Proteome Analysis of Skeletal Muscle From Obese and Morbidly Obese Women

Dustin S. Hittel,[1] Yetrib Hathout,[1] Eric P. Hoffman,[1] and Joseph A. Houmard[2]

Obesity-related diseases such as the metabolic syndrome and type 2 diabetes originate, in part, from the progressive metabolic deterioration of skeletal muscle. A preliminary proteomic survey of rectus abdominus muscle detected a statistically significant increase in adenylate kinase (AK)1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and aldolase A in obese/overweight and morbidly obese women relative to lean control subjects. AK1 is essential for the maintenance of cellular energy charge, and GAPDH and aldolase A are well known glycolytic enzymes. We found that muscle AK1 protein and enzymatic activity increased 2.9 and 90%, respectively, in obese women and 9.25 and 100%, respectively, in morbidly obese women. The total enzymatic activity of creatine kinase, which also regulates energy metabolism in muscle, was shown to increase 30% in obese/overweight women only. We propose that increased protein and enzymatic activity of AK1 is representative of a compensatory glycolytic drift to counteract reduced muscle mitochondrial function with the progression of obesity. This hypothesis is supported by increased abundance of the glycolytic enzymes GAPDH and aldolase A in obese and morbidly obese muscle. In summary, proteome analysis of muscle has helped us better describe the molecular etiology of obesity-related disease. *Diabetes* 54:1283–1288, 2005

A loss of systemic glucose and lipid homeostasis, which involves multiple organ systems, underlies obesity-related diseases such as the metabolic syndrome and type 2 diabetes. Specifically, defects are thought to occur in key metabolic processes that direct the entry, storage, and oxidative catabolism of glucose and fatty acids (1–4). It is now widely accepted that skeletal muscle plays a considerable role in regulating levels of circulating glucose and lipids and that this capacity is significantly depressed in obese and/or inactive individuals (2,3,5). In fact, recent studies of human skeletal muscle have revealed a decrease in the percentage of type I (oxidative) muscle fibers and parallel decreases in muscle glucose transport and lipid oxidation in obese individuals with and without type 2 diabetes relative to lean control subjects (2,3,5,6). These observations indicate significant metabolic dysfunction in muscle from obese individuals that contributes to the development of glucose intolerance, dyslipidemia, and the eventual onset of type 2 diabetes.

New techniques for the unbiased ascertainment of complex molecular events in diseased, damaged, and exercise-adapted skeletal muscle include the use of oligonucleotide microarrays and proteomic analysis using mass spectroscopy (7–10). Although there have been a number of microarray studies of diabetic and obese muscle across a variety of animal and human experimental models, the only real consensus is the apparent downregulation of genes encoding oxidative metabolism enzymes with obesity and diabetes (11). Although mRNA transcript levels respond acutely to metabolic and mechanical disruptions within muscle, they do not always immediately translate into changes in the abundance of their corresponding protein products (7,9). Consequently, there is growing support for the use of protein profiling techniques to help produce a more comprehensive molecular etiology of muscle remodeling and disease states.

The standardization of two-dimensional electrophoresis and increasingly rapid protein identification using mass spectroscopy has made the high-throughput assessment of protein expression changes both practical and cost-effective (9,12). In the present study, we analyzed skeletal muscle cytosolic proteins from lean, obese/overweight, and morbidly obese women to identify enzymes that may account for defects in muscle metabolism. This preliminary survey of soluble cytosolic extracts identified adenylate kinase (AK)1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and aldolase A as being preferentially expressed in muscle extracts from obese and morbidly obese individuals relative to lean control subjects. We feel that this discovery provides mechanistic insights into defects in muscle metabolism that underlie the progression of obesity-related metabolic diseases.

