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Identification of Novel Changes in Human Skeletal Muscle Proteome After Roux-en-Y Gastric Bypass Surgery

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PATHOPHYSIOLOGY

The mechanisms of metabolic improvements after Roux-en-Y gastric bypass (RYGB) surgery are not entirely clear. Therefore, the aim of our study was to investigate the role of obesity and RYGB on the human skeletal muscle proteome. Basal muscle biopsies were obtained from seven obese (BMI >40 kg/m^2) female subjects (45.1 ± 3.6 years) pre- and 3 months post-RYGB, and euglycemic-hyperinsulinemic clamps were used to assess insulin sensitivity. Four age-matched (48.5 ± 4.7 years) lean (BMI <25 kg/m^2) females served as control subjects. We performed quantitative mass spectrometry and microarray analyses on protein and RNA isolated from the muscle biopsies. Significant improvements in fasting plasma glucose (104.2 ± 7.8 vs. 86.7 ± 3.1 mg/dL) and BMI (42.1 ± 2.2 vs. 35.3 ± 1.8 kg/m^2) were demonstrated in the pre- versus post-RYGB, both $P < 0.05$. Proteomic analysis identified 2,877 quantifiable proteins. Of these, 395 proteins were significantly altered in obesity before surgery, and 280 proteins differed significantly post-RYGB. Post-RYGB, 49 proteins were returned to normal levels after surgery. KEGG pathway analysis revealed a decreased abundance in ribosomal and oxidative phosphorylation proteins in obesity, and a normalization of ribosomal proteins post-RYGB. The transcriptomic data confirmed the normalization of the ribosomal proteins. Our results provide evidence that obesity and RYGB have a dynamic effect on the skeletal muscle proteome.

Insulin resistance, type 2 diabetes, and defects in protein metabolism occur in obese insulin-resistant individuals (1,2). Bariatric surgery is an effective intervention for treating obesity and resolving comorbidities (3,4). Specifically, Roux-en-Y gastric bypass (RYGB) and biliopancreatic diversion procedures are highly effective at restoring glycemic control (5,6). After RYGB and biliopancreatic diversion, rapid resolution of type 2 diabetes and improved glycemia have been observed before any substantial weight loss has occurred (7).

In addition to impairments in glucose homeostasis, defects in protein metabolism are also observed in obese individuals (8–10). Skeletal muscle represents the primary site of insulin-stimulated glucose disposal. Obese insulin-resistant muscle has also been shown to have reduced amounts of mitochondrial and cytoskeletal proteins (11,12). The molecular basis of weight loss induced by RYGB in skeletal muscle is not well understood. There have been a number of studies that have identified genes or proteins that may explain the metabolic improvements observed in skeletal muscle after weight loss surgery (13–18). To the best of our knowledge, no study has performed a global analysis of protein abundance changes in combination with transcriptomic analyses in skeletal muscle pre- and post-RYGB.

Therefore, the goal of the current study was to decipher the mechanism of action of bariatric surgery on skeletal muscle metabolism. Specifically, we sought to determine the changes that occurred in the skeletal muscle proteome and transcriptome before and 3 months post-RYGB surgery. We hypothesized that gastric bypass surgery would *1*) result in weight loss and improvements in glucose metabolism and *2*) alter the skeletal muscle proteome and expression of proteins and genes in metabolic pathways. Conducting this

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study allowed us to perform global, unbiased analyses that may capture novel pathways that would then lead to new focused hypotheses on weight loss induced by surgery.

RESEARCH DESIGN AND METHODS

Subjects

Seven insulin-resistant obese (BMI >40 kg/m^2) female subjects (45.1 ± 3.6 years), including one with diabetes (treated with metformin), were studied before and 3 months post-RYGB. Four age-matched (48.5 ± 4.7 years), lean (BMI <25 kg/m^2), insulin-sensitive females served as control subjects. Studies were approved by the institutional review boards at the Mayo Clinic in Arizona and Arizona State University and were performed in the Clinical Studies Infusion Unit (CSIU) at the Mayo Clinic in Arizona. Each volunteer underwent a medical history, physical examination, screening laboratory tests, metabolic panel, bioelectrical impedance analysis, and a 75-g oral glucose tolerance test. On a separate day, all participants returned to the CSIU for a euglycemic-hyperinsulinemic clamp with basal muscle biopsies to assess insulin sensitivity, as previously described (19,20). Three months post-RYGB, the obese participants returned to the CSIU and a repeat euglycemic-hyperinsulinemic clamp with muscle biopsy was performed.

