Published in final edited form as:

Ann Surg. 2008 May ; 247(5): 779–790. doi:10.1097/SLA.0b013e318166fd5f.

Sustained Weight Loss After Roux-en-Y Gastric Bypass Is Characterized by Down Regulation of Endocannabinoids and Mitochondrial Function

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Abstract

Objective—To determine the physiologic importance of endocannabinoids and mitochondrial function in the long-term outcome using a rat model of Roux-en-Y gastric bypass (RYGB) surgery.

Background—Sixteen million people are morbidly obese and RYGB surgery is the most effective treatment. Endocannabinoids are implicated in appetite stimulation and regulation of peripheral energy metabolism. We hypothesize that down-regulation of endocannabinoids and alterations in mitochondrial function and hormones favoring catabolism contribute to sustained RYGB-induced weight loss.

Methods—Diet-induced obese Sprague-Dawley rats were randomized to sham-operated obese controls, RYGB, and sham-operated obese pair-fed rats. Body weight and food intake were recorded, and food efficiency was calculated. Endocannabinoid levels in skeletal muscle and liver, muscle mitochondrial respiratory complex I–V content, and hormones concentrations were determined 14 and 28 days postsurgery, reflecting rapid and sustained weight loss periods after RYGB, respectively.

Results—Compared with pair-fed controls, RYGB rats had significant reduction in body weight and food efficiency ($P < 0.001$). Increased cholecystokinin, reduced insulin, leptin, adiponectin, T3, and down-regulation of mitochondrial complex I were evident on day 14 postsurgery. On day 28, leptin, insulin, and T3 remained low, whereas adiponectin and cholecystokinin were normal. Along with complex I, the endocannabinoids anandamide in muscle ($P = 0.003$) and 2-arachidonoylglycerol in liver were significantly down-regulated ($P < 0.001$).

Conclusions—The attenuated caloric intake, reduced food efficiency, and normalization of hormonal levels on day 28 post-RYGB were associated with significant down-regulation of endocannabinoids anandamide and 2-arachidonoylglycerol in muscle and liver, respectively. These results suggest a role for endocannabinoids in the mechanism of sustained weight loss and RYGB success, and may have implications for treatment of morbid obesity.

In the United States 16 million persons are morbidly obese (body mass index (BMI) [weight (kg)/height (m²)] ≥ 40 kg/m²).¹ Roux-en-Y gastric bypass (RYGB) is currently the most effective therapy for morbid obesity because it achieves a substantial and sustained reduction in weight accompanied by reversal of associated metabolic comorbidities.^{2,3} In humans, RYGB slows nutrient digestion and absorption, and increases appetite-suppressing neuropeptides^{4,5} and induces catabolism.^{5,6} This leads to a biphasic weight loss pattern of an initially accelerated weight loss period lasting about 1 to 2 years followed by a sustained weight loss period during which weight loss is maintained.³ Other treatments for morbid obesity, including exercise, diet, and drug therapy, yield no more than 5% to 10% reduction in body weight⁷, and this is complicated by the fact that the weight loss is not maintained due to the temporal compensatory changes in energy intake and expenditure.⁸

A complex neuroendocrine network of central and peripheral mechanisms regulates food intake and adiposity⁹, and recent studies have established the endocannabinoid system as a major component of this metabolic regulatory network.^{10,11} Endocannabinoids stimulate caloric intake by interacting with the leptin-mediated neuroendocrine circuitry of appetite.^{12,13} Activation of the cannabinoid CB1 receptor in the brain either by the endocannabinoid anandamide (AEA) or synthetic analogues stimulate appetite and induce positive peripheral energy metabolism.^{10–12,14–19} Mice lacking CB1 receptors are resistant to diet-induced obesity,^{20,21} and treatment of obese human subjects with the CB1 antagonist Rimonabant (SR141716; which is undergoing human trial) resulted in sustained clinically beneficial weight loss, decreased waist circumference, and improved cardiometabolic risk factors of obesity.²² CB1 receptors are expressed in appetite centers in the brain, on cholecystokinin-1 receptor positive vagal afferent nerves,²³ and in a wide variety of peripheral tissues including adipose tissues, liver, pancreas, gastrointestinal tract, and thyroid.^{14,16,20,24–27} In the gastrointestinal tract, CB1 receptors mediate cannabinoid-induced delayed gastric emptying, suppression of small intestinal contraction and inhibition of intestinal motility.²⁸ In adipocytes, activation of CB1 receptor enhanced lipoprotein lipase activity and therefore lipogenesis.¹⁴ Furthermore, endocannabinoids have recently been found to modulate lipogenesis in the liver, where they also contribute to the development of fatty liver and obesity.²⁰ In the muscle, endocannabinoids modulate the expression of key genes involved in the regulation of fat and glucose oxidation.²⁹ However, to date there are no studies on the functional effects of the endocannabinoid system in weight loss surgery and how it impacts the maintenance of weight loss after RYGB.

Mitochondrial dysfunction is associated with the development of obesity and type 2 diabetes mellitus.³⁰ Both hepatic and skeletal muscle mitochondria function are also important for the global maintenance of energy homeostasis: reduced skeletal muscle mitochondrial mass and function are associated with the increased rate of fat recovery (catch-up fat) and insulin resistance; characteristic features of weight recovery after caloric restriction.³¹ Caloric restriction alters subcellular membrane lipid composition in the liver, cardiac, and skeletal muscles, and may therefore influence mitochondrial functionality and metabolic rate.^{32,33}

We have previously reported in our RYGB model in diet-induced obese rats differential central and peripheral expression of neuropeptides including NPY, alpha-melanocyte stimulating hormone, serotonin-1B-receptor, agouti-related protein, cocaine- and amphetamine-regulated transcript, corticotropin releasing factor, and peptide YY (PYY).^{34–38} These peptides have been implicated in the regulation of energy balance.^{9,39} Obese subjects display delayed satiety due to diminished activation of anorexigenic neuropeptides after meals. Hyperactive endocannabinoid signaling is associated with obesity, and treatment with the CB1 antagonist Rimonabant up-regulates central cocaine- and amphetamine-regulated transcript and leptin signaling.^{12,40} There are, however, patients who do not respond well to weight loss surgery and tend to regain weight after RYGB. In our model, 10 to 13 rat days is equivalent to 1 human

year,⁴¹ thus allowing us to analyze levels of various hormones and other regulators of metabolism and energy homeostasis at 14 and 28 days after surgery, which is equivalent to about 1 and 3 human years after RYGB. These time points are representative of the rapid and sustained weight loss periods.

