Proteomics Analysis of Human Skeletal Muscle Reveals Novel Abnormalities in Obesity and Type 2 Diabetes

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OBJECTIVE—Insulin resistance in skeletal muscle is an early phenomenon in the pathogenesis of type 2 diabetes. Studies of insulin resistance usually are highly focused. However, approaches that give a more global picture of abnormalities in insulin resistance are useful in pointing out new directions for research. In previous studies, gene expression analyses show a coordinated pattern of reduction in nuclear-encoded mitochondrial gene expression in insulin resistance. However, changes in mRNA levels may not predict changes in protein abundance. An approach to identify global protein abundance changes involving the use of proteomics was used here.

RESEARCH DESIGN AND METHODS—Muscle biopsies were obtained basally from lean, obese, and type 2 diabetic volunteers ($n = 8$ each); glucose clamps were used to assess insulin sensitivity. Muscle protein was subjected to mass spectrometry–based quantification using normalized spectral abundance factors.

RESULTS—Of 1,218 proteins assigned, 400 were present in at least half of all subjects. Of these, 92 were altered by a factor of 2 in insulin resistance, and of those, 15 were significantly increased or decreased by ANOVA ($P < 0.05$). Analysis of protein sets revealed patterns of decreased abundance in mitochondrial proteins and altered abundance of proteins involved with cytoskeletal structure (desmin and alpha actinin-2 both decreased), chaperone function (TCP-1 subunits increased), and proteasome subunits (increased).

CONCLUSIONS—The results confirm the reduction in mitochondrial proteins in insulin-resistant muscle and suggest that changes in muscle structure, protein degradation, and folding also characterize insulin resistance. ***Diabetes*** **59:33–42, 2010**

Skeletal muscle insulin resistance is critical to the pathogenesis of type 2 diabetes (1) and the metabolic syndrome (2). Numerous investigations over the past 4 decades have revealed an array of abnormalities in insulin action in skeletal muscle of obese and type 2 diabetic patients and have led to a better understanding of the molecular pathways involved in insulin and cytokine signaling, inflammation, and gene expression in skeletal muscle. In insulin-resistant muscle, there are defects in insulin signaling (3,4), activities of metabolic enzymes (5,6), and regulation of gene expression (6–9), among others.

Often these studies are highly focused. However, several groups have used approaches that give a more global picture of abnormalities, such as oligonucleotide microarray analysis (7–9). The value of global analysis is that it can yield a view of patterns of changes in pathways and generate new hypotheses that serve as foci for more detailed analysis. Global gene expression analyses conducted in insulin-resistant muscle have shown a pattern of reduced expression of nuclear-encoded mitochondrial genes (7–9). Such findings have spurred new avenues of research and fit well with observations of mitochondrial dysfunction in insulin resistance (10).

However, changes in mRNA levels for a gene may not be mirrored by changes in abundance of the proteins encoded by that gene, which are a closer reflection of the level of activity of a biological pathway. The advent of sensitive and accurate mass spectrometers has made mass spectrometry a valuable tool for biologists interested in protein identification and regulation of protein abundance in complex biological mixtures. Mass spectrometry–based proteomics also is a valuable tool for quantifying differences in protein abundance between, for example, cells cultured in vitro under different conditions or treatments (11–14). Assessment of differences in protein abundance using mass spectrometry analysis of human tissues, where in vivo stable isotope labeling of proteins is impractical, presents special challenges (15–18).

The challenge of comparative proteomics in small human tissue samples has resulted so far in studies that have revealed only a few differences in protein abundance in insulin-resistant muscle (18–20). These challenges were addressed here through the use of an initial separation technique of one-dimensional SDS-polyacrylamide gels, with protein assignment using high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry (HPLC-ESI-MS/MS) and with quantification using a normalized spectral abundance factor (NSAF) method (21–23). The purpose of this study was to develop a global view of protein abundance changes in insulin resistance, in order to provide data that can serve as a basis for generation of novel hypotheses concerning the molecular mechanisms of insulin resistance. Using these techniques, we assigned a total of 1,218 proteins in small percutaneous muscle biopsies taken from metabolically characterized lean and obese nondiabetic volunteers and type 2 diabetic patients. We used normalized spectral abundance factor methods to compare the abundance of

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400 of the proteins that were assigned in at least half of all subjects studied. These analyses reveal unexpected new patterns of changes in protein abundance in insulin-resistant muscle, including alterations in abundance of proteins involved in cytoskeletal structure and assembly and increases in proteasome and chaperone subunit abundance.