RESEARCH DESIGN AND METHODS

Eighteen women participated in this investigation, consisting of 6 normal-weight (age 45.1 ± 3.1 years; BMI 23.8 ± 0.58 kg/m^2; and homeostasis model assessment [HOMA] 1.10 ± 0.26), 6 overweight/obese (age 44.0 ± 2.8 years; BMI 30.2 ± 0.81 kg/m^2; HOMA 1.6 ± 0.47), and 6 extremely obese (age 37.9 ± 3.3 years; BMI 53.8 ± 3.5 kg/m^2; HOMA 3.6 ± 1.1) patients undergoing abdominal surgery, primarily gastric bypass and total abdominal hysterectomy (3). Research participants were categorized into their respective groups

From the [1]Research Center for Genetic Medicine, Children's National Medical Center, Washington, DC; and the [2]Department of Exercise and Sport Science and the Human Performance Laboratory and Diabetes/Obesity Center, East Carolina University, Greenville, North Carolina.

Address correspondence and reprint requests to Joseph A. Houmard, Department of Exercise and Sport Science and the Human Performance Laboratory and Diabetes/Obesity Center, East Carolina University, Greenville, NC 27858. E-mail: houmardj@mail.ecu.edu.

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AK, adenylate kinase; AMPK, AMP-activated protein kinase; CK, creatine kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HOMA, homeostasis model assessment; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; pI, isoelectric point; TOF, time-of-flight.

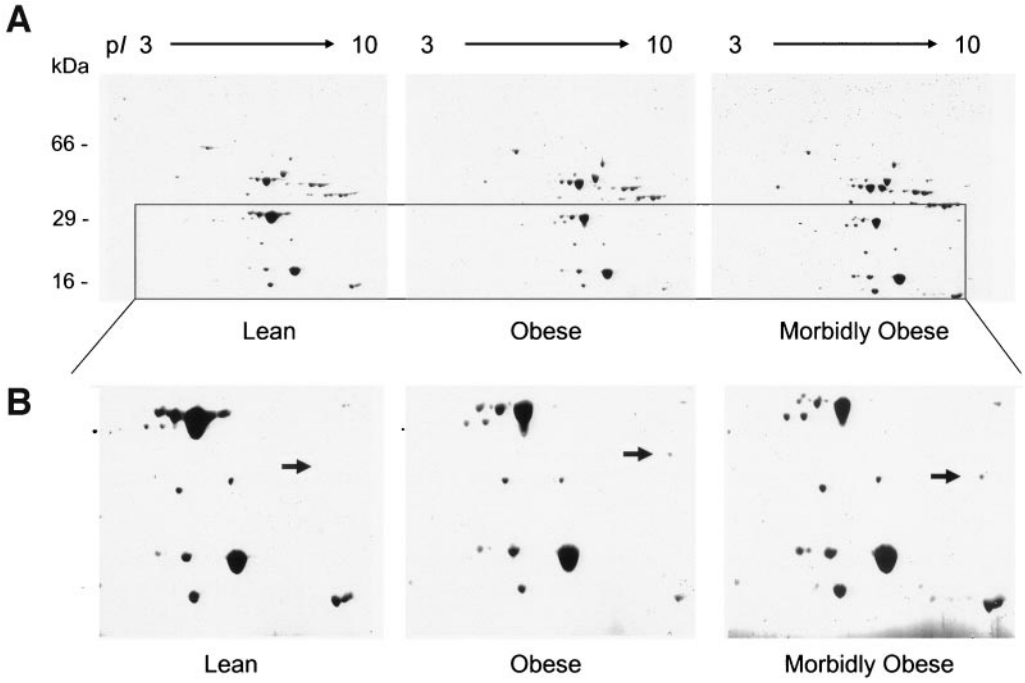

FIG. 1. Two-dimensional gel patterns of cytosolic proteins from lean, obese/overweight, and morbidly obese rectus abdominus muscle. *A*: Representative two-dimensional protein profiles of lean obese/overweight and morbidly obese muscle in which 100 μg of protein extract was separated by pI on a pH 3–10 gradient strip and then by molecular weight on an 8–15% SDS-PAGE gel. *B*: An expanded view of the same two-dimensional gels showing increased AK1 expression in obese and morbidly obese women. The location of AK1 within the gel (➡) is consistent with the known molecular weight (22 kDa) and pI (~8) of this protein. Proteins were visualized using a colloidal Coomassie stain.