Using this model, we tested our hypothesis that down-regulation of the peripheral endocannabinoid system along with alterations in mitochondrial function and hormones favoring catabolic processes contribute to sustained weight loss after RYGB surgery by counterbalancing the compensatory changes in energy intake and expenditure that occur with time. Thus, results from our study will provide surgeons with insight into the mechanism of successful sustained weight loss after RYGB in morbidly obese patients and could potentially lead to CB1 receptor antagonist becoming a postoperative adjunct drug for RYGB patients who fail to maintain reduced weight loss and to prevent weight regain.

MATERIALS AND METHODS

Rats, Study Conditions, Diet-induced Obesity

All animal studies were performed according to the guidelines and approval of the Committee for the Humane Use of Animals at SUNY Upstate Medical University, and in accordance with guidelines established by the National Institutes of Health.

Sprague-Dawley male rats (Charles River, Wilmington, MA), weighing 70.8 ± 0.7 g, were housed in wire mesh cages and acclimated in constant study environmental conditions: 12L:12D cycle (lights on at 6:00 am), $26^{\circ}\text{C} \pm 1^{\circ}\text{C}$ room temperature, and 45% humidity. Rats had free access to diet and municipal tap water.^{34,36,37,42,43} To induce obesity, rats were fed a high-energy diet (D12266, Research Diets, New Brunswick, NJ) for 16 weeks.³⁴ The high-energy diet consisted of 4.5 kcal/g (of which, 21% of the metabolizable energy content was protein, 31% fat, and 48% carbohydrate, consisting 50% sucrose).

Anesthesia, Analgesia, Surgical Procedures, and Postoperative Management

Preoperatively rats were anesthetized with a ketamine and xylazine mixture (100 mg:2 mg; 0.8 mL/kg, i.p.). Before operation rats were food-deprived for 12 hours. The RYGB procedure and the sham operation were described previously in detail and simulates that performed in morbidly obese humans.^{34,36,37,43} Postoperative care, including hydration and medication, was provided as previously described in detail.^{34,36,37,43} Bupropfen (Reckitt Benkiser Pharmaceuticals, Richmond, VA) 0.025 mg/kg was given for pain control postoperatively for 3 to 5 days. Rats were started on a liquid diet (Boost-Plus, Novartis, Fremont, MI), which was available for 7 to 10 days. Boost-Plus provides 1.5 kcal/mL. Its metabolizable energy is from protein: 15%, fat: 35%, and carbohydrate: 50%. On day 7 rats were offered a standard chow ad libitum (Diet #5008; Ralston Purina, St. Louis, MO), which was continued for the duration of this experiment.

Experimental Design

Diet-induced obese rats were stratified by weight to ensure similar mean weight for each of the 3 groups ($n = 8/\text{group}$) before the surgical procedures: sham-operated intact gastrointestinal tract obese rats (Obese), which continued to eat ad libitum; RYGB rats and sham-operated intact gastrointestinal tract obese rats that were pair-fed (PF) the same amount of food consumed the previous day by the RYGB rats. Cohorts of rats were killed at days 14 and 28 after operation.

Body Weight, Food Intake, and Food Efficiency

Postoperatively, daily weight and food intake were measured gravimetrically for 14 and 28 days. Daily food intake was computed as caloric intake because in the early postoperative days rats consumed a combination of liquid and solid diet. Food efficiency was calculated as grams of change in weight per kilocalorie of food eaten over the study period (14 or 28 days) and expressed as percentage [$\Delta BW \text{ (g)} \times 100/CI \text{ (g)}$]; thereby providing an index of how well a rat assimilated nutrients into body mass.

Morphologic Changes

At the end of either 14 or 28 days, rats were decapitated under isoflurane anesthesia. Mixed arterial and venous blood was collected into EDTA-rinsed tubes and was centrifuged at 3000 rpm for 10 minutes at 4°C for plasma. The plasma samples were stored for a limited time at -80°C before analysis. Retroperitoneal, epididymal, subcutaneous, and mesenteric fat pads were dissected out, weighed and computed together as total fat mass reflecting energy status. *Vastus lateralis*, gastrocnemius, and soleus muscles (right and left) were dissected out, weighed and computed together as skeletal muscle. Heart, pancreas, and liver were dissected out and weighed. Lean mass was calculated by subtracting the sum of the fat pads weight from total weight.

Biochemical Parameters

All samples were measured in duplicate. Plasma glucose, triglycerides, and free fatty acids (FFA) were measured by enzymatic colorimetric kits (WAKO, Richmond, VA). Plasma ghrelin, insulin, adiponectin (ALPCO, Windham, NH), free triiodothyronine 3 (T3) (Alpha Diagnostic, TX), cholecystokinin (CCK) (Phoenix, Belmont, CA) and leptin (R & D Systems, Inc., Minneapolis, MN) were measured by using enzyme-immunoassay kits.

Endocannabinoid Levels

Endocannabinoid levels were measured on 14 and 28 days after surgery in *vastus lateralis* and liver, which were weighed and stored at -80°C until ready for extraction.⁴⁴ Endocannabinoid concentrations were determined by liquid chromatography/mass spectrometry as described previously.⁴⁴

Blue Native Gels Electrophoresis of Skeletal Muscle Mitochondrial Protein Complexes