The RYGB was performed at the Bariatric Surgery Program at the Mayo Clinic in Arizona. Each participant that entered into the program met with a dietitian, one on one, at least two times prior to the surgery. Moreover, they were provided with 8 weeks of behavior modification classes, which included nutrition, exercise, and behavioral aspects of lifestyle change. Each participant had at minimum three sessions with the psychologist to work on behavior goals. The presurgery diet was a regular balanced diet with emphasis on portion control for modest weight loss prior to surgery. The average weight loss from the initial visit to the presurgery (average time prior to surgery in program was 5.3 ± 0.6 months) was 5.1 kg, with an average initial weight of 119.1 ± 8.9 kg and a presurgery weight of 114.0 ± 8.9 kg. All participants completed the 8 weeks of behavior modification classes prior to the presurgery biopsy. The postsurgery diet was a liquid diet for 3 weeks with a transitioning to mechanical soft after 3 weeks and then transition to normal textures after 6 weeks. All participants were taking daily multivitamin, calcium with vitamin D, B12 monthly injections, iron supplements, and protein supplementation.

Muscle Biopsy Processing

For protein analyses, ~50 mg of frozen muscle biopsy samples were homogenized on ice using a Polytron homogenizer (Brinkmann Instruments Westbury, NY) in detergent containing lysis buffer as previously described (21). For mRNA analyses, muscle biopsy specimens (50 mg) were homogenized directly in TRIzol solution (Invitrogen, Carlsbad, CA), using a Polytron homogenizer (Brinkmann Instruments Westbury, NY). Total RNA was purified with the RNeasy MinElute Cleanup Kit (Qiagen, Chatsworth, CA).

In-Gel Digestion and Mass Spectrometry

Muscle lysate protein (80 μg total protein) was separated on 4–20% TGX Criterion gradient SDS polyacrylamide gels (Bio-Rad, Berkeley, CA) and processed for mass spectrometry, as previously described (12). Mass spectrometry (liquid chromatography–electrospray ionization–tandem mass spectrometry [LC-ESI-MS/MS]) was performed on a Thermo Electron (San Jose, CA) Orbitrap Elite Velos Pro fitted with an EASY source (Thermo Electron, San Jose, CA). NanoLC was performed using a DIONEX/Thermo NCS-3500RS UltiMate 3000 with an EASY Spray column (Thermo Electron, San Jose, CA; 50 cm × 75 μm inner diameter, packed with PepMap RSLC C18 material, 2 μm). A "top 15" data-dependent MS/MS analysis was performed (acquisition of a full-scan spectrum followed by collision-induced dissociation mass spectra of the 15 most abundant ions in the survey scan).

Label-Free Peptide/Protein Quantification and Identification

Progenesis QI for proteomics software (version 2.0.5387.52102; Nonlinear Dynamics Ltd., Newcastle upon Tyne, U.K.) was used to perform ion intensity–based label-free quantification. Mascot .xml files were imported into Progenesis, allowing for peptide/protein assignment, and peptides with a Mascot ion score of <25 were not considered for further analysis. Protein quantification and logarithmic transformation was performed using only nonconflicting peptides, and precursor ion abundance values were normalized in a run to a reference run. Only proteins with two or more unique peptides were included in downstream analyses.

Immunoblotting

Muscle protein lysates (20–40 μg total protein) were resolved by TGX Criterion gradient SDS polyacrylamide gels (Bio-Rad, Berkeley, CA), transferred to polyvinylidene fluoride membrane, and probed using various antibodies. ImageJ was used for quantification of the blot (http://imagej.nih.gov/ij/index.html).

Transcriptomic Analysis

Transcriptomic analysis using the SurePrint G3 Human Gene Expression 8 × 60 K v2 Microarray was performed as per the manufacturer's instructions (Agilent Technologies, Palo Alto, CA). The data generated from the image analysis have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE76468. The data generated from the image analysis were imported into the R statistical program. Data were log transformed and quantile normalized using the Linear Models for Microarray Data (Limma) in R.