on the basis of BMI and the classifications of overweight and obesity set forth by the National Institutes of Health (13). BMI criteria for the normal-weight, overweight/obese, and extremely obese subjects were 24.9, 25.0–34.9, and ≥40 kg/m^2, respectively. The HOMA for insulin resistance and β-cell function was calculated from fasting plasma glucose and insulin concentrations (14). The experimental protocol was approved by the East Carolina University Policy and Review Committee on Human Research and was in accordance with the principles expressed in the Declaration of Helsinki. Informed consent was obtained from all patients. None of the subjects had any diseases or were taking any medications known to alter carbohydrate or lipid metabolism.

Digitonin extraction of cytosolic proteins. A sample of rectus abdominus tissue was obtained during surgery and treated as described previously (3). Cytosolic proteins were extracted from 25–30 mg of nitrogen-pulverized tissue using 500 μl of digitonin extraction buffer (10 mmol/l PIPES, 0.015% digitonin, 300 mmol/l sucrose, 100 mmol/l NaCl, 3 mmol/l $MgCl_2$, 5 mmol/l EDTA, and 1 mmol/l protease inhibitor [phenylmethylsulfonyl fluoride], pH 6.8) with gentle inversion at 4°C for 20 min (12). The cytosolic fraction was separated from the rest of cell pellet by centrifugation at 500*g* for 10 min. Cell pellets were flash frozen and stored at −80°C for future analysis. The supernatant containing cytosolic protein was transferred to a clean tube and centrifuged at 8,000*g* for 20 min. Protein concentration was determined in the final supernatant using the Bio-Rad protein assay dye reagent, following the manufacturer's instructions (Bio-Rad, Hercules, CA). The sample was then stored in aliquots of 500 μl in microcentrifuge tubes at −80°C.

Two-dimensional gel electrophoresis. Total cytosolic protein (100 μg) was buffer-exchanged (Tris-HCl, pH 7.4), concentrated, and then solubilized in isoelectric focusing rehydration buffer using a solution consisting of 7 mol/l urea, 2 mol/l thiourea, 1% (wt/vol) ASB-14, 0.5% (vol/vol) Triton X-100, 40 mmol/l Tris base, 30 mmol/l dithiothreitol, and 0.5% (vol/vol) Biolytes 3–10 (Amersham Life Sciences, Piscataway, NJ). Samples were then loaded onto an 11-cm, pH 3–10 IPG (Immobilized pH Gradient) strip (Bio-Rad) and left under mineral oil overnight. IEF was conducted for a total of 100,000 Vh (volt-hours) using a Multiphor II electrophoresis unit (Amersham Pharmacia Biotech) at 20°C. Before the second dimension, strips were incubated for 15 min in equilibration buffer (6 mol/l urea, 2% [wt/vol] SDS, 20% [vol/vol] glycerol, 0.15 mol/l bis-Tris, and 0.1 mol/l HCl) at 20°C, first with 65 mmol/l dithiothreitol (reducing agent) and second with 243 mmol/l idoacetamide (alkylating agent). Equilibrated strips were inserted into 8–16% Criterion SDS-PAGE gels (Bio-Rad) and sealed with 1% agarose (Bio-Rad) in running buffer, and two-dimensional gels were run at 200 V for 2 h (Bio-Rad Protean II Xi). After protein separation, the gel slabs were fixed for 30 min in a solution of methanol:water:acetic acid (45:50:5, vol/vol/vol) followed by three washes for 5 min in deionized water. Staining was preformed with Bio-Safe colloidal Coomassie Blue G-250 (Bio-Rad) for 1 h, followed by ample rinsing with deionized water until the desired contrast was obtained.

Two-dimensional gel quantification and analysis. Two-dimensional gels were analyzed using the Z3 software package (Compugen, Tel Aviv, Israel) using settings recommended by the manufacturer. Isoelectric point (pI) and molecular mass were calculated based on the position of each protein in the IPG strip and second dimension gel. Differential expressed proteins, average intensity values, and standard deviations were calculated for each spot and normalized to corresponding spots on the lean master gels.