To determine the relative amounts of mitochondrial respiratory complexes in skeletal muscle (*vastus lateralis*), frozen tissue (75 mg) samples from postoperative day 14 and 28 were homogenized in 2 mL of extraction buffer (440 mM sucrose, 20 mM MOPS, 1 mM Di-Na + EDTA, 0.2 mM PMSF, pH 7.2 at + 4°C), and centrifuged at 500g for 2 minutes. The pellet was discarded, and the supernatant was centrifuged at 14,000g for 10 minutes. The pellet from this spin was resuspended in 150 μ M of buffer (0.75 mM aminocaproic acid, 50 mM BisTris, pH7 at + 4°C) plus 22 μ M dodecyl- β -D-maltoside (10% wt/vol), and was kept on ice for 10 minutes. Then, the mixture was centrifuged at 14,000g for 10 minutes. The supernatant (100 μ M) was saved; 9 μ L of Coomassie blue suspension (0.5 M aminocaproic acid, 5% Coomassie Blue) was added and mixed before loading directly onto gel. Blue-native gel electrophoresis was performed as previously described with minor modifications.⁴⁵ Gels were run at a constant 40 V for 1 hour with Hi-Blue cathode buffer (50 mM Tricine, 15 mM BisTris, 0.02% Coomassie Blue, pH7 at + 4°C), and then at 20 V overnight with Lo-Blue cathode buffer (50 mM Tricine, 15 mM BisTris, 0.002% Coomassie Blue, pH7 at + 4°C). Gel was stained in solution of Coomassie Blue R-250 and G-250 (0.05 wt/vol each in 10% acetic acid and 25% isopropanol, pH7 at + 4°C) for 3 hours, and then destained in 10% acetic acid + 25% isopropanol. Gels were scanned in grayscale mode at 600 dpi resolution using a desktop scanner. Densitometry was performed using Scion Image software (a PC version of the freely available National Institutes

of Health image quantitation software), in which density profiles for individual lanes were generated and the baseline was subtracted by calibrating the density signal using a part of the gel that contained no protein. The relative amount of mitochondrial respiratory complexes contain within skeletal muscle sample was assessed by optical densitometry with Scion Image software (www.scioncorp.com), as previously described.⁴⁶ Thus, mitochondrial respiratory complexes were identified by their characteristic pattern on the blue native gel (ie, complex I, V, III, IV, II from top to bottom) via comparison with numerous publications on this topic, including articles from this laboratory,⁴⁵ and by molecular weight. This pattern of bands has been incisively identified previously by Western blotting.⁴⁶

Statistical Analysis

Comparisons of daily body weight, caloric intake, food efficiency, organ weights, hormones, and endocannabinoids levels among the 3 study groups were analyzed using one-way ANOVA followed by Student-Neuman-Keuls multiple-comparison test with significance set at $P \leq 0.05$.

RESULTS

Body Weight, Caloric Intake, and Food Efficiency

As shown in Figure 1A, at day 0 there were no differences in weight among groups. Body weight of RYGB rats 14 days postsurgery was lower than that of Obese and PF rats ($P < 0.001$), and no differences existed between Obese and PF rats in body weight. The percentage of weight reduction relative to Obese achieved by RYGB was $21.4\% \pm 3.1\%$, whereas that caused solely by caloric restriction (as shown by PF rats) was $6.1\% \pm 3.6\%$. On postoperative day 28, RYGB induced a significant decrease in weight compared with Obese ($P < 0.001$) and PF rats ($P = 0.01$), whereas PF versus Obese rats also had a lower weight ($P = 0.01$, Fig. 1A). RYGB rats weighed $34.6\% \pm 1.9\%$ less than Obese rats, whereas PF rats weighed $19.3\% \pm 2.9\%$ less than Obese. Both RYGB and PF rats had a decrease in weight on postoperative day 14 and 28 compared with day 0 ($P < 0.001$). RYGB rats also showed lower weight on postoperative day 28 versus postoperative day 14 ($P = 0.041$), whereas in Obese weight decreased at postoperative day 14 compared with day 0 ($P = 0.019$).

Cumulative caloric intake was significantly lower in RYGB and PF rats versus Obese on postoperative day 14 and 28 ($P < 0.001$; Fig. 1B). In all groups, cumulative caloric intake increased significantly from postoperative day 14 to postoperative day 28 ($P < 0.001$).

In all groups food efficiency was negative on postoperative day 14 and 28 and significantly higher at postoperative day 28 than at postoperative day 14 ($P < 0.001$, Fig. 1C). On postoperative day 14, RYGB rats had lower food efficiency than Obese and PF rats ($P < 0.001$), whereas PF rats also showed lower food efficiency compared with Obese ($P < 0.001$). At postoperative day 28, food efficiency in RYGB and PF rats remained lower than in Obese ($P < 0.001$) and in RYGB rats was also lower than in PF rats ($P = 0.003$).

Morphologic Changes

Total fat mass in RYGB and PF rats versus Obese did not change ($P = 0.191$ and $P = 0.575$) and was also similar in these 2 groups ($P = 0.315$) on postoperative day 14 (Table 1). On postoperative day 28, RYGB and PF rats had a reduction in fat weight versus Obese rats ($P = 0.006$ and $P = 0.033$, respectively). No differences were found between RYGB and PF rats at this time point ($P = 0.392$). On postoperative day 14 fat-free mass (Table 1) was similar in all groups ($P > 0.05$). On postoperative day 28 it was reduced in both RYGB ($P < 0.001$) and PF ($P = 0.016$) rats versus Obese rats and it was also lower in RYGB than in PF rats ($P = 0.019$). In RYGB rats, fat-free mass decreased significantly at postoperative day 28 versus postoperative day 14 ($P = 0.043$). The liver weight is shown in Table 1. No changes occurred

at postoperative day 14, whereas on postoperative day 28 both RYGB ($P = 0.001$) and PF ($P = 0.022$) rats had lower liver weight than Obese, which were similar between them ($P = 0.347$). On postoperative day 14 we did not find statistical differences between groups in heart weight ($P > 0.05$, Table 1), whereas on postoperative day 28 in RYGB rats versus Obese and PF rats a lower heart mass was found ($P = 0.002$ and $P = 0.023$, respectively). Pancreatic mass (Table 1) was significantly decreased in RYGB versus both Obese and PF rats ($P = 0.03$ and $P = 0.002$, respectively) on postoperative day 14. No differences were found between PF and Obese rats ($P = 0.234$) at this time. Pancreatic weight, 28 days after surgery, was lower in RYGB rats versus Obese and PF rats ($P = 0.005$), but no changes were found between PF and Obese groups ($P = 0.909$). As shown in Table 1, skeletal muscle weight was reduced in RYGB rats at both postoperative day 14 and 28 versus Obese and PF rats ($P < 0.001$). No differences were found between PF rats and Obese at any time ($P > 0.05$).