RESEARCH DESIGN AND METHODS

Lean nondiabetic control subjects, obese nondiabetic subjects, and patients with type 2 diabetes ($n = 8$ each) were studied. Studies were approved by the Institutional Review Board of the University of Texas Health Science Center at San Antonio and Arizona State University. After giving informed written consent, each volunteer underwent a medical history, physical examination, screening laboratory tests, and a 75-g oral glucose tolerance test and underwent a euglycemic-hyperinsulinemic clamp with basal muscle biopsies as described (3,24,25). Complete details are provided in the online appendix, which is available at http://diabetes.diabetesjournals.org/cgi/content/full/db09-0214/DC1.

Gel electrophoresis and mass spectrometry analysis. Muscle lysate proteins (60 μg total protein) were separated on 4–20% linear gradient SDS polyacrylamide gels and processed for mass spectrometry as described (26,27) and as outlined in supplemental Fig. 1 in the online appendix. A complete description of this analysis is provided in the online appendix.

Normalized spectral abundance factors. To quantify protein abundance, NSAFs were used (21–23). Tandem mass spectrometry (MS/MS) spectra assigned to a protein were normalized to the length of the protein (number of amino acids), resulting in a spectral abundance factor (SAF), where SAF = spectrum count/number AA. Each SAF was normalized against the sum of all SAFs in one sample, resulting in the NSAF value. For a protein, i, the normalized spectral abundance factor, *NSAF*, is calculated by

$$NSAF_i = SAF_i \Big/ \sum_{i=1}^{N} SAF_i$$

where N is the total number of proteins detected in a sample. Thus, NSAF values allow for direct comparison of a protein's abundance between individual runs in a fashion similar to microarray data analysis.

Reproducibility and linearity of NSAF method. To determine linearity, varying amounts of BSA tryptic digests (62–250 fmol) were added to 125 fmol cytochrome c digest. Mixtures were analyzed by MS/MS. To assess linearity in a "real life" complex mixture of proteins, muscle biopsy lysates were used. If NSAF provides an accurate reflection of the proportion of any particular protein in a complex protein sample, such as a muscle lysate, then the NSAF for any given protein should be equivalent, no matter how much total protein is analyzed. Complete methods for testing this and a description of the reproducibility experiment are described in the online appendix.

Single protein analysis. Although a large number of proteins were assigned in at least one of 24 subjects who were studied, a series of filters were used to narrow the number of proteins that were used in comparisons among groups. This approach is diagrammed in Fig. 1, with complete details provided in the online appendix.

Differential patterns of protein abundance among groups (protein sets). Differences in NSAF values for a set of proteins among groups were assessed as diagrammed in Fig. 2, with a complete description provided in the online appendix.

To better visualize patterns of differences among groups, protein abundance changes were visualized using the Gene Expression Dynamics Inspector (GEDI) (http://www.childrenshospital.org/research/ingber/GEDI/gedihome.htm) (28). GEDI uses an algorithm that groups individual proteins by patterns of group differences compared with an overall median. Proteins for a particular grouping that are generally lower in abundance than the global mean are depicted in shades of blue, whereas proteins that are increased are depicted in shades of orange and red, similar to a heat map.

Immunoblot analysis. To determine the relationship between NSAF quantification and relative protein abundance determined by immunoblot analysis, two proteins (desmin and alpha actinin-2) that differed significantly among the groups by NSAF analysis were subjected to immunoblot analysis. Only seven of the eight subjects in each group had sufficient muscle protein remaining to perform these analyses. Muscle protein lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed using antibodies against alpha actinin-2 or desmin (both from Santa Cruz Biotechnology, Santa Cruz, CA). PDLIM6 protein was used as a loading control (antibody from Santa Cruz Biotechnology). Blots were quantified by densitometry (VersaDoc 5000; Bio-Rad Laboratories, Hercules, CA).

