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GRB14, GPD1, and GDF8 as potential network collaborators in weight loss-induced improvements in insulin action in human skeletal muscle

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Park, Jung-Jun, Jason R. Berggren, Matthew W. Hulver, Joseph A Houmard, and Eric P. Hoffman. GRB14, GPD1, and GDF8 as potential network collaborators in weight loss-induced improvements in insulin action in human skeletal muscle. *Physiol Genomics* 27: 114–121, 2006. First published July 18, 2006; doi:10.1152/physiolgenomics.00045.2006.—Obesity is associated with insulin resistance in skeletal muscle; accordingly, weight loss dramatically improves insulin action. We sought to identify molecular remodeling of muscle commensurate with weight loss that could explain improvements in insulin action. Muscle from morbidly obese women was studied before and after gastric bypass surgery. Gastric bypass surgery significantly reduced body mass by ~45% and improved insulin action. We then assessed mRNA profiles using a stringent statistical analysis (statistical concordance with three probe set algorithms), with validation in a cross-sectional study of lean (n = 8) vs. morbidly obese (n = 8) muscle. Growth factor receptor-bound protein 14 (GRB14), glycerol-3-phosphate dehydrogenase 1 (GPD1), and growth differentiation factor 8 (GDF8; myostatin) significantly decreased ~2.4-, 2.2-, and 2.4-fold, respectively, after weight loss (gastric bypass). Increased expression of these transcripts was associated with increased obesity in the cross-sectional group (lean vs. morbidly obese muscle). Each transcript was validated by real-time quantitative RT-PCR assays in both study groups. Using Ingenuity Pathway Analysis, we show that all three transcripts are involved in the same regulatory network including AKT1, IGF1, TNF, PPARG, and INS. These results suggest that GRB14, GPD1, and GDF8 are weight loss-responsive genes in skeletal muscle and that the observed transcriptional modulation of these would be expected to improve insulin signaling, decrease triglyceride synthesis, and increase muscle mass, respectively, with weight loss. Thus our data provide a possible regulatory pathway involved in the development of insulin resistance in the morbidly obese state, and improvement of insulin resistance with weight loss.

obesity; gastric bypass; mRNA profiling; growth factor receptor-bound protein 14; glycerol-3-phosphate dehydrogenase 1; growth differentiation factor 8

THE PREVALENCE OF OBESITY has increased worldwide at an alarming rate, with a doubling of incidence over the last decade in most industrialized countries. Approximately 65% of US adults are overweight, 30% are obese, and 6% are morbidly obese (9). This increasing rate of obesity is independent of age, ethnicity, and sex and is associated with increased risk for many chronic health problems, including metabolic syndrome, Type 2 diabetes, and cardiovascular disease (33). In particular, the fact that ~75% of insulin-resistant individuals are overweight or obese indicates a strong relationship between obesity and insulin resistance (29).

Obesity results from a chronic imbalance between energy intake and energy expenditure, with excess calories stored as fat (32). As such, weight loss has long been considered as a primary goal of treatment for obesity. A well-known effect of weight loss in obese individuals is an improvement in insulin sensitivity (38). A typical weight loss program, consisting of calorie-restricted diet combined with behavior modification and exercise, results in significant improvement in insulin sensitivity (47). A surgical treatment of severe obesity such as gastric bypass provides more dramatic reductions in body weight ~35–50% and consequently increases insulin sensitivity approximately four- to fivefold (2, 12, 15, 35). Although a strong relationship between weight loss and improvements in insulin resistance has been consistently reported, the mechanism underlying this association remains unclear.

Under normal physiological conditions, >80% of insulin-mediated glucose metabolism occurs in skeletal muscle (37). We have previously demonstrated that weight loss induced by gastric bypass surgery increased insulin receptor content and decreased intramuscular lipids and long-chain fatty acyl-CoA in skeletal muscle of obese and morbidly obese individuals (12, 15, 35). Recently, we also reported that long-chain fatty acyl-CoA and stearoyl-CoA desaturase 1 expression and activity were higher in skeletal muscle from morbidly obese individuals than lean individuals (18, 19). These results suggest that skeletal muscle may play an important role in the regulation of insulin sensitivity in response to weight change.

The purpose of the current study was to discern whether alterations in distinct metabolic pathways in human skeletal muscle may explain, at least in part, the improvement in insulin action seen with weight loss in obese individuals. Here, we took a stringent approach, where two distinct subject populations were used. The test group was a longitudinal design, with morbidly obese women undergoing gastric bypass surgery, with serial muscle biopsies. The validation group was a cross-sectional study of lean and morbidly obese women, with a single muscle biopsy. As there is considerable debate concerning interpretation of microarrays, specifically with regard to probe set algorithm usage (39, 40), we used a stringent approach of requiring concordance between three common probe set algorithms in all analyses. Using this approach, we identified three key weight loss-responsive transcripts that participate in the same regulatory network.