Biochemical Parameters

Figure 2A, shows no differences in glucose levels among groups at postoperative days 14 or 28 ($P = 0.119$). However, plasma insulin (Fig. 2B) was significantly lower in RYGB rats versus Obese on postoperative days 14 and 28 ($P < 0.001$ and $P = 0.002$, respectively). It was also lower compared with PF rats on days 14 and 28 ($P < 0.001$ and $P = 0.007$, respectively). No differences were found between PF and Obese rats ($P = 0.257$ at postoperative day 14 and $P = 0.525$ at postoperative day 28), or with time in any of the studied groups. The glucose-to-insulin ratio, shown in Figure 2C, was significantly higher in RYGB versus Obese and PF rats ($P < 0.001$) at day 14, signifying enhanced insulin sensitivity. Similar results were found at postoperative day 28 ($P < 0.001$ vs. Obese and $P = 0.005$ vs. PF rats, respectively).

Changes in plasma triglycerides and FFA are summarized in Table 1. Plasma triglycerides were similar in all groups on postoperative 14 ($P > 0.05$). On day 28, RYGB rats showed lower levels versus Obese ($P = 0.014$) but similar levels to PF rats, although they tended to be lower ($P = 0.077$). There was a rise in plasma triglycerides in PF rats at day 28 versus postoperative day 14 ($P = 0.01$). On postoperative day 14 plasma FFA was similar in all groups ($P > 0.05$). On day 28, the FFA levels in RYGB were similar to Obese rats ($P = 0.772$) but higher than in PF rats ($P = 0.024$), which showed lower levels compared with Obese ($P = 0.021$). The FFA levels in PF rats were lower than in Obese ($P = 0.021$). Furthermore, FFA levels in PF rats decreased on postoperative day 28 compared with postoperative day 14 ($P = 0.026$).

Figure 3A shows that plasma leptin concentrations were significantly lower in RYGB versus Obese rats on days 14 and 28 ($P < 0.001$). In PF versus Obese rats, leptin levels were lower on days 14 and 28 ($P = 0.006$, and $P = 0.004$, respectively). On postoperative day 28, PF rats had significantly lower plasma leptin ($P = 0.008$). Leptin levels were not statistically different at any time in RYGB and PF rats, but they tended to be lower in RYGB rats at postoperative day 28 ($P = 0.055$). No changes were found with time in any of the groups ($P > 0.05$). As shown in Figure 3B, on postoperative day 14 plasma adiponectin was lower in RYGB rats versus Obese ($P = 0.024$) and PF rats ($P = 0.006$). No differences were found at this time between Obese and PF ($P = 0.506$). At day 28, there were not differences among groups ($P > 0.05$), but at this time RYGB rats had higher adiponectin levels than at postoperative day 14 ($P = 0.008$). Plasma T3 levels are shown in Figure 3C. RYGB rats had lower T3 than Obese and PF rats on both day 14 and 28 ($P = 0.018$ vs. Obese and $P = 0.028$ vs. PF rats at postoperative day 14; $P = 0.002$ vs. Obese and PF rats at postoperative day 28). No differences were found between Obese and PF rats on postoperative days 14 or 28 ($P = 0.830$) or postoperative day 28 ($P = 0.987$).

Table 1 shows that RYGB caused a marked increase in plasma CCK on postoperative day 14 versus Obese and PF rats ($P = 0.003$), whereas these 2 groups had similar CCK concentrations ($P = 0.912$). On day 28 all groups showed similar levels of plasma CCK ($P > 0.05$). No

significant differences were found among groups at any time point in plasma total ghrelin ($P = 0.641$; Table 1).

Endocannabinoid Levels in Skeletal Muscle

Figure 4, shows that no differences in the levels of AEA or 2-arachidonoylglycerol (2-AG) in skeletal muscle among groups on postoperative day 14 ($P > 0.05$). However, there was a significant reduction in the AEA levels in RYGB rats compared with Obese ($P = 0.003$) on postoperative day 28. At this time no differences were found in AEA content between RYGB and PF rats ($P = 0.118$) or PF rats and Obese ($P = 0.193$). On postoperative day 28 there were no differences in the content of 2-AG among groups ($P = 0.845$). The levels of any of these endocannabinoids studied did not change with time ($P > 0.05$).

Endocannabinoid Levels in Liver

Figure 5, shows hepatic AEA and 2-AG levels. AEA levels did not change in RYGB and PF rats compared with Obese and no differences were found between RYGB and PF rats ($P > 0.05$; Fig. 5A). In RYGB rats a significant decrease in hepatic AEA levels occurred on postoperative 28 versus postoperative 14 ($P = 0.031$). As shown in Figure 5B, 2-AG content was similar in all groups ($P > 0.05$) on postoperative day 14. On day 28 RYGB rats showed lower levels compared with PF rats ($P < 0.001$) but similar levels to Obese ($P = 0.402$). On day 28, PF rats showed higher levels of 2-AG compared with Obese ($P = 0.006$) and also compared with postoperative day 14 ($P < 0.001$).

Mitochondrial Complexes in Skeletal Muscle

Figure 6A shows a representative native blue gel electrophoresis of mitochondrial complexes pattern in skeletal muscle of the 3 groups studied on postoperative day 14. As shown in Figure 6B, on day 14 the amount of complex (Cx) I was significantly reduced in RYGB rats compared with Obese ($P = 0.048$) and PF rats ($P = 0.026$). No differences were found in Cx V ($P = 0.284$), Cx III ($P = 0.093$), Cx IV ($P = 0.380$), and Cx II ($P = 0.206$) among groups. In RYGB rats Cx V, Cx III, and Cx IV showed a statistical trend to be lower than in the Obese group. On day 28 there were no differences in any of the 5 mitochondrial complexes ($P > 0.05$), although the amount of Cx I tended to be lower than in Obese and PF rats (Fig. 6C).

DISCUSSION

The precise physiological mechanisms by which RYGB maintains weight loss is still a matter of intense research. In humans, however, RYGB is characterized by an initial accelerated weight loss lasting 1 to 2 years, followed by the sustained weight loss in the second and third postsurgical years.³ In our rat model, the 14- and 28-day time points are the equivalent of 1 to 3 human years,⁴¹ and reflect the rapid and the sustained weight loss periods. The nature of the weight-loss curve suggests interplay of several short-term and long-term metabolic processes that mediate weight loss and the weight maintenance phases post-RYGB surgery.