RESULTS

Clinical and metabolic characteristics. The diabetic subjects had greater fasting plasma glucose and A1C than the obese or lean control subjects ($P < 0.01$) (Table 1). Obese ($P < 0.01$) and diabetic ($P < 0.05$) subjects had a greater BMI. The diabetic subjects were slightly older than the lean group (48 ± 3 vs. 37 ± 4 years, $P < 0.05$), although the average age of the obese group was intermediate and did not differ significantly from either of the other groups. As expected, the rate of insulin-stimulated glucose uptake was significantly impaired in the obese nondiabetic group and further decreased in the diabetic subjects.

Linearity and reproducibility of NSAF technique. The relationship between the proportion of BSA spectra in a mixture of BSA and cytochrome c and BSA concentration fit a linear regression ($r^2 = 0.90$, $y = 3.23x - 0.19$). Using a complex mixture of proteins (muscle lysate) over a fourfold concentration range (30–120 μg protein), there was a linear relationship between NSAF values. The regression equations for these three comparisons (30 vs. 60, 30 vs. 120, and 60 vs. 120 μg protein) were $y = 0.61 - 0.05$, $y = 1.21x - 2.49$, and $y = 1.99x - 2.67$, with $r^2 = 0.99$, 0.94, and 0.96, respectively. Figure 3 shows the relationship between NSAF values obtained with 30 and 120 μg protein.

To assess reproducibility, three separate portions of a muscle biopsy from a single individual were individually homogenized, run in separate lanes on an SDS gel, and the gel slices trypsinized and processed for high-performance liquid chromatography electrospray ionization–tandem mass spectrometry as described above (20 slices per lane). A total of 334 proteins were assigned that were common to all three biopsy portions, and the average coefficient of variation for these proteins was 22.1%. Power analysis (NQuery Advisor; Statistical Solutions, Saugus, MA) revealed that, using a coefficient of variation of 22.1%, an ANOVA with eight subjects per group has a power of >90% to detect a difference of twofold at the 0.05 level.

Muscle proteome. When the results from all 24 volunteers were combined and redundant results were collapsed, 1,218 proteins were assigned in at least one of the 24 subjects. The complete list of proteins that were assigned is provided as supplemental Table 1, which is available in the online appendix. Proteins that were assigned in at least half (12 of 24) of all participants totaled 400 and are listed in supplemental Table 2. This set of 400 proteins was used for further analysis.

Single protein analysis. Out of 400 proteins that were assigned as present in at least 12 of 24 subjects, a total of 92 of these proteins also were increased or decreased by a factor of two in at least one of the three possible two-group comparisons (obese versus lean, diabetic versus lean, or diabetic versus obese) and are given in supplemental Table 3. Of these 92 proteins, 15 were significantly different among the three groups using one-way ANOVA ($P < 0.05$, Table 2). Because variances for NSAFs for about half the proteins analyzed were not homogeneous, we also used the nonparametric Kruskal-Wallis test to analyze these data. Of the 15 proteins that significantly differed by ANOVA, 10 also significantly differed by Kruskal-Wallis, and of the other five proteins, two had P values <0.10 and three had P values <0.17.

Of the 15 proteins that differed significantly by ANOVA among the groups, 9 were greater in abundance and 6 were