Preparation and mass spectrometry identification of proteins. Spots were excised with the tip of a clean polypropylene pipette and transferred to a microcentrifuge tube containing 100 μl of deionized water. Tryptic digestion was preformed as described previously (9), and peptides were extracted and then dried by vacuum centrifugation. Dried peptides were subsequently dissolved in 10 μl of 0.1% trifluoroacetic acid and desalted using C_{18} ZipTip micropipette tips (Millipore, Bedford, MA) following the manufacturer's instructions. Aliquots of peptide solutions were spotted on the matrix-assisted laser desorption/ionization (MALDI) plate. Mass spectrometry (MS) and tandem mass spectrometry (MS/MS) analyses were performed on a 4700 ABI time-of-flight (TOF)-TOF mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a Nd:YAG 200 Hz laser. The instrument was operated with delayed extraction in reflectron positive ion mode. A mixture of standard peptides was used to externally calibrate the instrument. Protein identification was carried out using GPS explorer software (Applied Biosystems) and the MASCOT database. Both MS and MS/MS data were used for protein identification.

Enzymatic assays. Standard assays for AK1 and creatine kinase (CK) were performed using established protocols that have been described previously (9,15,16). Briefly, frozen muscle samples were pulverized in liquid nitrogen and then extracted in a homogenization buffer containing 150 mmol/l NaCl, 60 mmol/l Tris-HCl, pH 7.5, 5 mmol/l EDTA, 0.2% Triton X-100, and a protease inhibitor cocktail. Total activities were measured using a coupled enzyme assay in a 96-well Labsystems Multiskan MCC/340 microplate reader (Fisher Scientific) at 340 nm. The number of subjects used to determine enzymatic activity was fewer than those used to assess two-dimensional gel protein patterns because the amount of tissue available was limiting. In addition, because muscle was obtained from some individuals undergoing hysterectomy (for various reasons) and others undergoing gastric bypass, we feel that this could be a potential confounding variable in our interpretation of the data.