Quantitative Real-Time PCR

Quantitative real-time PCR analyses for selected mRNAs (RNA28S5, RNA5-8S5, ATP5B, ACTB, CASQ2, COL6A1, COL6A2, EIF4A2, GAPDH, GAPVD1, IRS1, MAPK14, MYOD1, PDK2, PYGL, RPL12, RPL23A, RPS14, RPS16, SDHA, TUBB, and USP15) were performed as per the manufacturer's instructions (Life Technologies, Carlsbad, CA).

Other Analyses

The screening laboratory tests and metabolic panel were performed by the Biospecimens Accessioning and Processing Core at the Mayo Clinic in Arizona. Fasting plasma glucose (FPG) was measured by the YSI 2300 STAT plus (YSI Inc., Yellow Sprigs, OH) in the CSIU. Serum insulin was measured at the Immunochemical Core Laboratory at the Mayo Clinic in Rochester, NY. The d2-glucose enrichment data from the euglycemic-hyperinsulinemic clamp was measured at the Center for Clinical and Translational Science Metabolomics Core at the Mayo Clinic in Rochester.

Statistical Analysis

Statistical comparisons were performed using paired or unpaired Student *t* tests where appropriate. Pearson correlation was used for all correlations. KEGG pathway analysis was performed on the proteomics and transcriptomics data.

RESULTS

Subjects

Presurgery obese subjects had higher BMI, body fat percentage, hemoglobin A_{1c}, FPG, fasting serum insulin (FSI), and HOMA of insulin resistance (HOMA-IR) compared with lean controls (Table 1). Lower HDL levels and a reduced rate of insulin-stimulated glucose disposal were observed in the presurgery obese subjects compared with the lean subjects (Table 1). Three months postsurgery, significant improvements in BMI, body fat percentage, cholesterol, LDL, FSI, FPG, and HOMA-IR were observed compared with presurgery values (Table 1). No significant improvements in HDL levels or insulin-stimulated glucose disposal were observed 3 months postsurgery compared with presurgery (Table 1).

Proteomic Analyses

A summary of the proteomic analysis is shown in Supplementary Fig. 1. Proteomic analysis identified 2,877 quantifiable proteins. There were 260 and 135 proteins that were significantly ($P < 0.05$) decreased and increased in presurgery obese subjects versus lean subjects, respectively. KEGG pathway analysis of the 260 proteins that were decreased in presurgery obese subjects indicated an enrichment in ribosomal proteins and proteins involved in oxidative phosphorylation, muscle contraction, and the TCA cycle (Supplementary Table 1). There were no enriched pathways in the 135 proteins increased in the presurgery obese subjects compared with the lean subjects. There were 52 and 228 proteins that were significantly ($P < 0.05$) decreased and increased post- versus presurgery, respectively (Supplementary Table 1). KEGG analysis of the 52 decreased proteins in the postsurgery subjects revealed an enrichment for the proteasome pathway. The post- versus presurgery pathway analysis of the 228 increased proteins revealed that ribosome and spliceosome proteins were significantly enriched (Supplementary Table 1). However, there was no significant enrichment in oxidative phosphorylation, muscle contraction, or TCA cycle proteins postsurgery. Since one of the participants had type 2 diabetes and was treated with metformin, we reran the KEGG pathway analysis excluding this individual. The pathway analysis showed similar patterns as described above (data not shown).

We identified 49 proteins that post-RYGB surgery were normalized to levels similar to those observed in lean control subjects (Supplementary Table 2). In order to be considered normalized, we stipulated that proteins had to be significantly ($P < 0.05$) different in both the lean versus presurgery obese and pre- versus postsurgery analyses. KEGG pathway analysis of the 49 normalized proteins indicated a statistically significant enrichment (Bonferroni $P < 0.05$, 13-fold enrichment) for the ribosome pathway. The weight loss post-RYGB also revealed an increased abundance and normalization of insulin signaling proteins, including MAP kinase and proteins involved in vesicle transport/GLUT localization (Supplementary Table 2) relative to the lean healthy control subjects.