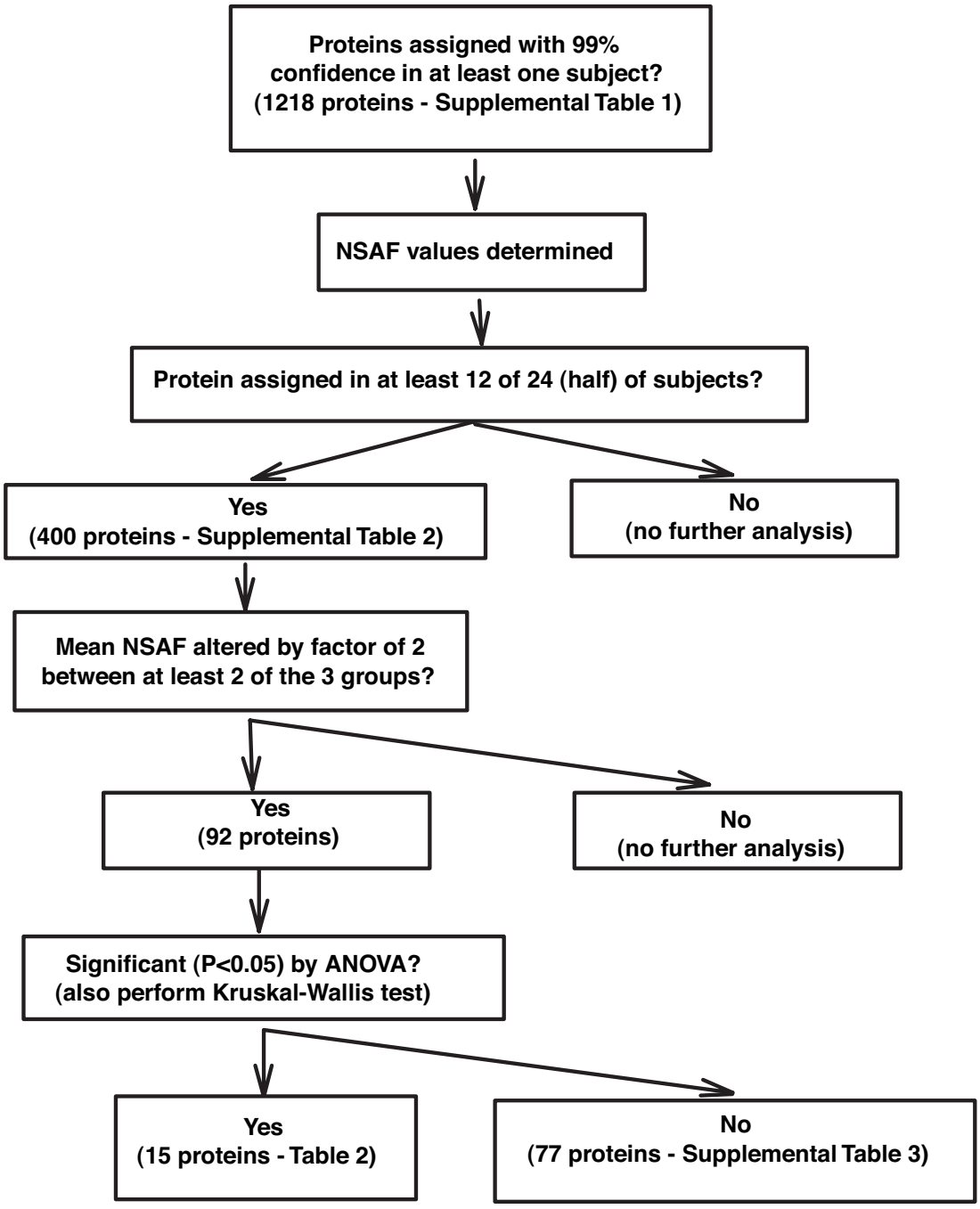

FIG. 1. Work flow of statistical analysis of single proteins.

lower in insulin resistance. Of the six that were lower, three were mitochondrial in location, cytochrome C oxidase VIC precursor (gene name COX6C), coiled-coil-helix-coiled-coil-helix domain-containing protein 3 (gene name CHCHD3), and ubiquinol-cytochrome C reductase complex ubiquinone-binding protein QP-C (gene name UQCRQ), while none of the nine proteins that increased were mitochondrial. Average NSAF values for these proteins are shown in Fig. 4*A*. The proportion of mitochondrial proteins in the proteins that decreased (3 of 6) was significantly greater than that in proteins that increased in abundance (0 of 9) ($P < 0.05$, Fisher exact probability test). Three nonmitochondrial proteins that were significantly higher in insulin resistance were proteins that are components of the cytoskeleton or structure of myocytes (alpha actinin 2, myozenin-1, and desmin; Fig. 4*B*).