All animals in the study groups lost weight after RYGB or sham operation as a result of the surgical stress and the inadequate caloric intake from the liquid diet. Although such postsurgical malaise is common, the RYGB and PF rats lost significantly more weight than the Obese rats. RYGB rats, which consumed a similar amount of calories as PF rats, displayed significantly greater weight loss at postoperative day 14 and 28, suggesting that, apart from the decreased caloric intake, the marked reduced food efficiency of the RYGB rats (Fig. 1) could also play a significant role in their greater postoperative weight loss. Because similar results were reported by us, as long as 90 days after RYGB,⁴⁷ it would appear that 28 days after surgery is the beginning of the metabolic milieu period leading to the weight maintenance period. At postoperative day 28, all groups had improved their food efficiency. However, food efficiency

remained lower in RYGB and PF rats compared with Obese (Fig. 1). The partial recovery of food efficiency at postoperative day 28 is attributed to the “starvation response,” aimed at conserving body energy stores and opposing weight loss by increasing food efficiency,⁴⁸ and is the result of a decline in leptin levels, which counterbalances the decreased energy storage.⁴⁹ In this model, we found a significant decrease in plasma leptin in RYGB rats as early as postoperative day 14, despite no significant reduction in their fat mass. This is in agreement with previous reports showing that not only loss of fat mass but also caloric restriction leads to the reduction of circulating leptin.⁵⁰ However, despite the lower levels of leptin in RYGB rats, food efficiency was significantly lower than in Obese and PF rats at postoperative day 14 and 28, suggesting that in RYGB rats this “starvation response” may be suppressed by other factors, resulting in a greater degree of weight loss. We found similar results in our previous 90-day study. Food efficiency increased with time in all groups but, as in the present study, even 90 days after surgery the RYGB rats had lower food efficiency than both Obese and PF rats, thereby contributing to sustained weight loss.⁴⁷

One of the factors that could contribute to counterbalance the decrease in plasma leptin levels, and therefore to the “starvation response,” could be the rise in plasma CCK at postoperative day 14 in RYGB rats versus both Obese and PF rats. CCK, acting on its CCK-1 receptors in the abdominal vagal afferent neurons of the stomach and the duodenum,⁵¹ induces the sensation of satiety, contributing to the RYGB’s lower caloric intake. The process of satiation induced by CCK-1 receptors is enhanced by activating synergistically with leptin on these vagal afferents.⁵² These satiety signals reach the nucleus of the solitary tract activating proopiomelanocortin⁵³ and neurons in the area postrema⁵⁴ where these elevated CCK levels could cause taste aversion because this area is involved in aversive stimuli,⁵⁵ contributing to a further decrease in food intake. The rise in CCK levels probably compensate for the drop in plasma leptin-inducing satiety because leptin reinforces the effects of CCK by altering the excitability of neurons in the nucleus of the solitary tract via stimulation of descending pathways from the hypothalamus⁵⁶ involving the gut-brain-gut signaling system. Moreover, elevated CCK could also compensate for the down-regulation of hypothalamic proopiomelanocortin neurons due to hypoleptinemia.⁵⁷ In this context, it is notable that some of the effects of endogenous cannabinoids on appetite and food intake are mediated by vagal afferent neurons, suggesting the involvement of the endocannabinoid system in modulating gut-brain signaling.⁵⁸

Cannabinoid CB1 receptors are expressed on CCK-1 receptors positive vagal afferent neurons and are up-regulated by fasting, when plasma CCK drops, and rapidly decrease by feeding and by CCK.²³ Furthermore, AEA levels rise in the rat intestine after an overnight food restriction.⁵⁸ Hence, the high concentrations of CCK found in RYGB rats 14 days after operation could down-regulate the expression of CB1 receptors, contributing to their lower caloric intake and higher weight loss. The absence of differences in CCK levels at both 28 (present study) and 90 days⁴⁷ after operation and in our previous study³⁴ between RYGB and PF rats after operation suggest that CCK return to normal levels with time and that after 14 days other mechanisms, such as the decrease in insulin, the down-regulation of endocannabinoids, and the increase in PYY, may underlie the lower weight, caloric intake, and food efficiency in RYGB rats.

We previously reported a several fold increase in plasma levels of anorectic PYY on postoperative days 14, 28, and 90 in RYGB rats.³⁴ Comparable findings were subsequently reported in humans after RYGB.⁴ This increase in plasma PYY may overcome the “starvation response” induced by decreased plasma leptin observed in RYGB rats. Also in high-fat diet-induced obese mice, exogenous PYY decreased food intake, weight, and increased fatty acid oxidation.⁵⁹ On the other hand, in PF rats, PYY levels were not that different from levels in the Obese rats. As a result, no compensation for the decreased leptin levels occurred. Hence,

only a small reduction in weight was seen (Fig. 1). The increased levels of systemic anorectic PYY after gastric bypass may contribute to the decrease in caloric intake in RYGB rats.^{34, 60} Furthermore, these elevated levels of systemic PYY after gastric bypass delays gastric emptying and intestinal transit time, further contributing to the decrease in caloric intake and weight loss in RYGB rats.³⁴ Additionally, the PYY induced sympathetic drive could contribute to the significant decrease in fat mass as a result of increased lipolytic function due to β_3 -adrenergic receptor activation and the increased expression of UCP1 and UCP2.⁶¹

No significant changes in plasma ghrelin occurred after RYGB on either postoperative day 14 or 28 (Table 1). This is consistent with our previous 90-day study,⁴⁷ suggesting that ghrelin probably does not play a significant role in stimulating appetite and food intake after RYGB. However, these data do not agree with the results of others⁶² in humans showing a decrease in plasma ghrelin, despite a decrease in weight. The inconsistent data may be due to confounding factors such as the size of the gastric pouch, the length of the afferent limb, current BMI, and the associated degree of weight loss, and the division of some versus all autonomic fibers innervating the stomach and the foregut.^{4,63} In fact, we have found a loss of vagal efferent innervation to the pouch after RYGB.⁴⁷ Ghrelin levels are also regulated by endocannabinoids. Blockade of cannabinoid CB1 receptors with antagonist SR141716 (Rimonabant) decreases circulating ghrelin in rats and diminishes the response of circulating ghrelin to fasting.⁶⁴ Hence, the down-regulation in peripheral endocannabinoids in RYGB rats could contribute to reduce plasma ghrelin counterbalancing its increase induced by weight loss.

