ C \) until further analysis. Sixteen weeks after surgery, rats were killed by decapitation after overnight fasting. To ensure comparability of the intestinal gene expression between animals, samples of the duodenum, jejunum, and ileum were taken \( 10 \) cm distal to the pylorus, \( 20 \) cm proximal to the cecum, and \( 60 \) cm proximal to the cecum, respectively.

Kidney and intestinal tissue was immediately frozen with liquid nitrogen and stored at \( -80^\circ C \) for analysis by quantitative PCR and Western blotting.

Blood, urine, and feces analysis. Serum total calcium and phosphorus were measured using the chemistry analyzer Cobas Integra 800 (Roche Diagnostic System, Basel, Switzerland). Serum-intact parathyroid hormone and osteocalcin levels and urine total deoxyyridine crosslinks were measured by two-site enzyme-linked immunosorbent assays (iPTH: Immutopics, San Clemente, CA; MicroVue osteocalcin and MicroVue total PPD: Quidel, San Diego, CA), according to the manufacturer’s protocols. Serum 25(OH)D and 1,25(OH)D levels were measured by radioimmunoassays (Immunodiagnostic Systems, Baldon, UK). Ammonium in urine was measured by the method of Berthelot \( (7) \). For pH measurements of feces, 1 g of fresh feces was extracted with \( 5 \) ml of distilled water and centrifuged \( (10600 \text{ g}, 20 \text{ min}) \). pH of the feces extracts and urine pH were determined with a Metrohm 744 pH meter. Calcium and phosphorus in urine and feces were measured with the chemistry analyzer Cobas Integra 800. Duplicates of feces samples were dried at 105°C for 3 h to constant weight and then reduced to ashes in a muffle oven for 16 h at 550°C. The ash was dissolved in \( 10 \text{ ml} \) \( 8 \%) \text{HCl} \), and the supernatant was used for analysis after centrifugation \( (3000 \text{ g}, 10 \text{ min}) \). Urine was acidified to \( \text{pH} < 2.0 \) with HCl before analysis. Relative intestinal calcium and phosphorus absorption were calculated as relative absorption \( = [\text{intake (mg)} - \text{fecal loss (mg)}]/\text{intake (mg)} \).

RNA isolation and cDNA synthesis. Total RNA from kidney and intestine was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany). Snap-frozen tissue was homogenized in a pestle homogenizer with \( 1 \) ml of precooled RLT buffer (Qiagen) supplemented with \( \beta \)-mercaptoethanol at a final concentration of \( 1\% \). Subsequently, \( 200 \mu l \) of the homogenate were used for RNA preparation, which was carried out according to the manufacturer’s protocol. DNase digestion was performed using the RNase-free DNAase set (Qiagen). Total RNA extraction was analyzed for quality, purity, and concentration using the NanoDrop ND-1000 spectrophotometer. RNA samples were diluted to a final concentration of \( 100 \) ng/\( \mu l \), and cDNA was prepared using the TaqMan reverse transcriptase reagent kit (Applied Biosystems/Roche, Foster City, CA).

In brief, in a reaction volume of \( 40 \) \( \mu l \), \( 300 \) ng of RNA was used as a template and mixed with the following final concentrations of RT buffer (\( 1 \times \)), MgCl\(_2\) (\( 5.5 \text{ mM} \)), random hexamers (\( 2.5 \text{ mM} \)), dNTP mix (\( 500 \text{ \mu M} \) each), RNase inhibitor \( (0.4 \text{ U/\mu l}) \), multiscribe reverse transcriptase \( (1.25 \text{ U/\mu l}) \), and RNase-free water. Reverse transcription was performed with thermocycling conditions set at \( 25^\circ C \) for \( 10 \) min, \( 48^\circ C \) for \( 30 \) min, and \( 95^\circ C \) for \( 5 \) min on a thermocycler (Bioter, Göttingen, Germany).