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Table 1. *Description of subjects for two study populations*

Variables	Gastric Bypass Test Data Set		Obesity Validation Data Set	
	Before	After	Lean	Morbidly Obese
n	3	3	8	8
Age, yr	36.0±4.2	37.0±4.2	45.1±3.1	37.9±3.3
Height, cm	163.6±4.0	165.1±3.9	161.0±2.4	164.3±2.9
Weight, kg	162.1±22.1	90.6±20.0*	62.0±3.3	145.5±10.9*
BMI, kg/m^2	60.6±7.7	32.9±6.2*	23.9±0.7	53.8±3.5*
Glucose, mg/dl	102.6±7.5	89.3±3.5	106.7±8.5	92.3±4.3
Insulin, μU/ml	20.9±3.7	4.8±1.6*	16.1±5.8	32.9±10.7
HOMA	5.3±0.6	1.1±0.3*	4.3±1.4	7.6±2.9

Values are means ± SE. BMI, body mass index; HOMA, homeostasis model assessment. *Significant difference at $P < 0.05$ (before vs. after gastric bypass surgery; lean vs. morbidly obese women).

RESEARCH DESIGN AND METHODS

Subjects. Two separate subject populations were studied as shown in Table 1. The test group was a longitudinal design with three morbidly obese women [body mass index (BMI) >40 kg/m^2] with muscle biopsies before and after gastric bypass surgery. Percutaneous skeletal muscle biopsies of the vastus lateralis were obtained by using a Bergstrom needle on patients under local anesthesia immediately before gastric bypass surgery and then a second biopsy taken ~1 yr after surgery, when weight had stabilized (15).

The second study population was a previously published cross-sectional study of eight lean women (BMI <25 kg/m^2) and eight morbidly obese women (BMI >40 kg/m^2) (18, 19). For this validation group (cross-sectional study design), skeletal muscle samples were obtained from the rectus abdominus during abdominal surgery as previously described (19). We used a different muscle group for validation to confirm that alterations of gene expression were not due to regional differences in muscle fiber type.

In both study populations, muscle biopsies were obtained after a 12-h overnight fast. The biopsy samples were cleaned and immediately frozen in liquid nitrogen and stored for gene expression analysis. All subjects were nondiabetic and not taking any medications known to affect carbohydrate or lipid metabolism. The experimental protocols were approved by the East Carolina University Policy and Review Committee on Human Research, and written informed consent was obtained from all subjects. A total of 22 muscle biopsies were profiled, from 19 subjects.

Insulin action. Insulin action was determined as previously described (12). Briefly, a fasting blood sample was drawn from an antecubital vein, and plasma was separated and frozen at −80°C. Plasma glucose level was measured with an oxidation reaction using a glucose analyzer (YSI 2300 STAT Plus; YSI, Yellow Springs, OH), and plasma insulin level was measured by immunoassay (Access Immunoassay System; Beckman Coulter, Fullerton, CA). Insulin action was determined by calculating a homeostasis model assessment (HOMA) [fasting glucose (mg/dl) × 0.05551 × fasting insulin (μU/ml)/22.1] (13).

RNA preparation and gene expression profiling. We performed mRNA expression profiling with Affymetrix microarrays for each subject for both study populations using standard operating procedures and quality controls as we have recently reported (44). Briefly, total RNA was extracted from the frozen muscle biopsy tissue homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA). Isolated total RNA was purified by using RNeasy Mini Kit (Qiagen, Santa Clara, CA) and used for cRNA synthesis. Preparation of cRNA and microarray processing were performed as previously described (5). Briefly, total RNA was converted to double-stranded cDNA using an oligo-dT primer that incorporates T7 RNA polymerase promoter. The cDNA was purified by phase lock gel (Eppendorf, Westbury, NY) with phenol-chloroform extraction and then used for in vitro transcription to make biotinylated cRNA using ENZO BioArray RNA transcript labeling kit (Enzo Life Sciences, Farmingdale, NY).

The longitudinal expression profiling data set was done using two-round amplification of cRNA, as we have previously described (14), whereas the cross-sectional data set was done using one-round amplification of cRNA. These two data sets were not directly compared, but rather only gene lists of *P* values and fold changes were compared.

Biotin-labeled cRNA was purified by RNeasy Mini Kit (Qiagen) and then fragmented and hybridized to GeneChip probe arrays. Affymetrix U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA) were used for the test group [6 arrays; Gene Expression Omnibus (GEO) accession numbers: GSM101299–101304], and U133A arrays (Affymetrix) for the validation group (16 arrays; GEO accession numbers: GSM 3979–3994). These U133 Plus 2.0 arrays contain all of the same probe sets as the U133A arrays; however, additional probe sets corresponding to the U133B array are also on the U133 Plus 2.0 array. The microarrays were washed and stained on the Affymetrix Fluidics Station 450 and then scanned using Affymetrix GeneChip Scanner 3000 as recommended by manufacturer (Affymetrix).