Similar to the landmark discovery by Pories⁶⁵ that insulin levels decreased precipitously after RYGB, in our model insulin levels also decreased (Fig. 2), thereby significantly increasing the glucose-to-insulin ratio, reflecting enhanced insulin sensitivity, and facilitating the mobilization of endogenous energy reserves. Insulin stimulates lipogenic genes to induce lipogenesis. Hence, in RYGB rats, the dramatic decrease in insulin levels (Fig. 2) may be a contributory mechanism to decreasing fat mass by facilitating the mobilization of stored substrates in liver and muscle.⁶⁶ In fact, we have found in a previous study a reduction in hepatic triglycerides³⁷ and a trend of lower intramyocellular triglycerides content (unpublished data) in RYGB rats. This reduction in insulin levels at both 14 and 28 postoperative days is consistent with our previous finding of decreased plasma insulin 90 days after RYGB.⁴⁷ The reduction in AEA levels in skeletal muscle at postoperative day 28 (Fig. 4) likely resulted in an increase in the rate of fat oxidation leading to the intramyocellular triglycerides reduction and eventually in a decrease of fat mass, given that skeletal muscle is the primary site of fat oxidation.⁶⁷ The lower plasma insulin and the higher insulin sensitivity found in RYGB rats could be related to the down-regulation of peripheral endocannabinoids. This novel system has been known to contribute to the physiological regulation of glucose metabolism.^{15,18,26,68} Oral treatment with SR141716 (Rimonabant) in diet-induced obese mice enhances glycolysis by inducing the expression of glycolytic enzymes and GLUT-4, which mediates the transport of glucose into the cells, thus improving insulin sensitivity.¹⁵ Furthermore, the chronic administration of SR141716 for 5 weeks in diet-induced obese mice corrected insulin resistance and lowered plasma insulin, leptin, and FFA.²¹ This is now supported by data showing that in humans studies, insulin resistance improved after 1 year of treatment with Rimonabant.²² The decreases in liver and muscle endocannabinoid levels at postoperative day 28 could contribute to improved insulin sensitivity in RYGB rats. At day 14, however, insulin sensitivity had improved despite the fact that no differences in endocannabinoid levels were observed. This could indicate that postoperative day 14 may be a transition point at which levels of endocannabinoids begin to change toward a down-regulation. Further studies are needed to understand this mechanism.

Deficient leptin signaling is associated with elevated hypothalamic levels of endocannabinoids. Acute leptin treatment of normal rats and leptin deficient *ob/ob* mice decreases hypothalamic

levels of AEA and 2-AG by approximately 40% to 50%.¹² If a similar regulation of endocannabinoids by leptin takes place at the peripheral level, we would expect an increase in AEA and 2-AG in skeletal muscle and liver concomitant to the marked reduction in leptin levels induced by both RYGB and caloric restriction by itself. However, this up-regulation induced by caloric restriction is completely reversed by RYGB, although RYGB induces a marked decrease in leptin levels. These low endocannabinoid levels accompanied by low leptin levels are in keeping with the fact that endocannabinoids may exert positive feedback on leptin production from adipose tissue, given that cannabinoid CB1 receptor-deficient mice has lower plasma levels than wild-type mice.²¹

We may expect that the lower food efficiency in RYGB rats was accompanied by higher T3 levels because T3 increases metabolic rate by up-regulating uncoupling proteins (UCPs) expression in skeletal muscle, heart, and white adipose tissue.^{69,70} Also, from the down-regulation of the peripheral endocannabinoids, an increase in T3 levels may be expected because intraperitoneal administration of WIN 55,212-2 (a CB receptor agonist) reduces serum T3 and T4 without changing thyroid-stimulating hormone, and this effect is antagonized by pretreatment with SR141716²⁷; whereas treatment with SR141716 (Rimonabant) enhances the expression of type II deiodinase and therefore the production of T3.¹⁵ But we have found a decrease in T3 in RYGB rats, in keeping with our findings at postoperative day 90,⁴⁷ suggesting a lower metabolic rate. The lower metabolic rate is consistent with the reduction in plasma T3, T4, and thyroid-stimulating hormone induced by caloric restriction, and also with the increase in T3 production, resulting from the conversion of T4 to T3 induced by intracerebroventricular administration of leptin in normal rats.⁷¹ From this we can deduce that lower leptin levels, along with caloric restriction as occurs in RYGB rats, contributes to a reduction in plasma T3 levels. Other mechanisms must also be involved given that PF rats had the same degree of caloric restriction and also reduced leptin levels but no reduction in T3. However, the down-regulation of endocannabinoid levels in muscle and liver could counterbalance the effect of the decrease in T3 on energy expenditure because SR141716 (Rimonabant) increases basal oxygen consumption and thermogenesis.¹⁸ Furthermore, treatment with SR141716 in diet-induced obese mice generates futile cycles in white adipose tissue, which contribute to increased energy expenditure.^{61,72} On the other hand, T3 up-regulates the expression of genes coding for lipogenic enzymes, including acetyl-coenzyme A carboxylase and glucose-6-phosphate dehydrogenase.⁷⁰ Overall, the lower T3 levels in RYGB rats could explain in part the reduction in fat mass and weight. However, the reduction in T3 may also be interpreted as a mechanism preventing an increase in energy expenditure in the negative energy balance state induced by RYGB as a result of the decreased weight.⁶

Both central and peripheral administration of AEA act on CB1 receptors to increase food intake in rodents and regulate peripheral energy homeostasis and weight.^{10,11,17} Furthermore, chronic administration of PF mice with the CB1 antagonist SR141716 (Rimonabant) caused a greater decrease in weight and in fat content.¹⁷ It is thus likely that the reduction in both hepatic and muscle endocannabinoids at postoperative day 28 in RYGB rats contributes to their lower fat and fat-free mass (Table 1). Endocannabinoids have direct peripheral actions being involved in peripheral signaling of nutritional status and lipogenesis.^{14,16,17,20} Both cannabinoids and ghrelin inhibit AMP-activated protein kinase (AMPK) activity in the liver and adipose tissue,⁷³ leading to the activation of ACC-1 and ACC-2, which results in the stimulation of fatty acid synthesis and the inhibition of fatty acid oxidation, respectively, and likely contributing to the development of obesity.^{29,73} The inhibition also affects mitochondria because AMPK regulates mitochondrial biogenesis.⁷⁴ Furthermore, treatment with AM251 results in an increase in AMPK α 1 expression in myotubes.²⁹ Hence, the down-regulation of endocannabinoid levels induced by RYGB could enhance the activity of AMPK leading to inhibition of downstream targets acetyl-CoA carboxylase-1 and -2 thereby inhibiting fatty acid synthesis and promoting fatty acid oxidation. Such an effect may contribute to the