This same pattern of decreased mitochondrial proteins also was present in the 77 proteins that differed by a factor of two but were not significant by ANOVA (supplemental Table 3). The *P* values determined using both ANOVA and Kruskal-Wallis are shown in this table also. The correlation between *P* values for the two methods was 0.76 ($P < 0.01$). Of those 77 proteins, 19 were lower in abundance in obese versus lean, diabetic versus lean, or diabetic versus obese subjects, and 58 were greater in abundance by a factor of 2 in either obese or diabetic subjects versus lean control subjects. Of the 19 that were lower in abundance in insulin resistance, 9 were mitochondrial; of the 58 that were higher in abundance, only 3 were

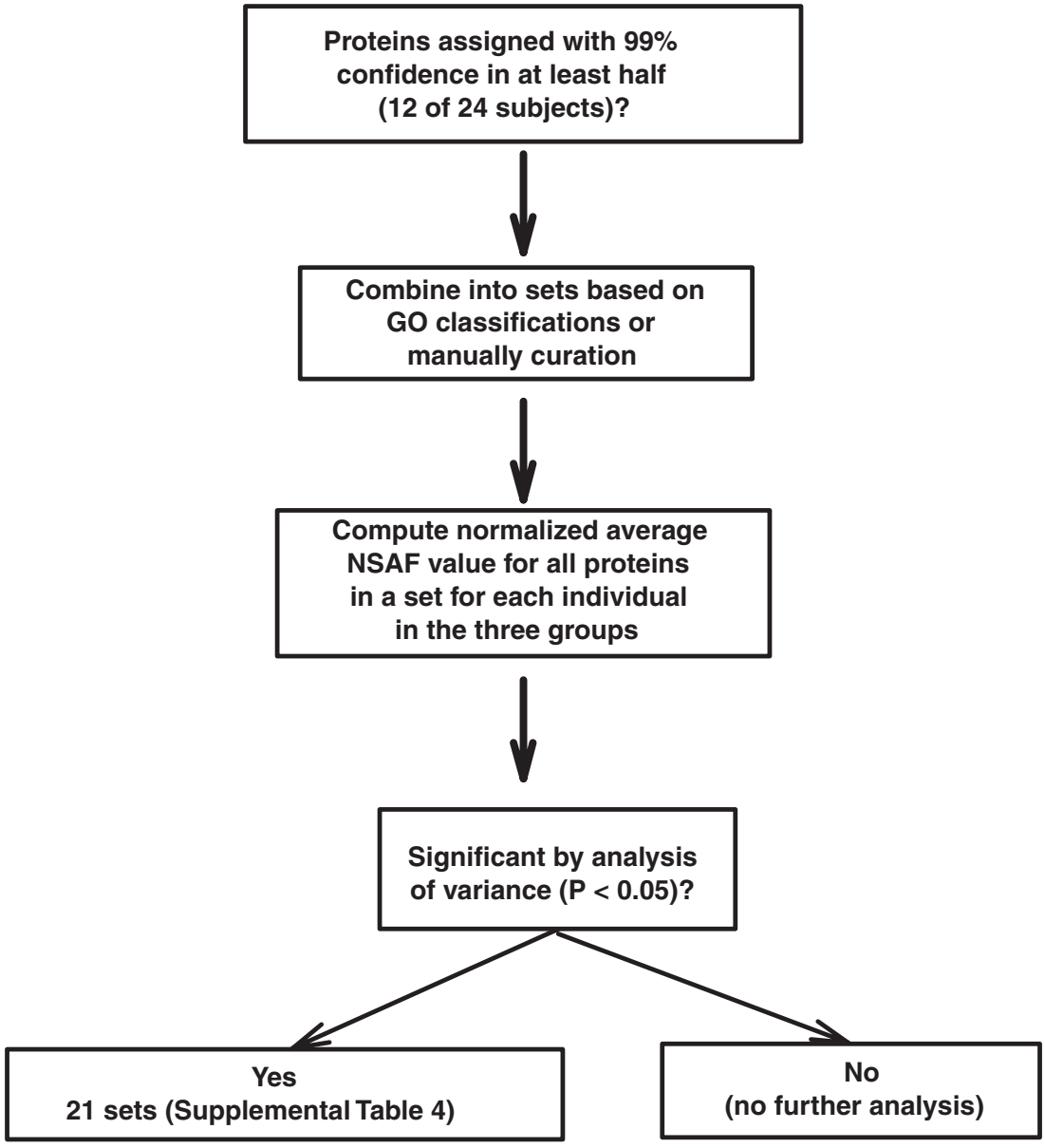

FIG. 2. Work flow of statistical analysis of protein sets.