Microarray data analysis. A total of 22 muscle biopsies were studied by microarray, two biopsies each in three subjects in the gastric bypass group, and one biopsy in each of 16 subjects in the cross-sectional study. All microarrays passed all quality control measures, including scaling factor ≤2, present calls >25%, and 3′/5′-GAPDH ratios <3 (44). We employed three distinct probe set algorithms (MAS5.0, RMA, and dCHIP difference model) for normalization and signal generation from each of the two projects separately (gastric bypass, cross-sectional study). For all probe set algorithms, we used a 30% "present call" filter (MAS5.0) to reduce the number of poorly performing probe sets in the analyses (41). To reduce array-wide variations, the intensity of each array was normalized to its 50th percentile. The intensity of each gene was also normalized to specific samples. In the test group (longitudinal design), each gene in post-surgery sample was normalized to the intensity of that gene in presurgery sample for each subject. In the validation group (cross-sectional design), each gene in the morbidly obese group was normalized to the average intensity of that gene in the lean group. Most microarray data analysis was done using GeneSpring 7.2 (Silicon Genetics, Redwood City, CA). Due to the extensive validation methods (independent microarray studies, and validation in both by RT-PCR), we did not correct for multiple testing.

There are many methods for the bioinformatic processing of microarray images to derive single "signals" for each gene and probe set (20). We have recently reported power calculation studies of human muscle microarray data and found that requiring concordance between different probe set algorithms is one approach toward reduction of false positives in data interpretation (40, 41). We analyzed both data sets with three distinct probe set algorithms [MAS5.0, RMA, and dCHIP difference model; see Seo et al. 2006 (40) for algorithm descriptions]. We then required that probe sets needed to show statistical significance ($P < 0.05$) with each of the three probe set algorithms, consistently in the same direction (increased vs. decreased expression).

Real-time quantitative RT-PCR. Real-time quantitative RT-PCR (qRT-PCR) was performed to validate expression levels of selected genes, showing an opposite direction of expression between the test group and validation group (decreased expression with weight loss in gastric bypass, and increased expression with increased obesity). cDNA was synthesized from newly isolated total RNA (validation data set) or remaining total RNA that was used for microarray assay (test data set) by using the SuperScript II Platinum Two-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA). For each target gene, Invitrogen LUX (Light Upon eXtension) primer pairs, including a fluorophore-labeled LUX primer and a corresponding unlabeled primer, were designed by web-based software, LUX Designer (Invitrogen, www.invitrogen.com/lux). For endogenous control, an hTBP (human TATA

box-binding protein) primer set, predesigned LUX primer set for housekeeping gene set (Invitrogen), was used because hTBP showed the most constant expression level in the microarray data. The primer sets for target gene and housekeeping gene were labeled FAM and JOE, respectively. The PCR reactions were prepared by using SuperScript II Platinum Two-Step qRT-PCR Kit (Invitrogen) and performed on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) as recommended by the manufacturers. All PCR amplifications were performed in triplicate wells with singleplex applications to minimize variations among samples and potential interference between target gene and housekeeping gene primer sets. To quantify the qRT-PCR results, we used the standard curve method according to a standard protocol of ABI (Applied Biosystems). For each sample, the threshold cycle (CT) value representing the number of PCR cycle at which fluorescence signal is increased above the automatically set threshold was measured by using Sequence Detection Software (SDS version 2.2, Applied Biosystems). On the basis of the CT value, the transcript quantities for target gene and endogenous control were determined from the appropriate standard curve for each. Then, the target gene quantity was divided by the endogenous control quantity to obtain a normalized target value.

Biological pathway analyses. To determine functional relationships among the identified genes, we used Ingenuity Pathways Analysis 3.1 (http://www.ingenuity.com) containing most literature knowledge of biological interactions between gene products. This web-based software application generated a set of molecular networks with a cutoff of 35 genes for each network based on interactions between uploaded genes and all other genes/proteins stored in the knowledge base. For each network, the probability of finding the uploaded genes in the network by random chance was calculated. The negative logarithm of the probability was then transferred to a score that represents how relevant this network was to the list of uploaded genes. For example, a score of 2 means there is a 1×10^{-2} probability that the genes in the network are only associated by chance, indicating a 99% confidence that the network is not generated by random chance. Therefore, we included only those networks showing a score >2.

Statistical analysis. For the longitudinal data set, a paired *t*-test with two-tailed distribution was used to analyze the changes in gene expression before and after gastric bypass surgery in morbidly obese individuals. For the cross-sectional data set, differently expressed genes in morbidly obese individuals compared with lean individuals were determined by an independent *t*-test with two-tailed distribution. We retained only those probe sets with $P < 0.05$ in both data sets, without correction for multiple testing.

RESULTS

Weight loss and insulin action. Subject characteristics for the test (gastric bypass) and validation (cross-sectional obesity) studies are shown in Table 1. As expected, gastric bypass significantly ($P < 0.01$) reduced body weight and BMI ~45 and 46%, respectively. Insulin level was also significantly ($P < 0.05$) decreased by ~76% with weight loss, while glucose level was not changed. As a result, there was a significant ($P < 0.05$) reduction in HOMA ~80%, indicating increased insulin action. In the validation study, morbidly obese women had significantly ($P < 0.001$) greater body weight and BMI than lean women. Insulin and HOMA values were also more than twofold higher in morbidly obese women, suggesting decreased insulin action. Although this difference was not statistically significant, the tendency was consistent with our previous data indicating that morbidly obese individuals are more insulin resistant (7, 8).