lower weight and lower fat mass in RYGB rats (Table 1). Activation of adipose AMPK up-regulates adiponectin and down-regulates TNF- α . Because endocannabinoids decrease AMPK in the liver, the decreased endocannabinoid levels observed at postoperative day 28 in RYGB rats could mediate the reduction in lipid storage, decreased fat mass, and decreased plasma triglyceride levels. This finding is supported by the fact that in our previous study RYGB was associated with reduced hepatic fat content, an indication of increased catabolic activity.³⁷ Furthermore, blocking the CB1 receptor with SR141716 stimulates adiponectin gene expression and synthesis in diet-induced obese mice,¹⁵ and activates AMPK. In addition, CB1 receptor activation increased the accumulation of lipid droplets and decreased adiponectin expression in adipocytes.⁶⁸ It is therefore likely that RYGB may down-regulate endocannabinoids thereby freeing up adiponectin to reduce fat mass, increase fatty acid oxidation, and weight loss.⁷⁵

Inbred transgenic C57BL/6 mice on a high-fat diet have decreased plasma adiponectin, lower energy expenditure, and higher food efficiency.⁷⁶ However, in endocannabinoid CB1 receptor knockout mice (CB1^{-/-}) food efficiency and adiponectin is unaffected by a high-fat diet, suggesting the involvement of endocannabinoids in promoting energy conservative processes such as an enhancement of food efficiency and a reduction of energy expenditure. On the other hand, chronic oral treatment with Rimonabant increases lipolysis in white adipose tissue via enhanced β -oxidation and an increase in energy expenditure, mainly through futile cycling (calcium and substrate).¹⁵ Chronic intraperitoneal administration of Rimonabant in leptin deficient *ob/ob* mice promotes a long-lasting weight loss independent of food intake, possibly through increased energy expenditure by way of enhanced basal oxygen consumption.¹⁸ SR141716 increases the expression of type II deiodinase, which is the enzyme that catalyzes the production of T3 to subsequently activate thermogenesis in brown adipose tissue. T3 activation also induces fatty acid CoA ligase and mitochondrial oxidative phosphorylation, and inhibits fat storage by promoting fat burning through mitochondrial-mediated heat production.¹⁵ A similar mechanism may be activated by the RYGB-mediated suppression of endocannabinoids.

The involvement of endocannabinoids in the regulation of mitochondrial function was shown by chronic treatment with SR141716, which induces the expression of carnitine acetyl transferase, carnitine palmityltransferase II, and enoyl CoA hydratase in white adipose tissue, indicating an increase in β -oxidation. Additionally, increases in the expression of 3 tricarboxylic acid (TCA) cycle enzymes also suggest increased fatty acid oxidation.¹⁵ Furthermore, blockade of CB1 receptors leads to an induction of cytochrome *c* oxidase subunit VIa, a subunit of the complex IV of the respiratory chain, and adenine nucleotide carrier, that exports ATP from the mitochondria while importing ADP.¹⁵ In brown adipose tissue, SR141716 induces the expression of enzymes of the oxidative phosphorylation pathway, including NADH ubiquinone oxidoreductase, ubiquinol cytochrome *c* reductase (complex III), and induces chaperonin 10, which modulates mitochondrial biogenesis.¹⁵ In a preliminary study (unpublished data) we found that after blocking of CB1 receptors with AM251, there was an up-regulation of the respiratory chain in skeletal muscle, as shown by a significant increase in proton leak. Such a metabolic event decreases the efficiency of oxidative phosphorylation, leading to more energy being dissipated as heat. We expected to find similar alterations in RYGB rats on postoperative day 28 because of the down-regulation of AEA and 2-AG, which occurred in skeletal muscle (Fig. 4) and liver (Fig. 5), respectively. Instead, we found a trend to a reduction of skeletal mitochondrial complexes at postoperative day 28 (Fig. 6), which indicates a decrease in the number of mitochondria, together with a reduction in the TCA activity. This conclusion is supported by the reduction of the amount of complex I, which implies a decrease in reduced nutrient substrate (eg, NADH) entering the mitochondria from the TCA cycle at postoperative day 28, when the concentrations of AEA and 2-AG were low. Alternatively, in the case of enhanced fat burning, the electron transfer flavoprotein of β -

oxidation may represent the major site of electron entry into the respiratory chain in these animals, thereby reducing the reliance on complexes I and II as more “classic” electron entry sites, leading to their down-regulation. However, the reduced AEA levels in skeletal muscle in RYGB rats could result in changes in the expression and/or activity of the proteins involved in entry of fatty acids into the mitochondria, such as acetyl-CoA carboxylase, leading to a more effective fat oxidation and to the subsequent reduction in muscle weight resulting from a decrease in the content of intramyocellular triglycerides. Such increase in key proteins expression could compensate the reduction in mitochondrial number.

The changes in mitochondrial complex amounts are interesting in the context of what also occurred at a later time point following RYGB, ie, 90 days later,⁴⁷ or the equivalent of 7 to 9 years post-RYGB. In the latter study, we showed that the decrease in the amount of mitochondrial complexes in RYGB was restricted to the subset of the RYGB rats that exhibited successful weight loss. In contrast, the rats that were unable to sustain weight loss and regained weight showed reversal of the mitochondrial complexes phenotype versus the RYGB rats that sustained weight loss. Together, these studies suggest that changes in mitochondrial complexes are an early event in RYGB and that maintenance of this modified mitochondrial phenotype is essential to ensure continued and sustained weight loss. Reversal of mitochondrial phenotype may be a factor that drives failure in those rats that regain weight at a later time point.