Confirmation of Mass Spectrometry–Based Protein Quantification by Immunoblot Analysis

Figure 1*A* shows the mass spectrometry–normalized ion abundance data for desmin, mitochondrial citrate synthase, cytochrome *c*, and superoxide dismutase (SOD2) relative to GAPDH. Consistent with the mass spectrometry results, quantification and normalization of Western blots for desmin, citrate synthase, cytochrome *c*, and SOD2 relative to GAPDH showed decreases in all four proteins for both pre- and postsurgery samples, compared with lean (Fig. 1*B*).

Transcriptomic Analyses

In addition to identifying the changes in skeletal muscle proteins after bariatric surgery, we also examined changes in gene expression using whole genome microarrays. There were 3,047 and 2,643 probes that were significantly (uncorrected $P < 0.05$) increased and decreased in gene expression post- versus presurgery, respectively. KEGG pathway analysis of the microarray data identified that the ribosome pathway was the most significantly enriched group in the genes that were increased postsurgery compared with presurgery (Supplementary Table 3). There was a decreased transcription of genes involved in oxidative phosphorylation and the TCA cycle pathways postsurgery compared with presurgery (Supplementary Table 3).

Confirmation of Transcriptomics Data Using Quantitative Real-Time PCR

Microarray data were validated using quantitative real-time PCR. Twenty-two genes were chosen for validation of the microarray technique. For all genes selected, PCR fold changes correlated ($R = 0.695$, $P < 0.001$) with the microarray data (Supplementary Fig. 2).

Ribosomal Protein Correlation Analysis

Since we observed that the ribosomal proteins were the only pathway significantly increased post-RYGB for both "omics" analyses, we selected all ribosomes identified by

Table 1—Subject characteristics

	Lean	Presurgery obese	Postsurgery obese	*P* value lean vs. pre	*P* value pre vs. post	*P* value lean vs. post
Sex	4 female	7 female	7 female			
Age (years)	48.5 ± 4.7	45.1 ± 3.6	45.3 ± 3.5	NS	NS	NS
BMI (kg/m^2)	23.6 ± 0.7	42.1 ± 2.2	35.3 ± 1.8	<0.001	<0.001	<0.001
Body fat (%)	30.6 ± 1.6	46.4 ± 1.2	40.6 ± 1.3	<0.001	<0.01	<0.01
Systolic blood pressure (mmHg)	116.3 ± 2.4	125.1 ± 3.9	119.1 ± 4.6	NS	NS	NS
Diastolic blood pressure (mmHg)	67.5 ± 6.3	71.7 ± 2.0	75.1 ± 1.7	NS	NS	NS
Triglycerides (mg/dL)	75.5 ± 13.3	121.9 ± 17.5	107.7 ± 11.2	NS	NS	NS
Cholesterol (mg/dL)	173.3 ± 22.9	181.4 ± 13.2	151.5 ± 11.2	NS	<0.01	NS
HDL (mg/dL)	60.8 ± 5.0	45.0 ± 2.7	45.0 ± 2.5	<0.05	NS	<0.05
LDL (mg/dL)	97.3 ± 15.5	112.1 ± 11.9	84.8 ± 10.5	NS	<0.01	NS
Hemoglobin A_{1c}, % (mmol/mol)	5.5 ± 0.1 (36.5 ± 1.4)	6.0 ± 0.2 (42.3 ± 1.7)	5.7 ± 0.1 (39.1 ± 1.4)	<0.05	NS	NS
FPG (mg/dL)	84.9 ± 1.7	104.2 ± 7.8	86.7 ± 3.1	<0.05	<0.05	NS
FSI (μIU/mL)	4.3 ± 0.5	18.2 ± 2.7	7.5 ± 1.0	<0.001	<0.01	<0.05
M value (mg/kg/min)	7.8 ± 0.5	2.4 ± 0.3	2.9 ± 0.4	<0.001	NS	<0.001
M value (mg/kg FFM/min)	11.2 ± 0.7	4.4 ± 0.6	4.9 ± 0.6	<0.001	NS	<0.001
HOMA-IR	1.1 ± 0.1	4.4 ± 0.8	1.6 ± 0.3	<0.01	<0.05	NS

Data are means ± SEM. FFM, fat-free mass.