Real-time RT-PCR analysis. Semiquantitative real-time qRT-PCR was performed on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Primers for all genes of interest were designed using Primer Express Software \( (v.2.0.; \text{Applied Biosystems}) \), and primers were tested by PCR with cDNA and always resulted in a single product of the expected size. Primers were chosen to result in amplicons no longer than 150 bp spanning intron-exon boundaries to exclude genomic DNA contamination (Table 1). Probes were labeled with the reporter dye FAM at the 5’ end and the quencher dye TAMRA at the 3’ end (Microsynth, Balgach, Switzerland). Real-time PCR reactions were performed using the TaqMan Universal PCR Master Mix (Applied Biosystems). Briefly, \( 3.5 \text{ \mu l} \) cDNA, \( 1 \text{ \mu l} \) of each

<table>
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<tr>
<th>Gene1</th>
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<th>Forward Primer (5’ - 3’) Reverse Primer (3’ - 5’)</th>
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1 TRPV5/6, transient receptor potential vanillic acid 5/6; Calb1, calbindin D28k; Cyp24a1, 1,25-dihydroxysteroid D 24-hydroxylase; Cyp27bl1, 25-hydroxysteroid D 1-alpha-hydroxylase; Slc9a3, solute carrier family 9 (sodium/hydrogen exchanger) member 3; Slc4a4, solute carrier family 4 (sodium/bicarbonate cotransporter) member 4; HPR1, hypoxanthine guanine phosphoribosyl transferase. 2 NCBI Nucleotide Database (https://www.ncbi.nlm.nih.gov/nucleotide).
BMI decreases after RYGB surgery. BMD was significantly reduced in RYG rats already 2 wk after surgery and decreased further between weeks 2 and 7; thereafter, the difference in BMD between RYG and AL rats remained unchanged until week 14. There was no difference in BMD between AL and BWm rats at any time point, which suggests that the decrease of BMD in RYG rats was not directly caused by body weight loss (Fig. 2A). The results obtained by CT were confirmed by bone histomorphometry, which showed reduced bone volume and trabecular thickness in vertebrae and reduced bone volume in tibiae of RYG, but not BWm rats (Fig. 2, B–F).

RYGB leads to transient calcium malabsorption and increased urinary calcium excretion. To determine the potential role of malabsorption in the observed bone changes, calcium and phosphorus absorption was calculated from 24-h intake and fecal losses. Two weeks after surgery, both the absorption relative to intake and the absolute absorption of calcium were lower in RYG rats compared with sham controls. However, there was no evidence of calcium malabsorption in RYG rats 7 and 14 wk after surgery (Fig. 3A). The absorption of phosphorus was not impaired at any time point in RYG rats (Fig. 3B). Fourteen weeks after surgery, there was a decrease in calcium and phosphorus absorption compared with the earlier time points in all rats. We speculate that this was because rats had reached the age when linear growth stopped (Fig. 1), since it is known that mineral absorption is markedly decreased in older rats (3).

We expected RYG rats to compensate for the initial calcium malabsorption by decreasing urinary calcium excretion. Paradoxically, urinary calcium and phosphorus concentrations, as well as the absolute 24-h excretions, were increased in RYG rats throughout the entire study (Fig. 3, C and D).

RYGB causes chronic metabolic acidosis. Because overall bone metabolism and renal calcium excretion are strongly affected by acid-base imbalances, we investigated systemic acid-base status after RYG surgery. Throughout the study,

RESULTS

RYGB surgery leads to body weight loss and maintenance of a lower body weight. RYG rats lost ~15% of their original body weight within the first 2 wk after surgery and maintained a significantly lower body weight compared with AL rats from postoperative day 4 (P < 0.05). From week 2 after surgery, body weight was significantly lower in BWm rats compared with AL rats (P < 0.001), but there was no difference between RYG and BWm rats (Fig. 1). We have previously shown that BWm rats require significantly less food than RYG rats to maintain a similar body weight and that the lower food efficiency in our RYG model is not caused by nutrient malabsorption, but it is associated with an increase in energy expenditure (11).