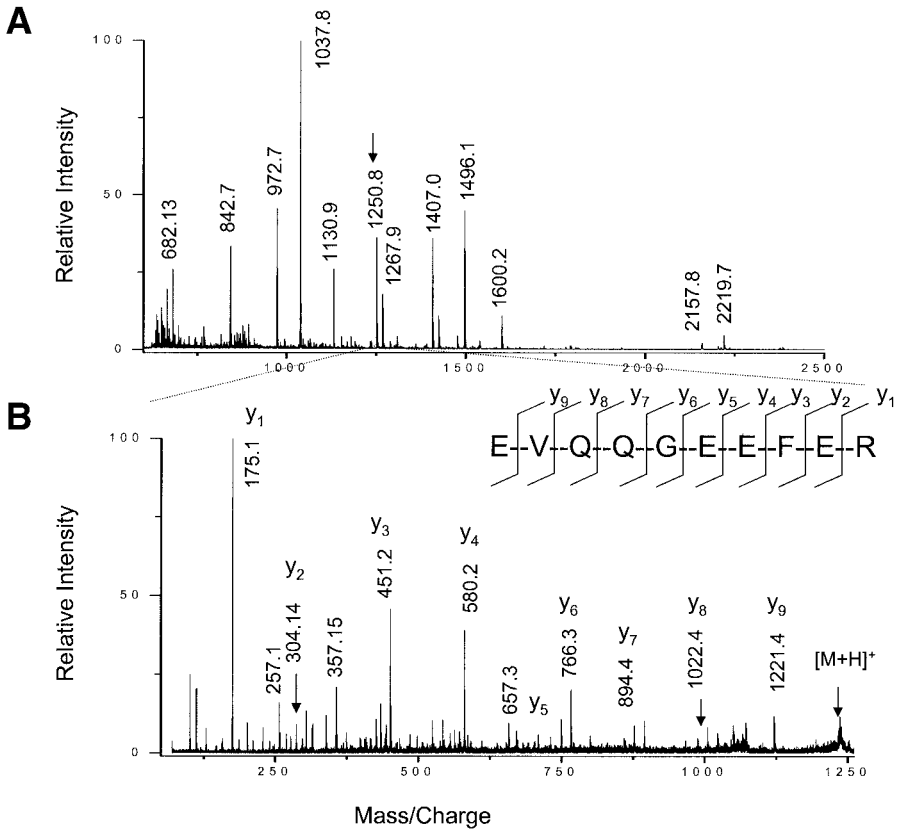

FIG. 2. Identification of AK1 by MALDI-TOF. *A*: MALDI-MS tryptic mass fingerprint of AK1 from in-gel digestion of a spot using Coomassie stain. *B*: MALDI-TOF fragmentation of a unique peptide ion at *m/z* 1250.8 Da from the same tryptic in-gel digest reveals proper identification of this peptide, which is unique to AK1.

Statistical analysis. Results of protein quantification and enzymatic assays were expressed as means ± SE. Student's *t* test was used for all statistical analyses, and $P \leq 0.05$ was considered for biological significance. Tests for statistical significance were corrected for multitesting

RESULTS

Proteomic analysis of obese skeletal muscle. Cytosolic proteins were extracted from rectus abdominus muscle of lean, obese/overweight, and morbidly obese women and analyzed for significant differences with the progression of obesity. Protein profiles were analyzed using two-dimensional gel electrophoresis coupled to mass spectroscopy to identify differentially expressed proteins. A sequential extraction method was used because it was thought that the proportionately high content of contractile proteins in skeletal muscle would introduce significant variability, making the qualitative and quantitative ascertainment of differential expression difficult. The broad pI range of 3–10 was chosen to include as many proteins as possible. Figure 1*A* shows representative two-dimensional protein profiles of lean ($n = 6$), obese/overweight ($n = 6$), and morbidly obese ($n = 6$) rectus abdominus cytosolic proteins (100 μg/gel). These two-dimensional gels exhibited highly reproducible protein profiles between different individuals and different BMI categories, indicating a low level of intraindividual and experimental variation that could confound data interpretation. No significant changes in protein abundance were detected between the individuals in each BMI category.

Comparative visual and software-guided analyses of these two-dimensional protein profiles revealed a consistent increase in the abundance of a number of proteins in obese and morbidly obese women, the most striking of which was a ~22-kDa protein with a pI of ~8 that increased 2.9-fold in obese/overweight and 9.25-fold in morbidly obese muscle relative to lean control subjects. Figure 1*B* is an expanded view of the region of the two-dimensional gels in which increased expression of this protein (subsequently identified as AK1) was clearly shown in obese/overweight and morbidly obese muscle.

Differentially expressed and control protein spots of various intensities were excised, digested in-gel with trypsin, and then analyzed using MALDI-TOF for initial protein identification. As an example, the peptide masses obtained from the AK1 MALDI-MS were used to search the entire genome database using the MASCOT search engine (Matrix Science, Boston, MA). In addition, MALDI-TOF fragmentation of a unique peptide ion at m/z 1,250.8 Da from the same tryptic in-gel digest revealed that this peptide is unique to AK1 (Fig. 2*B*). The observed pI and molecular weight of the identified protein were consistent with the calculated values of AK1 (pI 8.73, molecular weight 21,735). Table 1 is a list of differentially expressed and control proteins, their calculated fold change, fold change *P* values (relative to lean control subjects), theoretical pI, and molecular weight. In addition to AK1, the glycolytic proteins GAPDH and aldolase A were significantly increased in obese/overweight and morbidly obese muscle relative to lean control individuals. It was particularly interesting that GAPDH was found to increase because it is often used as a internal normalization control. In

TABLE 1
Differentially expressed and control proteins, their calculated fold change, fold change *P* values, theoretical and experimental pI, and molecular weight