Gene expression profiling. Muscle from morbidly obese women was obtained before and after gastric bypass surgery, and expression was profiled using U133 Plus 2.0 microarrays containing 54,000 probe sets (1,300,000 oligonucleotides). We used a 30% "present call" filter to reduce the number of poorly performing probe sets and signals near background and then used a *P* value cut-off of $P < 0.05$ without multiple testing correction. We also used three probe set algorithms, MAS5.0, RMA, and dCHIP difference model, and subsequent analysis was limited to probe sets showing significant paired *t*-test *P* values by all three algorithms in the same direction of changes in expression. This resulted in 23 probe sets defined as "differently expressed" after gastric bypass surgery in skeletal muscle of morbidly obese individuals. These probe sets represent 20 genes, and all of these genes showed decreased expression (Table 2). Of these, 14 genes are involved in metabolism: two genes (G6PT1 and ACYP2) in carbohydrate/glucose metabolism, five genes (GPD1, FABP7, AKR1C1, AKR1C2, and AKR1C3) in lipid/fatty acid metabolism, three genes (AGTPBP1, CTH, and FBXO31) in protein/amino acid metabolism, and four genes (OXCT1, MT1X, MT1G, and HIBCH) in cellular metabolism. The remaining six genes (GRB14, GDF8, LRP12, FEZ2, GPR49, and DKK-2) are involved in signal transduction.

Validation of transcripts using a cross-sectional lean and morbidly obese subject population. The expression patterns of the 20 transcripts identified in the gastric bypass subjects were validated with muscle from lean and morbidly obese women. Our model for diet-responsive transcripts required that transcripts that were decreased by dietary restriction (gastric bypass) would be increased by obesity in the cross-sectional group. For this validation set, U133A microarrays containing 24,000 probe sets (528,000 oligonucleotides) were used, with the same array data analysis methods used for the test set (concordance of three probe set algorithms, same data-filtering methods). Of the 20 transcripts derived from analysis of the gastric bypass data, three of these, growth factor receptor-bound protein 14, glycerol-3-phosphate dehydrogenase 1, and growth differentiation factor 8 (GRB14, GPD1, and GDF8, respectively), showed significantly altered expression in morbidly obese women compared with lean women, whereas 17 genes showed no differences (Supplemental Table 1; the online version of this article contains supplemental data). Additionally, the expression levels of these three genes were all increased in morbidly obese women (GRB14, 1.4–2.7 fold; GPD1, 1.3–1.5 fold; GDF8, 1.4–1.9 fold), suggesting that these genes are "weight loss-responsive" genes.

Confirmation of GRB14, GPD1, and GDF8 expression by qRT-PCR. To confirm microarray data, GRB14, GPD1, and GDF8 expression were measured by qRT-PCR. These genes were selected based on the validation result, showing an opposite direction of expression between the test group and validation group (e.g., diet-responsive). As shown in Fig. 1*A*, GRB14 expression was significantly decreased by 2.7-fold ($P = 0.027$) after gastric bypass surgery and increased by 2.0-fold ($P = 0.003$) in morbidly obese women. Similarly, GPD1 expression was significantly decreased by 1.4-fold ($P = 0.04$) and increased by 1.8-fold ($P = 0.04$) after gastric bypass surgery and in morbidly obese women, respectively (Fig. 1*B*). These results confirmed microarray data for both the test data set and validation data set. On the other hand, GDF8 expression confirmed microarray data for only the test group (Fig. 1*C*). It was significantly decreased after gastric bypass surgery

Table 2. *Differently expressed probe sets before and after gastric bypass surgery*