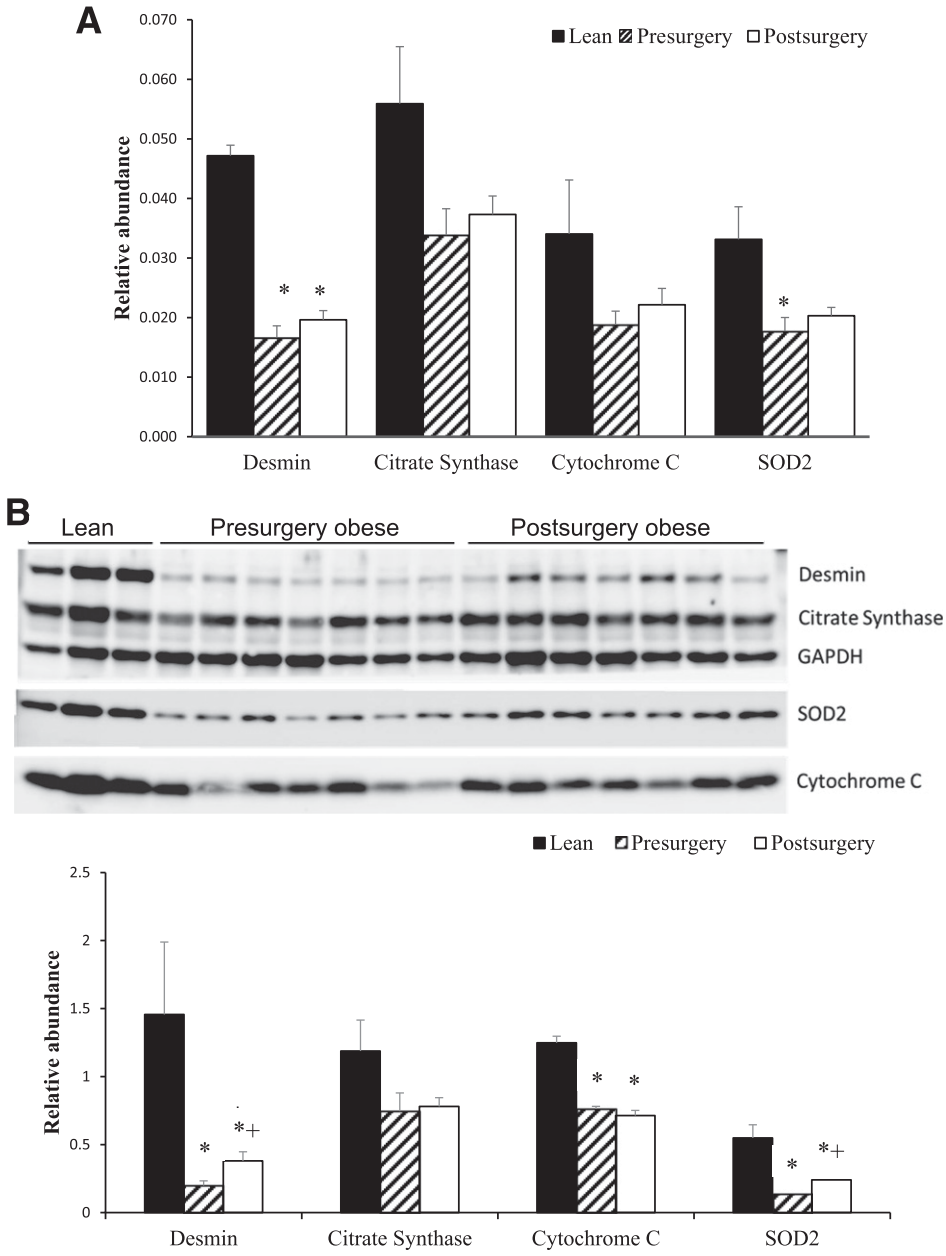

Figure 1—Proteomics data validated by immunoblotting analyses. The normalized ion abundance data for desmin, citrate synthase, cytochrome c, and SOD2 expressed relative to GAPDH taken from mass spectrometry (*A*) and immunoblot analysis comparing abundance of desmin, citrate synthase, cytochrome c, and SOD2 expressed relative to GAPDH protein in skeletal muscle biopsies from lean (n = 4), presurgery obese (n = 7), and postsurgery obese subjects (*B*). Data are means ± SEM. *P < 0.05, vs. lean control; +P < 0.05, postsurgery vs. presurgery.