	Lean-to-obese ratio	Lean–to–morbidly obese ratio	*P* value*	M_r (t)	M_r (ex)	pI (t)	pI (ex)
AK1	2.90	9.25	0.007	21.7	23.2	8.7	9.1
Aldolase A	1.76	2.60	0.001	39.3	41.2	8.4	8.2
GAPDH	1.81	2.00	0.005	35.9	32.2	6.6	7.0
Myoglobin	1.12	1.41	0.065	17.1	17.2	7.3	7.6
Carbonic anhydrase 3	0.93	0.61	0.072	29.4	28.9	6.9	6.9
Fatty acid–binding protein 3	1.78	3.75	0.078	14.7	14.3	6.3	6.1
Pyruvate kinase M	1.70	1.79	0.088	57.8	58.1	8.0	8.1
Prostatic-binding protein	0.81	1.28	NS	20.9	22.3	7.4	7.6
β-Enolase	1.26	1.25	NS	46.9	48.1	7.7	7.5
Actin	0.59	1.23	NS	41.7	43.1	5.3	5.1
CK M	0.92	0.85	NS	43.1	45.2	6.8	6.9
Albumin	1.60	0.71	NS	69.4	66.1	5.9	5.7
Hemoglobin A	0.39	0.08	NS	15.1	15.0	8.7	7.8

*Calculated relative to lean control subjects. ex, experimental, t, theoretical.

addition, a number of proteins exhibited strong differential expression trends such as myoglobin, fatty acid-binding protein 3, and pyruvate kinase, which increased with increased BMI, and carbonic anyhydrase 3 decreased with increasing BMI.

AK activity. AK is an important enzyme in the regulation of cellular energy metabolism, particularly in tissues that may experience rapid changes in energy flux (17). To determine whether changes in AK1 protein reflect changes in AK total enzymatic activity, we measured AK activity in muscle of four lean, four obese/overweight, and four morbidly obese women (Table 2).

Total lean muscle AK activity was significantly lower in muscle from lean women (1,430 ± 376 nmol ATP · min^{-1} · mg $protein^{-1}$) than in obese/overweight individuals (2,668 ± 387 nmol ATP · min^{-1} · mg $protein^{-1}$) by 90% (P = 0.02), whereas in morbidly obese muscle, AK activity was elevated (2,873 ± 555 nmol ATP · min^{-1} · mg $protein^{-1}$) 100% (P = 0.04) compared with the lean control subjects. The disparity between the increase in protein content and enzymatic activity for AK1 may be due to previously described difficulties in the estimation of total muscle protein and/or preferential enrichment of AK1 using the digitonin extraction method.

CK activity. As with AK, CK contributes significantly to the balance of nucleotide ratios and to the regulation of energy metabolism in skeletal muscle (17). It has also been demonstrated that CK activity is reciprocally responsive to the activity and amount of AK1 and vice versa in human and mouse skeletal and cardiac muscle (9,18–21). We therefore decided to assay total CK activity in skeletal muscle from the same subject cohort to establish whether a similar reciprocal trend could be observed (Table 2). Total CK activity from lean muscle (n = 4) was 7,826 ± 603 nmol ATP · min^{-1} · mg $protein^{-1}$, whereas total CK activity increased by 30% to 9,830 ± 440 nmol ATP · min^{-1} · mg $protein^{-1}$ in obese muscle (n = 4, P = 0.02) and then dropped back to 8,907 ± 270 nmol ATP · min^{-1} · mg $protein^{-1}$ in morbidly obese muscle.

DISCUSSION

Contributing to the current epidemic levels of obesity in the population are our permissive or ad libitum eating habits and declining physical activity levels. It has been speculated that these environmental factors unmask a genetic susceptibly to obesity, particularly in those individuals with a thrifty metabolic genotype (11,13). Regardless of the cause, obesity is an independent risk factor for the development of cardiovascular disease, insulin resistance, and the eventual onset of type 2 diabetes (13).

Skeletal muscle contributes significantly to the maintenance of systemic substrate and energy balance and, when this balance is lost, to the pathophysiology of obesity-related diseases (2–6,10,11,22,23). This makes muscle an attractive target organ for the identification of diagnostic biomarkers for the onset and progression of these disease states. The findings of this study provide additional information about changes in metabolic enzymes in muscle from obese individuals using protein profiling.

