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

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1Institute of Veterinary Physiology, Vetsuisse Faculty University of Zurich, Zurich, Switzerland; 2Institute of Physiology, University of Zurich, Zurich, Switzerland; 3Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland; 4Institute of Animal Nutrition, Vetsuisse Faculty University of Zurich, Zurich, Switzerland; 5Department of Surgery, Division of Visceral and Transplantation Surgery, University Hospital Zurich, Zurich, Switzerland; and 6Institute of Laboratory Animal Science, Vetsuisse Faculty University of Zurich, Zurich, Switzerland

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Abegg K, Gehring N, Wagner CA, Liesegang A, Schiesser M, Bueter M, Lutz TA. 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 in 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 (PHT) 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 shams (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°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 ~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 40 cm from the cecum, creating the common channel. The stomach was transected ~5 mm below the gastroesophageal junction, creating a gastric pouch of a size of no more than 2–3% of original stomach size. The Roux-en-Y reconstruction was completed by connecting the distal end of the small bowel to the gastric pouch leading to the formation of the alimentary limb. For sham operations, an anterior gastrotomy and a jejunotomy with subsequent closures were performed.

**Microcomputed tomography scans.** Two, seven, and fourteen weeks after surgery, BMD of lumbar vertebrae 1 to 6 was measured by quantitative microcomputed tomography (CT) with a LaTheta LCT-100A scanner (Hitachi-Aloka Medical, Tokyo, Japan). Rats were anesthetized with 2–3% isoflurane in oxygen (0.5 liter per min) during the measurement. Continuous 2-mm slice images with a pixel size of 250 × 250 μm were utilized for calculation of BMD using LaTheta software (version 2.10).

**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°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°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 deoxypyridinoline 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 radioimmunnoassays (Immuno-diagnostic 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 g, 20 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 ml 8% HCl, and the supernatant was used for analysis after centrifugation (3000 g, 10 min). Urine was acidified to pH <2.0 with HCl before analysis. Relative intestinal calcium and phosphorus absorption were calculated as relative absorption = [intake (mg) – fecal loss (mg)]/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 β-mercaptoethanol at a final concentration of 1%. Subsequently, 200 μl 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 DNase set (Qiagen). Total RNA extractions were analyzed for quality, purity, and concentration using the NanoDrop ND-1000 spectrophotometer. RNA samples were diluted to a final concentration of 100 ng/μl, and cDNA was prepared using the iScript cDNA synthesis kit (BioRad, Hercules, CA).

In brief, in a reaction volume of 40 μl, 300 ng of RNA was used as a template and mixed with the following reagents: 1× iScript cDNA synthesis reaction buffer, 5 mM MgCl2, 5 mM random hexamers (2.5 μM), dNTP mix (500 μM each), RNase inhibitor (0.4 U/μl), multiscribe reverse transcriptase (1.25 U/μl), and iScript reverse transcriptase (1.25 U/μl). Reverse transcription was performed with thermocycling conditions set at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min on a thermocycler (Biometra, Göttingen, Germany).

**Real-time RT-PCR analysis.** Semi-quantitative 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.; 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 amplifications 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 μl cDNA, 1 μl of each forward and reverse primer, and 10 μl of the Master Mix were mixed in a total reaction volume of 20 μl.

**Table 1. Primers used for semi-quantitative real-time PCR**

<table>
<thead>
<tr>
<th>Gene(^1)</th>
<th>Accession No.(^2)</th>
<th>Forward Primer (5’ - 3’) Reverse Primer (3’ - 5’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TRPV5</strong></td>
<td>NM_053787</td>
<td>AGA GCA GCC AAC GAA ATT GA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TAG CAG CAT GCA GGT GTA CA</td>
</tr>
<tr>
<td><strong>TRPV6</strong></td>
<td>NM_053686</td>
<td>GCC GCA GAA GAA GAT CCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGG GCA GCT ATG TGA AGT GC</td>
</tr>
<tr>
<td><strong>Calb1</strong></td>
<td>NM_031984</td>
<td>CCA CTT GCA GTC ATC TCA GCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCT GCT TGA AGT GTC TCA</td>
</tr>
<tr>
<td><strong>Cyp24a1</strong></td>
<td>NM_201635</td>
<td>TTG AAA GCA TCT GCC TTG TG</td>
</tr>
<tr>
<td></td>
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<td>GGG GTG ACC ATC TTC CCG</td>
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<tr>
<td><strong>Cyp27b1</strong></td>
<td>NM_012654</td>
<td>GAC GCA GCA GCG TGG GCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GTG ATC TGG TGC CCA GTG T</td>
</tr>
<tr>
<td><strong>Slc4a4</strong> (<strong>NBE1</strong>)</td>
<td>NM_053424</td>
<td>GTG GAT GCT ACT CCG CAG CTA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATT CAA AGG GGG CCA</td>
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<tr>
<td><strong>HPRT</strong></td>
<td>NM_012583</td>
<td>AAA CAG GGG CCG CCA</td>
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</table>