	MAS5		dChip		RMA		
Affy ID	Fold	*P*	Fold	*P*	Fold	*P*	Gene Name
206204_at	−2.29	0.018	−2.42	0.021	−2.37	0.02	GRB14 (growth factor receptor-bound protein 14)
213706_at	−2.50	0.015	−2.19	0.038	−1.95	0.03	GPD1 (glycerol-3-phosphate dehydrogenase 1)
207145_at	−2.42	0.021	−2.21	0.02	−2.46	0.016	GDF8 (growth differentiation factor 8)
202830_s_at	−1.84	0.014	−1.50	0.028	−1.26	0.029	G6PT1 (glucose-6-phosphate transporter 1)
205030_at	−2.51	0.007	−2.93	0.009	−2.77	0.011	FABP7 (fatty acid binding protein 7, brain)
202780_at	−2.07	0.029	−2.02	0.007	−1.82	0.019	OXCT1 (3-oxoacid coa transferase 1)
219631_at	−1.28	0.043	−1.17	0.038	−1.21	0.044	LRP12 (low density lipoprotein-related protein 12)
204151_x_at	−1.89	0.024	−1.88	0.024	−1.69	0.013	AKR1C1 (aldo-keto reductase family 1, member C1)
216594_x_at	−1.96	0.029	−1.91	0.026	−1.76	0.011	AKR1C1 (aldo-keto reductase family 1, member C1)
211653_x_at	−2.27	0.007	−1.86	0.038	−1.73	0.027	AKR1C2 (aldo-keto reductase family 1, member C2)
209160_at	−2.92	0.008	−2.64	0.007	−2.69	0.008	AKR1C3 (aldo-keto reductase family 1, member C3)
202305_s_at	−2.10	0.003	−1.90	0.044	−1.83	0.039	FEZ2 (fasciculation and elongation protein zeta 2)
215000_s_at	−1.90	0.01	−1.81	0.044	−1.83	0.037	FEZ2 (fasciculation and elongation protein zeta 2)
204326_x_at	−1.71	0.003	−1.43	0.012	−1.59	0.007	MT1X (metallothionein 1X)
208581_x_at	−1.66	0.004	−1.64	0.015	−1.50	0.019	MT1X (metallothionein 1X)
203711_s_at	−1.83	0.005	−1.50	0.026	−1.57	0.028	HIBCH (3-hydroxyisobutyryl-coenzyme A hydrolase)
204500_s_at	−1.44	0.036	−1.53	0.05	−1.66	0.019	AGTPBP1 (ATP/GTP binding protein 1)
204745_x_at	−1.34	0.037	−1.26	0.025	−1.24	0.042	MT1G (metallothionein 1G)
206833_s_at	−1.50	0.016	−1.50	0.03	−1.47	0.048	ACYP2 (acylphosphatase 2, muscle type)
213880_at	−2.24	0.039	−2.28	0.019	−2.31	0.028	GPR49 (G protein-coupled receptor 49)
217127_at	−2.19	0.028	−1.43	0.026	−1.32	0.048	CTH (cystathionine gamma-lyase)
219785_s_at	−1.72	0.017	−1.47	0.021	−1.31	0.024	FBXO31 (F-box protein 31)
219908_at	−2.78	0.02	−3.11	0.028	−2.62	0.043	DKK-2 (Dickkopf homolog 2)

Affy ID, probe set identification number for a specific isoform of the corresponding gene.

by 2.2-fold ($P = 0.04$) and increased in morbidly obese muscle (1.7-fold); however, the latter was not statistically significant due to a relatively large variance in the three subjects available for validation. Also, previous studies have shown an association of GDF8 expression and obesity in adipocytes (45).

Molecular networks of differently expressed genes after gastric bypass surgery. To determine functional relationships between differently expressed genes after gastric bypass surgery, we uploaded the 20 identified genes into Ingenuity Pathways Analysis. This revealed a total of six molecular networks showing a score >2 (Supplemental Table 2). These networks collectively contained 14 identified genes, including all three validated genes, GRB14, GPD1, and GDF8, and other genes involved in carbohydrate and lipid metabolism, such as G6PT1, FABP7, AKR1C1, AKR1C2, and AKR1C3, that were defined as differentially regulated in the gastric bypass by our stringent microarray analyses (concordance with three probe set algorithms; paired *t*-test). We combined all six networks to create a composite network and then simplified this network to focus on only the three validated genes and others showing direct literature-supported relationships to these genes (Fig. 2). This showed that GRB14, GPD1, and GDF8 could all be placed within the same regulatory network, via direct interactions with shared network members. This network contained PPARG, INS, AKT1, IGF1, and TNF, each of which is known to have a key role in glucose metabolism or insulin signaling pathway but was not differentially regulated in our microarray analysis.

DISCUSSION

The purpose of the present study was to identify weight loss-responsive genes in skeletal muscle that could relate to improvement of insulin sensitivity after weight loss induced by gastric bypass surgery. To our knowledge, this is the first study investigating mRNA expression profiling in skeletal muscle of obese individuals in response to weight loss. Here, we report that GRB14, GPD1, and GDF8 are the key weight loss-responsive genes in skeletal muscle; each was downregulated in vastus lateralis muscle biopsies after weight loss (gastric bypass) and increased in rectus abdominus muscle biopsies of morbidly obese women compared with lean women. Using Ingenuity Pathway Analysis, we found that these three transcripts are potential collaborators in the same molecular network (Fig. 2). This network includes proteins well documented to be related to insulin action such as AKT1, IGF1, TNF, PPARG, and INS. The data presented suggest that GRB14, GPD1, and GDF8 may play an important role in the diet-responsive modulation of insulin sensitivity of muscle in morbidly obese individuals.