We detected a statistically significant increase in the abundance and activity of AK1 and in the abundance of the glycolytic proteins aldolase A and GAPDH in muscle from obese/overweight and morbidly obese women relative to

TABLE 2
Total AK and CK activity in lean, obese/overweight, and morbidly obese muscle

	Lean	Obese	Morbidly obese
AK activity	1,430.868 ± 376.469	2,668.81 ± 387.705*	2,873.794 ± 555.878*
CK activity	7,826.21 ± 603.86	9,830.65 ± 440.3*	8,907.26 ± 270.84

Data are means ± SE. Units of activity are nmol ATP · min^{-1} · mg $protein^{-1}$. Total AK activity increased significantly in obese/overweight and morbidly obese muscle extracts relative to lean control subjects. Total CK activity increased in obese/overweight muscle only relative to lean control subjects. Results were obtained in duplicate from total muscle homogenate of four lean, four obese, and four morbidly obese women. *Significant difference between relative to lean control muscle.

lean control subjects (Table 1). AK catalyzes the near-equilibrium interconversion of two ADPs to an ATP and an AMP and is a key enzyme in the maintenance of intracellular adenine nucleotide ratios, particularly in tissues with a high ATP flux rate (17). Of the three known AK isoenzymes, the cytosolic is most highly expressed in muscle, whereas AK2 and AK3 are restricted to the intermembrane space and the mitochondrial matrix, respectively (17,21). AK is integrated with CK and the glycolytic phosphotransferases in large intracellular flux networks, the purpose of which is to rapidly transfer high-energy phosphate groups from the site of production to the site of use (17). Mouse AK knockout models and specific AK inhibitor studies have demonstrated that CK and AK reciprocally compensate for each other (18,19). AK1 knockout mice also exhibit molecular and structural adaptations, most notably an increased mitochondrial volume, increased glycolytic enzymes, and increased CK activity (21,24,25). It is speculated that these adaptations enhance the surface area of ATP-producing structures to overcome the diffusion barrier for ATP imposed by the disruption of intracellular phosphotransferase networks. This is relevant to our study because inactive and obese muscle exhibit both reduced mitochondrial volume and function and increased glycolytic enzymes (10,22,23).

It was initially surprising to us that CK activity increased by 30% in obese/overweight women because we expected that lower CK activity would follow the elevation of AK activity in obese muscle. An increase in CK activity was also unexpected because we detected no significant change in CK M protein (Table 1); however, quantification of this spot was difficult because it was present at saturating levels (data not shown). It is also possible that increased CK activity in muscle of obese women is the result of post-translational modifications or allosteric effects on enzyme activity. We surmise that reduced muscle mitochondrial content and function with increasing obesity would lower the total cellular ATP yield, necessitating increased AK, CK, and glycolytic enzymes (20). It is possible also that increases in AK1, aldolase A, and GAPDH may be associated with reported differences in muscle fiber type with obesity (4,22). This is supported by the fact that AK1 is thought to be preferentially expressed in glycolytic (fast-twitch) muscle fibers (21).

The production of AMP is particularly important because it is a potent allosteric activator of numerous glycolytic enzymes and of AMP-activated protein kinase (AMPK) (26,27). Activated AMPK plays a key role in the regulation of energy homeostasis, particularly in regulating the uptake of both glucose and fatty acids (26,27). Because AK activity is primarily responsible for the production of AMP in actively contracting skeletal muscle, we speculate that increased AK activity could lead to altered intracellular AMP levels in muscle from obese and morbidly obese women. In the near future, we plan to investigate the role of AMPK in regulating downstream metabolic targets such as acetyl-CoA carboxylase and the regulation of gene expression in muscle from obese and morbidly obese individuals.

In summary, AK1, aldolase A, and GAPDH proteins are increased in obese/overweight and morbidly obese skeletal muscle of women relative to lean control subjects. It is hypothesized that these changes compensate for the progressive decrease in muscle mitochondrial function in obese individuals, which over time, and in the absence of dietary and exercise intervention, contributes to the loss of glucose and lipid homeostasis and to the eventual development of obesity-related diseases such as type 2 diabetes.

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