Western blot analysis. Snap-frozen kidneys were homogenized in ice-cold RIPA buffer containing 1 mM PMSF, 2 μg/ml aprotinin, 10 μg/ml leupeptin, and 5 mM EDTA. After measurement of the protein concentration using the method of Pierce (45), 50 μg of proteins were solubilized in loading buffer containing β-mercaptoethanol and separated on 10% polyacrylamide gels. For immunoblotting, proteins were transferred electrophoretically to nitrocellulose membranes. After blocking with 5% BSA in PBS/0.1% Tween-20 for 60 min, blots were incubated with the primary antibodies: mouse monoclonal anti-VDR D6 (51 kDa; Santa Cruz Biotechnology, Santa Cruz, CA; 1:100) (51) and mouse monoclonal horseradish peroxidase (HRP)-conjugated anti-GAPDH (40 kDa; Abcam, Cambridge, UK; 1:10,000) overnight at 4°C. Membranes were then incubated for 1 h at room temperature with secondary goat anti-mouse antibodies 1:5,000 linked to HRP (Santa Cruz Biotechnology). The protein signal was detected with the appropriate substrates from Bio-Rad using the LAS-3000 chemiluminescence detection system (FujiFilm, Tokyo, Japan). All images were analyzed using the software ImageJ (20) to calculate the VDR/GAPDH ratio.

Bone histology. Bone specimens were fixed in 40% ethanol at 4°C for 3 days and further dehydrated in an ascending series of ethanol (50%, 70%, 80%, 90% 96%, and 100% at 4°C, 2 days per concentration) before being defatted in xylene under vacuum for 4 days. Embedding of specimens in methylmethacrylate was performed in customized Teflon forms. Tibiae and L3 vertebrae bodies were embedded in a position that allowed mid-sagittal and coronal sectioning, respectively. After polymerization, the blocks were mounted on plastic frames and cut with a precision saw (Leica SP 1600, Leica Biosystems, Nussloch, Germany). Ground sections were mounted on acrylic glass slides (Perspex GS Acrylglas Opal 1013; Wachendorf AG, Basel, Switzerland), polished to 200-μm sections, and surface stained with toluidine blue. Sections were digitally recorded (Axiovision, Carl Zeiss AG, Oberkochen, Germany; magnification 10 × 4); bone volume fraction and trabecular thickness were quantified with the software ImageJ and the plug-in BoneJ (15, 17) in vertebral bodies and proximal tibiae. To exclude the primary spongosia from the analysis, cancellous bone within 0.5 mm from the growth plate and within 0.25 mm from the endocortical bone surface was excluded (18).

For the measurement of growth plate width, images of the total growth plate were obtained at a magnification of 10 × 10. Seven vertical (perpendicular to chondro-osseous junction) lines were drawn throughout the growth plate and measured; growth plate width was calculated as an average of these seven measurements (23).

Statistical analysis. Data were analyzed with one-way ANOVA and are expressed as means ± SE. Following a significant F ratio, Bonferroni-Hochberg post hoc tests were used to determine differences between groups. Significance was established at P < 0.05.

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venous blood pH was significantly decreased in RYGB compared with sham rats, and the anion gap and lactate levels were increased (Fig. 4A). There was a significant negative correlation between lactate levels and blood pH in RYGB rats ($R = -0.667$), but not in AL or BWm rats, suggesting that increased lactate levels at least partly accounted for the metabolic acidosis of RYGB rats.

Chronic metabolic acidosis (CMA) leads to an activation of compensatory mechanisms in the kidney and intestine, including increased renal ammonium excretion and upregulation of the intestinal ion transporters sodium-hydrogen exchanger 3 (NHE3) and sodium-bicarbonate cotransporter 1 (NBCe1) (31). Accordingly, urinary and fecal pH were decreased (Fig. 4B) in RYGB rats, while urinary ammonium excretion was increased (Fig. 4C). mRNA expression of NHE3 was increased in the jejunum and of NBCe1 in the jejunum and ileum of RYGB rats, respectively (Fig. 4D). Together, these results indicate an adaptive response to the CMA in RYGB rats; however, this response seemed to be insufficient to fully compensate for the increased lactate levels and systemic acidosis.