Our experimental approach is novel in a number of ways. We used two different human patient clinical studies, using distinct experimental designs, as independent validation sets. The first group of subjects was a small longitudinal gastric bypass patient series ($n = 3$), where vastus lateralis muscle biopsies were analyzed by microarray using a paired *t*-test (each individual serving as their own control; pre- vs. postgastric bypass). The longitudinal design is better able to diminish the interindividual variability at baseline, thus providing better sensitivity for transcripts responding to weight loss. The second group of subjects was a larger cross-sectional study of rectus abdominus muscle biopsies from lean ($n = 8$) and morbidly obese ($n = 8$) women. We required that diet-responsive transcripts in muscle show significant changes in expression in the opposite direction in these two patient groups (e.g., decreased expression in gastric bypass and increased expression in morbidly obesity). We also utilized a novel bioinformatics approach, where we required all transcripts to show statistical significance by three distinct probe set algorithms for

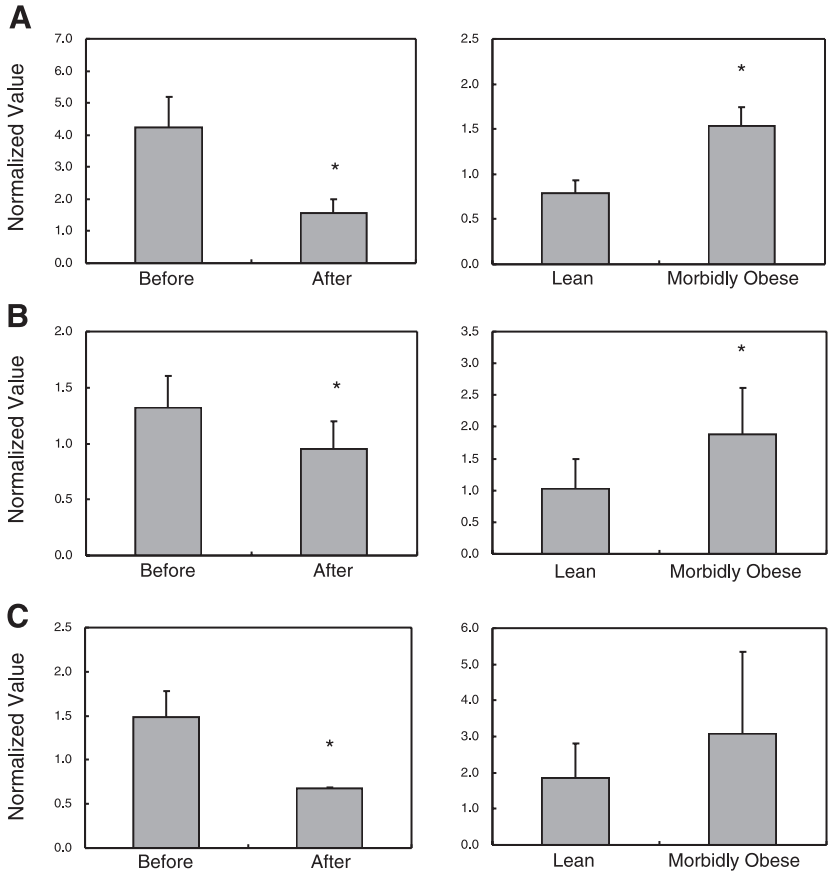

Fig. 1. Growth factor receptor-bound protein (GRB) 14, glycerol-3-phosphate dehydrogenase (GPD) 1, and growth differentiation factor (GDF) 8 show decreased expression in skeletal muscle following gastric bypass and increased expression in obese skeletal muscle. Shown is quantitative RT-PCR in the test group (before and after gastric bypass surgery in morbidly obese women) and validation group (lean and morbidly obese women). The target gene quantity was divided by the endogenous control quantity to obtain a normalized target value. *A*: GRB14 expression, *B*: GPD1 expression, *C*: GDF8 expression; values are means ± SD. *Significant difference at $P < 0.05$ (before vs. after gastric bypass surgery; lean vs. morbidly obese women).

signal generation from the microarrays (MAS5.0, RMA, and dCHIP difference model). It is well established that very different microarray data interpretations are obtained with different probe set algorithms from the same microarray data (39–41). Our use of concordance for three distinct probe set algorithms would be expected to detect highly specific (robust) gene expression changes, although this approach is also relatively insensitive (stringent). Finally, transcripts surviving the validation data sets and all three probe set algorithms were further validated by RT-PCR.

There is considerable pre-existing functional data supporting involvement of the three transcripts (GRB14, GPD1, and GDF8) and the proposed insulin-responsive regulatory network (Fig. 2) in metabolism. GRB14 is a member of the GRB7 family of adaptor proteins and specifically expressed in insulin target tissues such as skeletal muscle and other insulin-sensitive organs (4). It binds to the insulin receptor and inhibits insulin signal transduction through repression of tyrosine phosphorylation (1). Thus GRB14 is considered as a negative regulator of insulin signaling. Indeed, GRB14-deficient mice showed improvements in insulin-stimulated glucose uptake and glycogen synthesis in skeletal muscle as well as significant reductions in body weight (6). No previous reports have addressed GRB14 expression in skeletal muscle of obese individuals. Here, we describe the novel finding that GRB14 expression is significantly elevated in skeletal muscle of morbidly obese individuals and, furthermore, that GRB14 levels decrease with weight loss (gastric bypass). Consistent with previous mouse studies, we found that decreased GRB14 expression was associated with increased insulin action in both our gastric bypass and cross-sectional obesity groups. Although insulin action was not significantly reduced in morbidly obese individuals in our validation group, it showed the same trend with previously reported data from our laboratory in which we demonstrated the presence of insulin resistance in obese populations (7, 8). Thus our data suggest that GRB14 may be altered by energy balance or metabolic states and may play a key role in obesity-induced insulin resistance and weight loss-induced increase in insulin sensitivity in skeletal muscle.