mass spectrometry that had corresponding transcript data (n = 111) (Supplementary Table 4). We categorized the proteins as cytoplasmic (n = 75) or mitochondrial (n = 36) and revealed a strong correlation between protein and transcript changes for the cytoplasmic ribosome population (R = 0.489, $P < 0.001$) (Fig. 2*A*) but not for the mitochondrial ribosomes (R = 0.133, $P < 0.439$) (Fig. 2*B*). Immunoblot analysis for two of the cytoplasmic ribosomes (RPL23A and RPS14) confirmed the pattern of recovery of these proteins postsurgery (Fig. 3). Correlation analysis of the cytoplasmic ribosome transcript data with BMI, our most significantly altered phenotype post-RYGB, revealed significant and inversely correlated associations for RPL3L, RPS6KA3, and RPS21 (r = −0.94, −0.91, and −0.90, respectively). For the protein data, we identified that RPS11 was significantly and inversely correlate with BMI (r = −0.81).

DISCUSSION

The current study was undertaken to decipher the molecular mechanisms through which weight loss induced by RYGB alters skeletal muscle metabolism in morbidly obese patients. We believe our study is the first to perform proteome

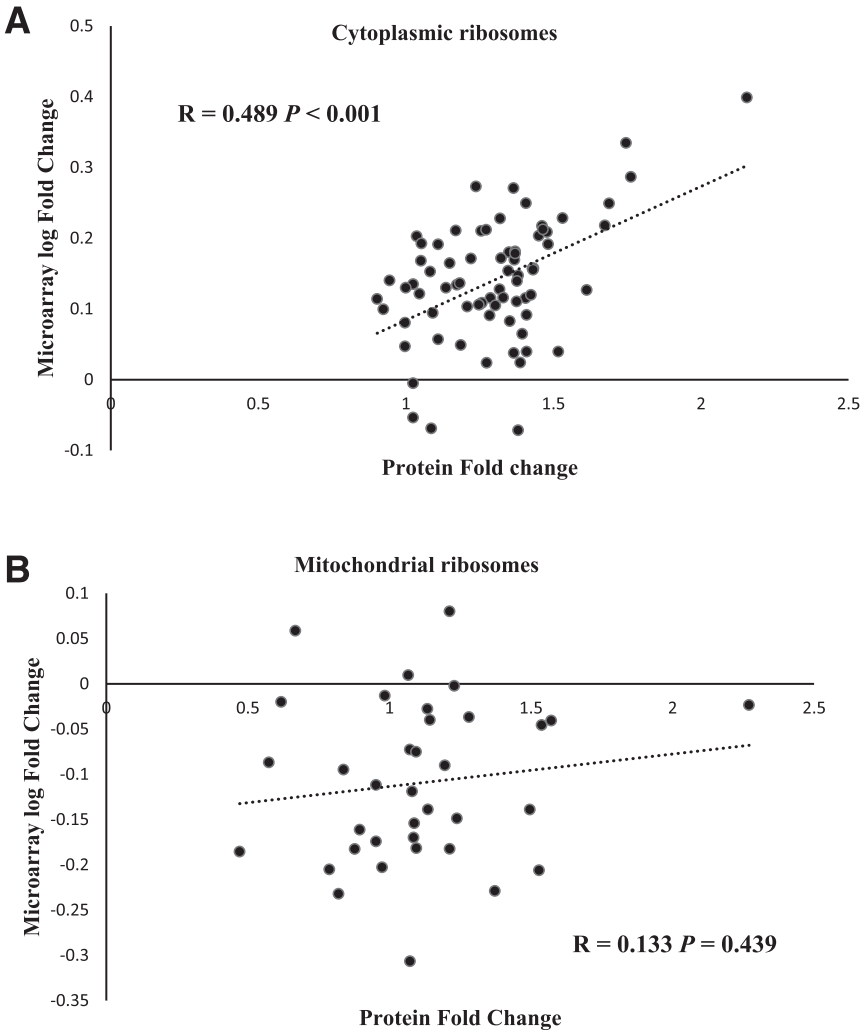

Figure 2—Correlation analysis of protein fold changes and microarray log2 fold changes for the cytoplasmic ribosomes in the pre- vs. postsurgery subjects (*A*) and correlation analysis of protein fold changes and microarray log2 fold changes for the mitochondrial ribosomes in the pre- vs. postsurgery subjects (*B*).

and transcriptome analyses on skeletal muscle from patients before and 3 months post-RYGB surgery. Although we demonstrated reductions in body weight, FPG, FSI, and HOMA-IR levels post-RYGB, there were no significant improvements in glucose disposal as determined by the euglycemic-hyperinsulinemic clamp. In our study, the BMI post-RYGB surgery was on average 35 kg/m^2, which still is considered obese. Other studies have shown that improvements in glucose disposal occur 6–12 months postsurgery (16,17). Therefore, the lack of improvement in peripheral insulin sensitivity may be more pronounced when weight loss is more extensive.