Changes in parameters of mineral homeostasis and bone metabolism after RYGB surgery. Total and ionized serum calcium levels were unchanged in RYGB rats except for a small decrease after 14 wk compared with AL rats. Serum phosphorus levels were increased compared with BWm, but not AL rats 2 wk after surgery. There was no difference 7 or 14 wk after surgery (Fig. 5A). Surprisingly, there was no significant difference between PTH levels of RYGB and sham-operated rats at any time point (Fig. 5B).

Serum osteocalcin levels and urinary deoxypyridinoline (DPD) excretion were measured as markers of bone formation and resorption, respectively. While osteocalcin was unchanged throughout the study, DPD excretion was increased compared with BWm rats at 2 and 7 wk after surgery, suggesting increased bone resorption at these time points, at least compared with weight-matched animals (Fig. 5C).

Renal activation of vitamin D is increased, but vitamin D-induced gene upregulation in the kidney is lower after RYGB. Consistent with human studies reporting 25(OH)D deficiency in patients after RYGB surgery, RYGB rats had significantly lower levels of 25(OH)D than both AL and
BWm control rats. However, active 1,25(OH)₂D was increased more than twofold (Fig. 6A). Consistent with the circulating blood levels, RYGB rats displayed both increased renal expression of the vitamin D-activating enzyme 1α-hydroxylase (CYP27B1) mRNA and decreased renal expression of the vitamin D-inactivating enzyme 24-hydroxylase (CYP24A1) mRNA (Fig. 6B), suggesting that both increased activation and decreased inactivation contributed to elevated circulating 1,25(OH)₂D levels.

The vitamin D receptor (VDR) is a nuclear transcription factor that is expressed in various tissues (51). Binding of 1,25(OH)₂D to the VDR leads to upregulation of genes involved in calcium homeostasis, including the VDR itself, to increase intestinal calcium absorption and decrease renal calcium excretion. In the kidney, the membrane calcium channel transient receptor potential vanilloid 5 (TRPV5) and the cytosolic calcium buffer calbindin D28k (CALB1) are upregulated by 1,25(OH)₂D (53); VDR binding in the intestine leads to upregulation of TRPV6 under normal conditions (28). Nevertheless, mRNA expression of TRPV5 and CALB1 in RYGB rats was unchanged (Fig. 6C), and VDR protein levels were lower in the kidney of RYGB rats (Fig. 6D). In contrast, 1,25(OH)₂D signaling in the intestine seemed to be intact because TRPV6 mRNA expression was increased in the duo-

Fig. 3. Relative and absolute 24-h intestinal absorption of calcium (A) and phosphorus (B), 24-h urinary concentration and absolute urinary loss of calcium (C) and phosphorus (D) in AL, BWm, and RYGB rats 2, 7, and 14 wk after surgery. Data are expressed as means ± SE (*P < 0.05; **P < 0.01; ***P < 0.001).

Fig. 4. Venous blood pH, anion gap, and lactate levels (A), pH of urine and feces extracts (B), and 24-h urinary ammonium (NH₄⁺) excretion (C) in AL, BWm, and RYGB rats 2, 7, and 14 wk after surgery. D: mRNA expression of the apical sodium-hydrogen exchanger NHE3 and the basolateral sodium-bicarbonate cotransporter NBCe1 in duodenum (biliopancreatic limb in RYGB), jejunum (alimentary limb in RYGB), and ileum (common channel in RYGB) of AL, BWm, and RYGB rats 16 wk after surgery. Data are expressed as means ± SE (*P < 0.05; **P < 0.01; ***P < 0.001).
and jejunum of RYGB rats, as expected. TRPV6 expression was also upregulated in the duodenum of BWm rats, which may potentially be considered an adaptive response to reduced dietary calcium intake (Fig. 6E).