In conclusion, RYGB induces a coordinated down-regulation of the endogenous cannabinoid system and enhanced mitochondrial function, leading to normalization of the dysregulated physiological processes associated with obesity. Furthermore, these results also suggest that the RYGB-induced down-regulation of endocannabinoids may serve as a permissive effect to suppress anabolic processes and up-regulate catabolic neuroendocrine mediators of stored energy mobilization such as mitochondrial-mediated increased fatty acid oxidation and the subsequent decrease in fat mass. Furthermore, in this model the successful down-regulation of endocannabinoids occurs with the preservation of the vagal nerve, which is quite important to facilitate the beneficial effects of RYGB. Pharmacological antagonism of the endocannabinoid system with Rimonabant may have implications for the treatment of morbid obesity to maintain weight loss and prevent weight regain post-RYGB.

Acknowledgements

This study was supported in part by a SUNY Upstate Intramural Fund (130230-30), American Diabetes Association (1-05-RA-81) and the American Society for Metabolic and Bariatric Surgery (A1). Douglas Osei-Hyiaman is supported by the NIH Intramural Research Program. Paul S. Brooks is funded by NIH-HL071158.

The authors thank Karen Hughes, AAS, for her technical assistance.

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FIGURE 1.

Body weight (BW, panel A), cumulative caloric intake (CI, panel B) representing the total kilocalories eaten over either postoperative day 14 or 28 (POD) and food efficiency (panel C), calculated as grams of body weight change per kilocalorie of food eaten and expressed as percentage. Data are expressed as mean $\pm$ SEM. * $P < 0.05$ and *** $P < 0.005$ compared with Obese; ## $P < 0.01$ and ### $P < 0.005$ compared with PF rats; §§§ $P < 0.005$ compared with day 0.

FIGURE 2.

Plasma concentrations of glucose (panel A), insulin (panel B) and the ratio glucose-to-insulin (panel C). Data are expressed as mean $\pm$ SEM. * P < 0.05 and *** P < 0.005 compared with Obese; # P < 0.05 and ## P < 0.01 compared with PF rats; ††† P < 0.005 compared with postoperative day (POD) 14.

FIGURE 3.

Plasma concentrations of leptin (panel A), adiponectin (panel B), and T3 (Panel C). Data are expressed as mean $\pm$ SEM. * $P < 0.05$ and *** $P < 0.005$ compared with Obese; # $P < 0.05$, ### $P < 0.01$, and #### $P < 0.005$ compared with PF rats; and †† $P < 0.001$ compared with postoperative day (POD) 14.

FIGURE 4. Concentrations of anandamide (AEA; panel A) and 2-arachidonoylglycerol (2-AG; panel B) in skeletal muscle. Data are expressed as mean $\pm$ SEM. *** $P < 0.005$ compared with Obese.

FIGURE 5. Concentrations of anandamide (AEA; panel A) and 2-arachidonoylglycerol (2-AG; panel B) in the liver. Data are expressed as mean $\pm$ SEM. ** $P < 0.01$ compared with Obese; ### $P < 0.005$ compared with PF rats; $\dagger P < 0.05$ and $\dagger\dagger\dagger P < 0.005$ compared with postoperative day (POD) 14.

FIGURE 6.

Representative picture of mitochondrial respiratory complexes separated by blue-native gel electrophoresis (panel A) and quantitative analysis of mitochondrial respiratory complexes in skeletal muscle at postoperative day (POD) 14 (panel B) and at POD 28 (panel C). Results are expressed as mean $\pm$ SEM of 4 independent experiments. * $P < 0.05$ compared with Obese; # $P < 0.05$ compared with PF.

TABLE 1
Body Composition, Selected Plasma Substrates, and Hormones in Obese, RYGB, and PF Rats at 14 and 28 Days After Operation

	Obese		RYGB		PF	
	Day 14	Day 28	Day 14	Day 28	Day 14	Day 28
Total fat mass (g)	79.51 ± 12.51	68.31 ± 7.93	51.74 ± 8.30	20.89 ± 4.02 [†]	71.81 ± 15.13	32.68 ± 4.94 [*]
Fat-free mass (g)	565.74 ± 30.28	607.57 ± 17.51	489.90 ± 21.79	419.64 ± 10.95 ^{‡§**}	550.32 ± 25.08	512.57 ± 16.19 [*]
Liver mass (g)	16.78 ± 1.32	18.33 ± 0.80	13.07 ± 1.32	11.76 ± 1.06 [‡]	14.71 ± 1.12	13.85 ± 0.34 [*]
Heart mass (g)	1.91 ± 0.09	1.88 ± 0.06	1.52 ± 0.08	1.19 ± 0.03 ^{‡§}	1.84 ± 0.20	1.66 ± 0.17
Pancreas mass (g)	1.35 ± 0.11	1.84 ± 0.12	0.77 ± 0.13 ^{*//}	1.03 ± 0.18 ^{‡//}	1.61 ± 0.23	1.81 ± 0.10
Skeletal muscle mass (g)	7.63 ± 0.38	8.25 ± 0.39	4.55 ± 0.44 ^{‡//}	5.40 ± 0.17 ^{‡//}	8.40 ± 0.42	7.43 ± 0.27
Triglycerides (mg/mL)	53.22 ± 8.60	76.94 ± 8.39	44.28 ± 9.77	41.28 ± 4.98 [*]	30.80 ± 2.06	67.71 ± 8.37 ^{††}
FFA (mEq/L)	0.40 ± 0.02	0.32 ± 0.03	0.35 ± 0.04	0.29 ± 0.04 [§]	0.31 ± 0.01	0.21 ± 0.02 ^{*,**}
CCK (ng/mL)	0.19 ± 0.02	0.30 ± 0.03	0.54 ± 0.11 ^{‡//}	0.30 ± 0.03	0.21 ± 0.04	0.22 ± 0.03
Total ghrelin (ng/mL)	1.75 ± 0.13	1.77 ± 0.18	1.85 ± 0.20	1.77 ± 0.30	1.55 ± 0.17	1.41 ± 0.13

Total fat mass was calculated as the sum of four different fat pads (retroperitoneal, epididymal, mesenteric, and subcutaneous). Fat-free mass was calculated by subtracting the total weight of the fat pads from total body weight. Skeletal muscle mass represents the total weight of *vasus lateralis*, gastrocnemius, and soleus from both legs.

Data are expressed as mean ± SEM.

^{*} $P < 0.05$,

[†] $P < 0.01$, and

[‡] $P < 0.005$ compared with Obese.

[§] $P < 0.05$ and

^{//} $P < 0.005$ compared with PF rats.

^{**} $P < 0.05$ and

^{††} $P < 0.01$ compared with postoperative day 14.