GPD1 is a NAD(H)-dependent cytosolic enzyme, catalyzing the conversion of dihydroxyacetone phosphate derived from glucose to glycerol-3-phosphate, which is finally acylated to form triglycerides. GPD1, therefore, makes an important link between the glycolytic pathway and triglyceride biosynthesis and, thereby, carbohydrate and lipid metabolism. Although this reaction is reversible, production of glycerol-3-phosphate is favored under normal physiological conditions (3). In fact, it has been demonstrated that chronic glucose oversupply leads to increased triglyceride accumulation in skeletal muscle, likely via the GPD1 pathway (25, 26). Although increased GPD1 expression and activity have been reported in adipocytes of obese human and rats (28, 42, 45), no data are available for GPD1 expression in muscle. Here, we report that GPD1 expression is increased in skeletal muscle of morbidly obese individuals and that this increase can be reversed by weight loss. GPD1 is responsible for triglyceride synthesis because fatty acids are esterified to the hydroxyl groups of the glycerol molecules to form triglyceride (23, 42). Thus our findings

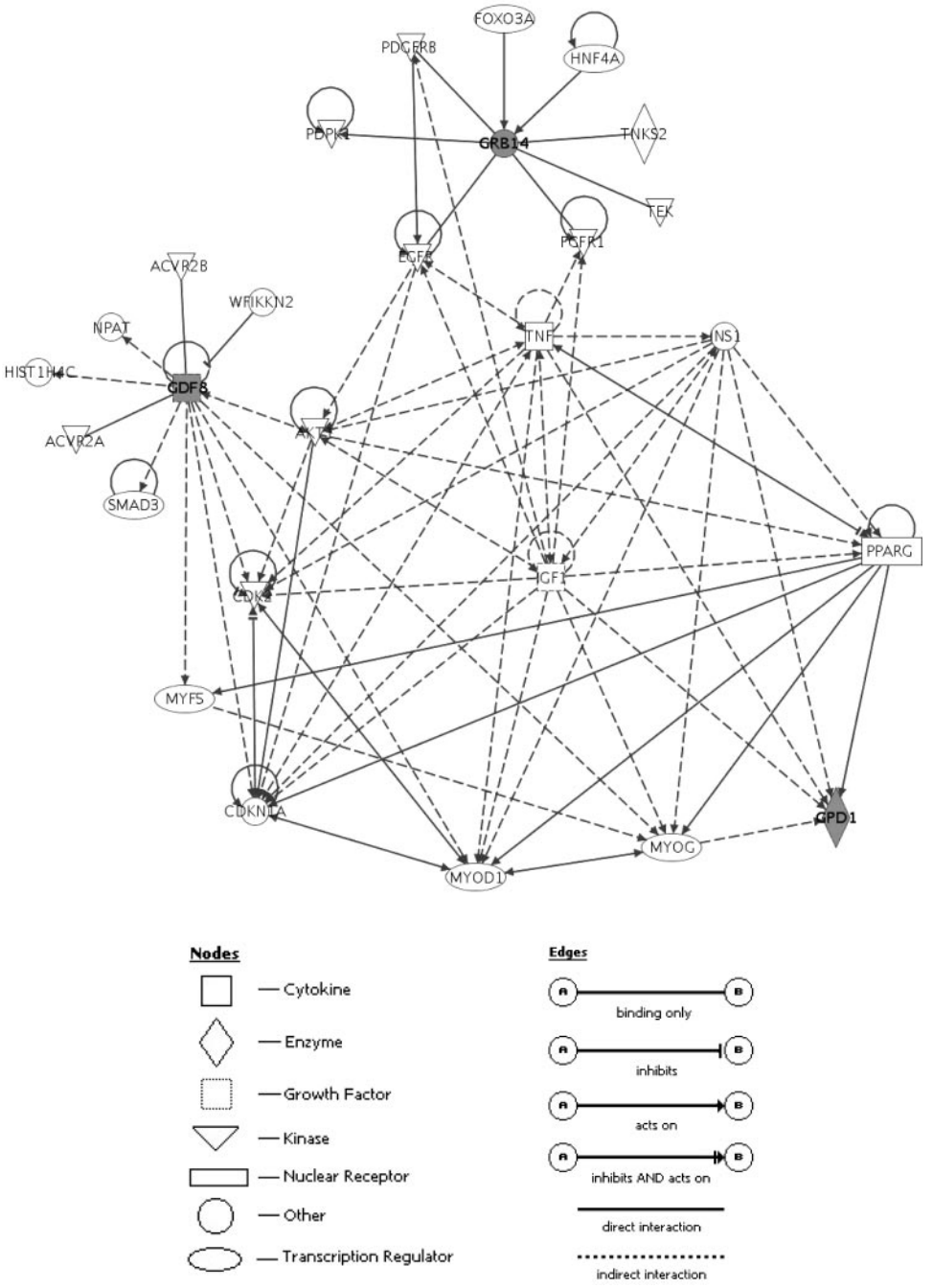

Fig. 2. An insulin regulatory network including weight loss-responsive genes, GRB14, GPD1, and GDF8. Shown is the simplified network derived from 6 of the top-ranked networks (score >2; confidence >99%) detected by the Ingenuity Pathway Analysis program. This network focuses on GRB14, GPD1, and GDF8 and others showing direct literature-supported relationships to these genes. Loci for GRB14, GPD1, and GDF8 indicate significantly downregulated genes by weight loss.

suggest that increased GPD1 expression in the muscle of obese individuals may play a role in the increased conversion of excess digestible carbohydrates into triglyceride. This hypothesis is supported by the fact that intramuscular triglyceride is increased in obesity and decreased by weight loss (11, 21). Additionally, triglyceride and intramuscular lipid accumulation in skeletal muscle have been strongly associated with insulin resistance (21). Taken together, these results suggest that GPD1 plays an important role in the accumulation of triglyceride and lipid in skeletal muscle and, thereby, development of insulin resistance in obesity.

GDF8, also called myostatin, is a member of the transforming growth factor-β family of signal transduction proteins that negatively regulates skeletal muscle mass through antianabolic effects (10). GDF8-deficient mice have increased muscle mass along with a significant reduction in fat accumulation, despite normal food intake (27). On the other hand, GDF8 expression in skeletal muscle has been shown to be increased by nutrient supply (22). More interestingly, a recent study found that serum withdrawal conditions resulted in dramatic reduction in GDF8 expression in cultured murine myoblasts (36). Consistent with these findings, a human study recently reported that GDF8 expression is decreased with weight loss in skeletal muscle of obese individuals (31). These results correspond to our data showing that GDF8 expression is increased in skeletal muscle of morbidly obese individuals and decreased by weight

loss, suggesting a potential role of GDF8 in regulation of energy homoeostasis in skeletal muscle. Consistent with this model, genetically engineered GDF8 null mice also showed suppressed glucose metabolism and increased insulin signaling (30, 49).