Increased 1,25(OH)2D levels in RYGB rats potentially affect growth plate maturation. Vitamin D deficiency or lack of the VDR lead to characteristic changes in the epiphyseal growth plates, i.e., growth plate widening with disorganized structure and accumulation of immature, unmineralized bone mass (8). 1,25(OH)2D on the other hand reduces growth plate width in young rats (23). In our study, the histological evaluation of the epiphyseal growth plate in the proximal tibia revealed a trend toward narrowing of the growth plates in adult RYGB rats (P < 0.1; Fig. 7).

**DISCUSSION**

We performed the first comprehensive longitudinal investigation of changes in bone metabolism after RYGB surgery in a rat model. Postoperative bone loss has repeatedly been described in RYGB patients and is often associated with low 25(OH)D levels and 2° HPT (6, 14, 25). To our knowledge, however, no causal relationship among vitamin D status, PTH levels, and bone loss after RYGB surgery has been demon-

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Fig. 5. Serum levels of total calcium, ionized calcium, and phosphorus (A) and of parathyroid hormone (PTH) (B). C: serum levels of osteocalcin and urinary deoxypyridinoline (DPD) excretion in AL, BWm, and RYGB rats 2, 7, and 14 wk after surgery. Data represent mean values ± SE (*P < 0.05; **P < 0.01; ***P < 0.001).

Fig. 6. Altered vitamin D metabolism and signaling after RYGB surgery. A: serum levels of 25-hydroxyvitamin D [25(OH)D] and 1,25-dihydroxyvitamin D [1,25(OH)2D] in AL, BWm, and RYGB rats 2, 7, and 14 wk after surgery. B–E: renal mRNA expression of CYP24A1 and CYP27B1, the genes encoding the 24-hydroxylase and the 1-α-hydroxylase, respectively (B), renal transient receptor potential vanilloid 5 (TRPV5), and calbindin D28k (CALB1) mRNA expression (C) and renal vitamin D receptor (VDR) protein expression (D) in AL, BWm, and RYGB rats 16 wk after surgery. E: TRPV6 mRNA expression in duodenum, jejunum, and ileum of AL, BWm, and RYGB rats 16 wk after surgery. Data represent mean values ± SE (*P < 0.05; **P < 0.01; ***P < 0.001).
strated. Since vitamin D deficiency and 2\textsuperscript{o} HPT often occur in obese patients even before any weight loss intervention, it is difficult to dissect the specific contribution of RYGB surgery to these conditions. Using a well-established RYGB rat model with standardized light exposure and access to standard laboratory chow without calcium or vitamin D supplementation, we showed that BMD decreased progressively for several weeks after surgery. Bone loss was initially associated with calcium malabsorption; however, no restoration of bone mass was detectable in RYGB rats even after normalization of intestinal calcium absorption. No signs of 2\textsuperscript{o} HPT were detected, but elevated active vitamin D levels and metabolic acidosis caused by increased lactate levels occurred throughout, which may have adversely affected bone metabolism. These data suggest that initially, reduced calcium absorption after RYGB may lead to calcium mobilization from bone; however, this seems to be independent of vitamin D malabsorption. In contrast, RYGB-induced metabolic acidosis may interfere with the hormonal control of calcium homeostasis and bone mass. Importantly, these changes were independent of body weight loss, since they did not occur in the food-restricted, body weight-matched control group.