\(^1\)TRPV5/6, transient receptor potential vanilloid 5/6; Calb1, calbindin D28k; Cyp24a1, 1,25-dihydroxyvitamin D 24-hydroxylase; Cyp27b1, 25-hydroxyvitamin D 1-alpha-hydroxylase; Slc4a4, solute carrier family 4 (sodium bicarbonate cotransporter) member 4; HPRT, hypoxanthine guanine phosphoribosyltransferase. \(^2\)NCBI Entrez Nucleotide Database (https://www.ncbi.nlm.nih.gov/nuccore).
primer (25 μM), 0.5 μl labeled probe (5 μM), 6.5 μl RNase free water, and 12.5 μl TaqMan Universal PCR Master Mix reached 25 μl of final reaction volume. Reaction conditions were the following: denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing/elongation at 60°C for 60 s with auto ramp time. All reactions were run in duplicate. For analyzing the data, the threshold was set to 0.06, as this value had been determined to be in the linear range of the amplification curves for all mRNAs in all experimental runs. The expression of the gene of interest was calculated in relation to hypoxanthine guanine phosphoribosyl transferase (HPRT). Relative expression ratios were calculated as \( R = 2^{\Delta\Delta Ct} \), where Ct represents the cycle number at the threshold 0.06 (35).

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%, 95%, and 100% at 4°C, 2 days per concentration) before being defatted in xylene under vacuum for 4 days. Embedding of specimens in methylmetacrylate was performed in customized Teflon molds. Tibiae and L3 vertebral 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 Acrylglass 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 spongiosa 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 \( P < 0.05 \).

RESULTS

RYGB surgery leads to body weight loss and maintenance of a lower body weight. RYGB 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 RYGB and BWm rats (Fig. 1). We have previously shown that BWm rats require significantly less food than RYGB rats to maintain a similar body weight and that the lower food efficiency in our RYGB model is not caused by nutrient malabsorption, but it is associated with an increase in energy expenditure (11).

BMD decreases after RYGB surgery. BMD was significantly reduced in RYGB rats already 2 wk after surgery and decreased further between weeks 2 and 7; thereafter, the difference in BMD between RYGB 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 RYGB 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 RYGB, 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 RYGB rats compared with sham controls. However, there was no evidence of calcium malabsorption in RYGB rats 7 and 14 wk after surgery (Fig. 3A). The absorption of phosphorus was not impaired at any time point in RYGB 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 RYGB 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 RYGB 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 RYGB surgery. Throughout the study,
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-
denum 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-
increased 1,25(OH)2D levels. In the distal small intestine, expressed in the duodenum and is upregulated in response to conditions, most of dietary calcium is absorbed in the duodenum and jejunum of RYGB rats remains responsive to elevated 1,25(OH)2D 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)2D 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)2D-mediated TRPV6 expression (48, 52); it is, therefore, surprising that the food-restricted BWm rats did not display increased 1,25(OH)2D 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)2D 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 25(OH)D deficiency in patients after 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)2D were markedly increased. Even though 1,25(OH)2D 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 calciresis 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 jejunoojejunostomy 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 in women (24).

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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 absorption, which compensates for the decreased active absorption in the duodenum. The normalization of intestinal calcium malabsorption does not 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-
sis have been described (7a). However, the most common
bolic acidosis in humans, even though cases of D-lactic acido-
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'
'surgery on skeleton development during growth. 1,25(OH)2D
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
treatment causes altered maturation and narrowing of the epiph-
ysosomal changes in acid-base status. Our data suggest that future
development of an acidotic 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 envi-
ronmental 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 activa-
tion 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 sur-
gery 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 postsur-
gical 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