mitochondrial ($P < 0.002$, Fisher exact probability test), including citrate synthase. Among the mitochondrial proteins that decreased by a factor of 2 but were not

TABLE 1
Subject characteristics

	Lean	Obese	Diabetic
n	8	8	8
Sex (F/M)	4/4	4/4	4/4
Age (years)	37 ± 4	44 ± 3	48 ± 3*
BMI (kg/m^2)	24.3 ± 0.8	31.6 ± 0.8†	29.0 ± 1.1*
Body fat (%)	26.8 ± 2.9	33.7 ± 2.8	34.2 ± 3.5
M [mg/(kg · min)]	8.2 ± 0.7	3.8 ± 0.2†	1.6 ± 0.5†
Fasting blood glucose (mg/dl)	94 ± 5	97 ± 1	148 ± 25†
A1C (%)	4.8 ± 0.2	5.1 ± 0.2	8.3 ± 1.1†
Insulin (μU/ml)	4.9 ± 0.4	12.6 ± 3.4†	9.7 ± 2.9

Data are means ± SE. *$P < 0.05$, †$P < 0.01$ vs. lean control subjects. M, rate of glucose metabolism.

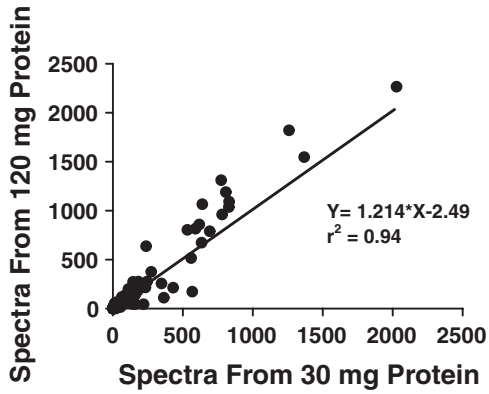

FIG. 3. Linearity of the relationships between spectral abundance factors observed when 30 and 120 μg protein were analyzed, as described in RESEARCH DESIGN AND METHODS. Similar data obtained for 30 vs. 60 and 60 vs. 120 μg protein loading were compared (data not shown).

TABLE 2
Proteins differing significantly among groups

Protein name	NSAF					Number of subjects with protein assigned			Total spectra per group		
	Lean	Obese	Diabetic	P_{ANOVA}	$P_{K\text{-}W}$	Lean	Obese	Diabetic	Lean	Obese	Diabetic
HSP90 co-chaperone CDC37	0.22 ± 0.22	1.92 ± 0.50†	0.99 ± 0.41	0.02‡	0.03	1	6	5	3	18	11
Myosin-15	0.10 ± 0.1	0.83 ± 0.21*	0.54 ± 0.22*	0.03	0.03	1	6	5	5	40	27
55-kDa protein; protein disulfide-isomerase A3 precursor	0.29 ± 0.21	1.71 ± 0.61*	0.66 ± 0.13	0.04‡	0.05	2	6	7	4	22	9
Cullin homolog 5	0.17 ± 0.08	0.78 ± 0.21	0.87 ± 0.28	0.05‡	0.08	3	6	5	4	17	18
N^G,N^G-dimethylarginine dimethylaminohydrolase 1	0.80 ± 0.55	3.28 ± 0.58*	2.32 ± 0.63	0.02	0.03	2	8	6	6	24	17
Chaperonin containing TCP1, subunit 8 (theta) variant; T-complex protein 1 subunit theta	0.78 ± 0.19	1.88 ± 0.51	2.50 ± 0.52*	0.03‡	0.04	7	7	8	13	27	35
T-complex protein 1 subunit delta	0.85 ± 0.16	1.95 ± 0.39*	2.00 ± 0.34*	0.03	0.02	7	7	8	13	27	28
Glutaminyl-TRNA synthetase	0.32 ± 0.16	0.60 ± 0.20	1.30 ± 0.42*	0.05	0.17	3	5	5	7	12	27
Proteasome subunit beta type 3	0.78 ± 0.32	0.92 ± 0.37	2.05 ± 0.55*	0.01	0.16	4	4	6	5	5	11
Alpha actinin-2	18.2 ± 3.3	10.6 ± 3.2*	8.18 ± 1.22*	0.04‡	0.05	8	8	8	508	282	195
Cytochrome C oxidase polypeptide VIC precursor	72.1 ± 15.1	38.2 ± 10.6*	27.1 ± 5.9*	0.03‡	0.10	7	8	7	166	74	57
Coiled-coil-helix-coiled-coil-helix domain-containing protein 3	5.57 ± 1.25	2.51 ± 0.56*	2.07 ± 0.91	0.03	0.03	8	7	4	36	15	12
Myozenin-1	6.65 ± 1.73	2.70 ± 1.78*	1.56 ± 0.59	0.05‡	0.13	6	2	5	68	27	13
Desmin; mutant desmin	3.98 ± 0.96	1.45 ± 0.52*	1.86 ± 0.56*	0.04	0.07	7	5	6	62	21	24
Ubiquinol-cytochrome C reductase complex ubiquinone-binding protein QP-C	21.4 ± 5.70	4.03 ± 2.70†	4.81 ± 2.10†	0.006*	0.04	6	2	4	57	12	11