Our data show that after an initial decrease in BMD, the difference between AL and RYGB rats remained unchanged between postoperative weeks 7 and 14. Together with the normalization of intestinal calcium absorption at these time points, this suggests that impaired intestinal calcium absorption contributed to bone loss, at least initially. Under physiological conditions, most of dietary calcium is absorbed in the duodenum (42) by active transcellular transport, which requires the conditions, most of dietary calcium is absorbed in the duodenum and jejunum of RYGB rats indicates a compensatory response to the sudden decrease in calcium availability. This response, however, is futile since the proximal small intestine is excluded from nutrient flow; nonetheless, the data show that the duodenum of RYGB rats remains responsive to elevated 1,25(OH)\textsubscript{2}D levels, even in the absence of direct nutrient contact. The compensation by increased passive absorption in the distal small intestine seems to be insufficient shortly after surgery, potentially due to a lack of adaptation time of the gut to the altered anatomy. Duodenal TRPV6 expression was also increased in BWm rats; however, their 1,25(OH)\textsubscript{2}D levels were comparable to AL rats. It has been described in animals on a calcium-deficient diet that reduced dietary calcium intake leads to increased 1,25(OH)\textsubscript{2}D-mediated TRPV6 expression (48, 52); it is, therefore, surprising that the food-restricted BWm rats did not display increased 1,25(OH)\textsubscript{2}D levels. It must be noted, however, that in contrast to animals fed a calcium-deficient diet ad libitum, our animals were fed a restricted amount of a diet with normal calcium content. This might physiologically represent a completely different situation since, unlike a calcium-deficient diet, our approach presumably does not influence the luminal calcium concentration in the intestine. To our knowledge, the effects of chronic food restriction on 1,25(OH)\textsubscript{2}D levels and on intestinal calcium transporter gene expression have never been investigated. Therefore, we speculate that in this situation, either a vitamin D-independent mechanism or a change in vitamin D receptor sensitivity led to duodenal TRPV6 upregulation.

The finding of increased PTH levels in many patients after RYGB surgery has led to the assumption that PTH-induced bone resorption is the main cause of reduced BMD. However, the high prevalence of 2\textsuperscript{o} HPT in obese people already before RYGB surgery complicates the assessment of a potential causal link between PTH and postsurgical bone loss. Interestingly, we did not find a significant increase in PTH levels in RYGB rats, which suggests that PTH-independent mechanisms causing bone loss seem to prevail in the first weeks after surgery.

Consistent with previous studies reporting 25(OH)D deficiency in patients after RYGB surgery, our RYGB rats had decreased 25(OH)D levels. Importantly, however, we found that levels of the hormonally active metabolite 1,25(OH)\textsubscript{2}D were markedly increased. Even though 1,25(OH)\textsubscript{2}D increases...
active calcium absorption under physiological conditions, we cannot exclude that in combination with intestinal calcium malabsorption, 1,25(OH)2D may, in fact, have negative effects on bone mass. A recent study by Lieben et al. (30) showed that specific disruption of intestinal vitamin D signaling in intestine-specific VDR knockout mice increased 1,25(OH)2D levels, which contributed to maintaining normocalcemia by mobilization of skeletal calcium and inhibition of bone mineralization. Because the main physiological effect of intestinal vitamin D signaling is to increase active calcium absorption in the proximal intestine, intestine-specific VDR knockout mice may, to some extent, be comparable to the situation after RYGB surgery, where duodenal vitamin D signaling, although intact, remains ineffective, due to the altered gut anatomy. We, therefore, speculate that increased 1,25(OH)2D levels contribute to bone loss in the initial phase of intestinal calcium malabsorption and to the insufficient restoration of bone mass after normalization of calcium absorption (22).

The exact mechanism behind increased 1,25(OH)2D levels in our RYGB rats is not clear. The fact that 1,25(OH)2D levels remained elevated after normalization of calcium absorption suggest that the increased vitamin D activation may not only be related to serum calcium levels. Our data show that RYGB surgery led to severe systemic CMA that was at least partly due to increased plasma lactate levels. We did not determine the source of increased lactate levels; however, we speculate that alterations in the bacterial flora of the intestine (54) led to increased lactate production after carbohydrate digestion, a phenomenon frequently observed in patients with short-bowel syndrome or jejuno-ileal bypass surgery (40). CMA leads to a PTH-independent increase in 1,25(OH)2D production (27) and could, therefore, play a role in vitamin D metabolism after RYGB surgery. In addition, it has previously been speculated that enterocytes may have the capacity to sense changes in dietary calcium intake and to increase renal 1α-hydroxylase activity via an unknown signal in response to decreased intestinal calcium concentrations (32). The sudden absence of luminal calcium in the proximal small intestine after the RYGB procedure could, thereby, directly induce an increase in 1,25(OH)2D levels.