Unique to our study was the identification of ribosomal proteins as being decreased in abundance in presurgery obese subjects compared with lean subjects, and subsequently increased and normalized in abundance post-RYGB surgery. Our transcriptomic analysis in the pre- and postsurgery analyses suggests that this could be transcriptionally mediated. The transcriptional fold changes correlated significantly with protein fold changes for cytoplasmic ribosomes. ETS family transcription factors, including Elk1, have been shown to be a regulator of genes encoding ribosomal proteins (22). Elk1 has also been shown to be activated in response to insulin signaling via the mitogen-activated protein kinase signaling pathway (23,24). As Akt activates ribosomal protein S6 kinase, translation may also be playing a role in increased ribosome abundance postsurgery (25). However, a study by Albers et al. (16), showed no increase in Akt activity in normoglycemic individuals at 3 months postsurgery. Additional studies would be necessary to explore the factors that regulate the gene expression of ribosomal proteins.

It is well known that obesity and insulin resistance in skeletal muscle is, in part, due to mitochondrial dysfunction (2). In the current study, we observed a reduction in oxidative phosphorylation genes in the obese subjects compared with the lean control subjects for the proteomic analyses. However, post-RYGB surgery, we did not see a

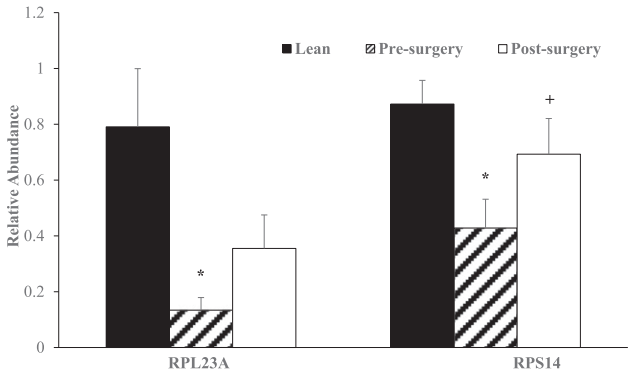

Figure 3—Immunoblot analysis of RPL23A and RPS14 expressed relative to GAPDH protein in skeletal muscle biopsies from lean (n = 4), presurgery obese (n = 7), and postsurgery obese subjects. Data are means ± SEM. *P < 0.05, vs. lean control; +P < 0.05, post-surgery vs. presurgery.

recovery of these genes or proteins. In contrast, Gastaldi et al. (14) demonstrated an upregulation of the mRNA for PPARGC1A at 3 and 12 months post-RYGB. In that study, there was an improvement in insulin sensitivity at 3 months for their patients, which may explain the inconsistency (14).

In summary, our findings suggest that there is a wide array of changes in protein abundance in skeletal muscle pre- and post-RYGB surgery. There are several pathways that emerge from our global "omics" approach for additional follow-up studies. Ribosomal proteins appear to recover post-RYGB and normalize to the levels observed in lean, healthy control subjects. The role of these ribosomal proteins, specifically the cytosolic ribosomes, warrants further analysis. Moreover, oxidative phosphorylation proteins/genes do not recover at 3 months post-RYGB, and normalization of these proteins at this time point may have occurred with an exercise intervention program. A limitation of our study is that we did not record daily exercise in our patients. Therefore, the contribution of daily physical activity on the degree of weight loss and "omics" findings after bariatric surgery is unclear. In addition, we have a small number of subjects in our study and that limits our power. Despite these limitations, we identified interesting patterns of regulation in ribosomal pathways post-RYGB. Collectively, our results provide evidence that obesity and RYGB have a dynamic effect on the skeletal muscle proteome.

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