A key finding in our study is the placement of GRB14, GPD1, and GDF8 into the same molecular network (Fig. 2). This network shows functional relationships between these genes and other gene products based on direct literature-supported biological interactions. Indeed, this network includes signaling proteins that are well known to be involved in insulin signaling pathways and cellular metabolism (e.g., AKT1, IGF1, TNF, PPARG, and INS). Many of these signaling molecules have been shown to be modulated by nutritional status or obesity state in adipocytes (16, 46). It is important to note that not all members of the pathway were found to be differentially regulated at the transcriptional level in this current study. However, our validation and statistical approach was designed to be stringent and the sample numbers small (particularly in the gastric bypass); both of these would result in decreased sensitivity for transcriptional regulation. More importantly, the pathways shown are considered primarily signaling pathways regulated at the protein level. Thus expression profiling is a blunt instrument by which to sensitively monitor alterations in these pathways.

Although there are limited studies of the proposed network in muscle (Fig. 2), there is growing support for the importance of network members in energy balance and metabolism in fat tissue and adipocytes. For example, it has been shown that PPARG increases GPD1 expression in adipocytes of mice (34), and PPARG expression increased in adipocytes of obese individuals and decreased with weight loss (46). Another example is that TNF negatively regulates PPARG and GPD1. TNF has been shown to inhibit adipogenesis, which is accompanied by suppression of PPARG and GPD1 expression in cultured human adipocytes (17, 48). Interestingly, TNF is also known to cause insulin resistance in adipocytes by decreasing insulin signaling pathway through in part inhibition of AKT phosphorylation (43). Because elevated TNF expression is often observed in adipocytes of obese individuals, it has been hypothesized that TNF plays a role in development of obesity-linked systemic insulin resistance (16). Other important relationships in the network may include that AKT phosphorylation is increased in GDF8 knockout mice and decreased by overexpression of GRB14 (24, 49). However, it should be noted that this network was built using all available literature knowledge, without selection for species or tissue. Thus edges (biological relationships) between nodes (gene products) in the network would need to be further studied specifically in human skeletal muscle.

In summary, the results of the present study clearly show that GRB14, GPD1, and GDF8 expressions in skeletal muscle are increased with obesity and decreased by weight loss, along with increased insulin action. These are novel findings, particularly in skeletal muscle of morbidly obese individuals without diabetes. In addition, these genes are revealed to be involved in the same regulatory network including AKT1, IGF1, TNF, PPARG, and INS. Therefore, our data define key weight loss-responsive genes and regulatory pathway involved in the development of insulin resistance in obesity, suggesting that inhibition of this pathway may be useful for treatment of obesity and insulin resistance.

GRANTS

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