An unexpected finding of our study was that renal calcium excretion was increased in RYGB rats; this was already seen in the initial phase of the study with intestinal calcium malabsorption and massive bone loss, but also later in the study. A decrease in intestinal calcium availability normally leads to 1,25(OH)2D-mediated upregulation of renal CALB1 and TRPV5 expression (53) to minimize urinary calcium loss. Despite higher 1,25(OH)2D levels, urinary calcium concentration and absolute calcium excretion were increased in RYGB rats throughout the study, and there was no change in renal CALB1 and TRPV5 expression when examined at study end. This suggests that additional factors may have counteracted the control of renal calcium metabolism by 1,25(OH)2D. CMA has been shown to enhance calcitriol synthesis by decreasing the expression of CALB1 and TRPV5 in the kidney (34); therefore, the CMA detected in RYGB rats is one possible explanation for increased urinary calcium loss. CMA has additional direct effects on bone metabolism, including a stimulatory effect of H+ on osteoclast activation (4) and an inhibition of bone formation and mineralization (9). CMA may, therefore, play an important facilitator role in bone resorption after RYGB surgery and in the prevention of bone mass restoration after normalization of calcium absorption.

Since the original submission of our paper, Stemmer et al. (47) published data on the effect of RYGB surgery on bone mass in rats. Our data are largely consistent with their findings. Similar to our results, Stemmer et al. (47) found that bone mass was reduced by RYGB 8 wk after surgery, which was associated with decreased 25(OH)D levels. A calcium and vitamin D-enriched diet attenuated the effects of RYGB surgery on bone mass; however, it did not fully prevent bone loss, which further suggests that additional factors to calcium and vitamin D malabsorption may be involved in RYGB-induced bone loss. In addition to their findings, we now report that lower 25(OH)D may not be a pathophysiologically relevant factor because increased conversion to the active metabolite may compensate for lower absorption. Hence, our data nicely complement the findings by Stemmer et al. (47) and suggest further mechanisms that may contribute to the significant bone loss that occurs after RYGB in rats.

Relevance to human disease. The current study provides valuable new insights into the effects of RYGB surgery on bone metabolism that may be of high clinical interest (Fig. 8). The increased levels of active 1,25(OH)2D in our RYGB rats indicate that vitamin D deficiency may not be the sole cause of RYGB-induced bone loss (44) because insufficient 25(OH)D absorption may be compensated by a higher proportion of transformation to the active 1,25(OH)2D form of vitamin D. This finding suggests that the need of vitamin D supplementation after surgery should be evaluated on the basis of measured 25(OH)D and 1,25(OH)2D levels. Further, we believe that calcium supplementation may have beneficial effects on bone mass, especially in the first months after the procedure, because calcium malabsorption was transient and because there was no further bone loss in our RYGB rats after normalization of calcium absorption. It has to be mentioned in this context that the jejunojejunosotomy in our rat model is performed more distally than in most human RYGB procedures. This could potentially contribute to the reported calcium malabsorption; however, it has previously been shown that the true fractional calcium absorption also decreases after RYGB surgery in humans (41), suggesting that this observation is not specific to our model but of general relevance. Finally, metabolic acidosis should be monitored and treated with alkaline supplementations, if necessary. Alkaline treatment has been shown to increase bone mass, even in nonacidotic postmenopausal women (24).

Even though the clinical relevance of bone loss after RYGB surgery in humans is still unclear, there are at least two patient subpopulations that are of particular interest for potential future studies. First, a majority of patients undergoing RYGB surgery are women, and many of them are perimenopausal or postmenopausal. We have recently established an RYGB model in female rats and have shown that the reproductive axis function may influence the outcome of RYGB surgery in women (5). Given the strong connection between menopause and osteoporosis, it will be of high interest to determine whether the postmenopausal state directly influences bone loss after RYGB surgery and whether there are potential interactions between the mechanisms of postsurgical and postmenopausal bone loss in women.
Fig. 8. Proposed mechanisms for bone loss and changes in calcium and vitamin D metabolism early after RYGB surgery (A) and after compensation of initial calcium malabsorption (B). A: active intestinal calcium absorption decreases early after Roux-en-Y gastric bypass surgery due to the exclusion of the duodenum from nutrient flow. Subsequently, increased conversion of 25-hydroxyvitamin D to active 1,25-dihydroxyvitamin D [1,25(OH)₂D] may be further supported by metabolic acidosis and direct signaling from calcium-sensing enterocytes in the duodenum. Compensatory upregulation of the intestinal calcium channel TRPV6 by 1,25(OH)₂D is futile since TRPV6 is mainly expressed in the duodenum. Calcium mobilization from bones ensures stable blood calcium levels, which may be partly mediated by elevated 1,25(OH)₂D. In addition, metabolic acidosis potentially contributes to bone resorption and leads to decreased renal calcium reabsorption. B: after adaptation to the altered anatomy of the gastrointestinal tract, direct delivery of nutrients to the distal small intestine facilitates passive calcium absorption, which compensates for the decreased active absorption in the duodenum. The normalization of intestinal calcium malabsorption does not lead to a restoration of bone mass due to inhibitory effects of metabolic acidosis and potentially 1,25(OH)₂D on bone formation. Vitamin D activation and renal calcium loss remain increased. Thick arrows represent the proposed predominant mechanisms.

Second, bariatric surgery has become an important treatment option for morbid obesity in adolescents in recent years (36). Besides the loss of bone mass that has been described in adult patients, there may be additional negative effects of RYGB surgery on skeleton development during growth. 1,25(OH)₂D levels detected in RYGB rats and also previously yseal growth plate in rats (23). The chronically elevated treatment causes altered maturation and narrowing of the epiph- sial bone loss in humans and the question of whether there are relevant functional consequences may still be disputed, although recent evidence suggests a connection between RYGB surgery and fracture incidence (33). However, as already mentioned, the evaluation of changes in bone and calcium metabolism after RYGB surgery in humans is very challenging because of preexisting conditions, such as 25(OH)D deficiency and 2° HPT. In addition, most patients are supplemented with vitamin D and calcium after the surgery, but there is often no information about vitamin D and calcium levels prior to supplementation, there is no standardized replacement protocol, and information about compliance is difficult to obtain.

Another potential limitation of our study is that there are currently no data showing that RYGB surgery leads to metabolic acidosis in humans, even though cases of D-lactic acidosis have been described (7a). However, the most common calcium supplements, calcium citrate and calcium carbonate, also confer alkali load that could unintentionally prevent the development of an acidic state; long-term assessment of acid-base imbalances in RYGB patients is, therefore, similarly challenging to the investigation of bone metabolism.

Lastly, we want to point out that the major advantages of using animal models such as ours are the close controllability of environmental factors, the standardization among groups, and the inclusion of different control groups, in particular, a group matched for body weight loss. We can, therefore, use the new evidence provided by experiments in animal models to improve and refine the design of studies involving human RYGB patients.

Perspectives and Significance

Our data point to a complex disruption of bone homeostasis after RYGB that is not a simple consequence of alterations in vitamin D and calcium levels. By the use of a body weight-matched control group, we demonstrated that chronic food restriction in sham-operated rats did not have comparable effects on bone metabolism. We showed that increased activation of vitamin D may compensate for intestinal vitamin D malabsorption in RYGB rats. Together with the absence of 2° HPT, this questions the role of vitamin D deficiency and 2° HPT in RYGB-induced bone loss. Interestingly, RYGB surgery led to increased lactate levels and chronic metabolic acidosis, which may have contributed to both the initial bone loss and the insufficient subsequent restoration of bone mass. Even though cases of D-lactic acidosis have been described in humans after RYGB surgery, there are no studies on postsurgical changes in acid-base status. Our data suggest that future studies should include consideration of metabolic acidosis.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