Data are for NSAF values × 10,000 and are means ± SE. *P* value listed is for overall ANOVA (P_{ANOVA}) and Kruskal-Wallis test ($P_{K\text{-}W}$) among groups. Total spectra = sum of spectra across eight subjects in each group. Individual group a posteriori comparisons: *$P < 0.05$, †$P < 0.01$ vs. lean control subjects, by a posteriori tests after ANOVA. ‡Variances not homogeneous.

statistically significant by ANOVA were five additional subunits involved in the electron transport chain, including the ATP synthase β-subunit, and two additional proteins involved in import of proteins into mitochondria, mitochondrial import inner membrane translocase subunit TIM13, and sorting and assembly machinery component homolog SAM50.

Regarding proteins that were increased in insulin resistance, nine proteins were greater by at least twofold (obese versus lean and/or diabetic versus lean) and also were significant by ANOVA. Of those nine, three were chaperones or co-chaperones (HSP90 co-chaperone CDC37, T-complex protein 1 θ (TCP1 θ), and T-complex protein 1 δ (TCP1 δ). HSP90 co-chaperone CDC37 was increased by a factor of four- to eightfold in insulin resistant muscle, while the TCP1 subunits both were increased two- to threefold (Fig. 4*C*). Two proteins that significantly increased are involved in protein ubiquitination and degradation (cullin homolog 5 and proteasome subunit beta type 3). One protein, myosin-15, is a molecular motor that was increased four- to eightfold in insulin resistance. Another protein, protein disulfide isomerase a3, which stabilizes proteins with disulfide bonds, was increased two- to fourfold in insulin resistance. Of note is N^G,N^G-dimethylarginine dimethylaminohydrolase 1, which was higher by fourfold in obesity and nearly threefold in the diabetic group.

Among the 77 proteins that were altered by a factor of 2 in insulin-resistant subjects but that were not statistically significantly different, 58 were greater in abundance in either obese or diabetic, or both, versus lean control subjects. The 58 that were greater included a number of proteins related to those that did increase significantly by ANOVA. Perhaps the most pronounced example of this is the TCP1 proteins, of which TCP1 α and η subunits were increased by twofold or more. NSAF values for the TCP1 subunits that were assigned in at least half the subjects are given in Fig. 4*C*. In addition a number of proteasome subunits also were higher by at least twofold in insulin resistance, including 26S proteasome regulatory subunit 3, 26S proteasome regulatory subunit 4, proteasome subunit beta type 4, proteasome 26S subunit 11 variant, isoform 1 of proteasome subunit alpha type 7, and 26S proteasome regulatory subunit 7. NSAF values for the proteasome subunits are shown in Fig. 4*D*. Other proteins, related to the cytoskeleton or cell structure that were at least twofold greater but were not significant by ANOVA, included actin, alpha actinin-3, kinesin, and troponin.

Protein set analysis. Patterns of changes in proteins were analyzed by comparing differences in abundance of sets of proteins included in various gene ontology (GO) classifications and selected hand-curated sets (electron transport chain complexes I–V and TCP1 subunits). Only sets containing five or more proteins were analyzed to avoid undue influence of changes in only one or two proteins in a set. Supplemental Table 4 shows representative sets that were significantly different ($P < 0.05$ by

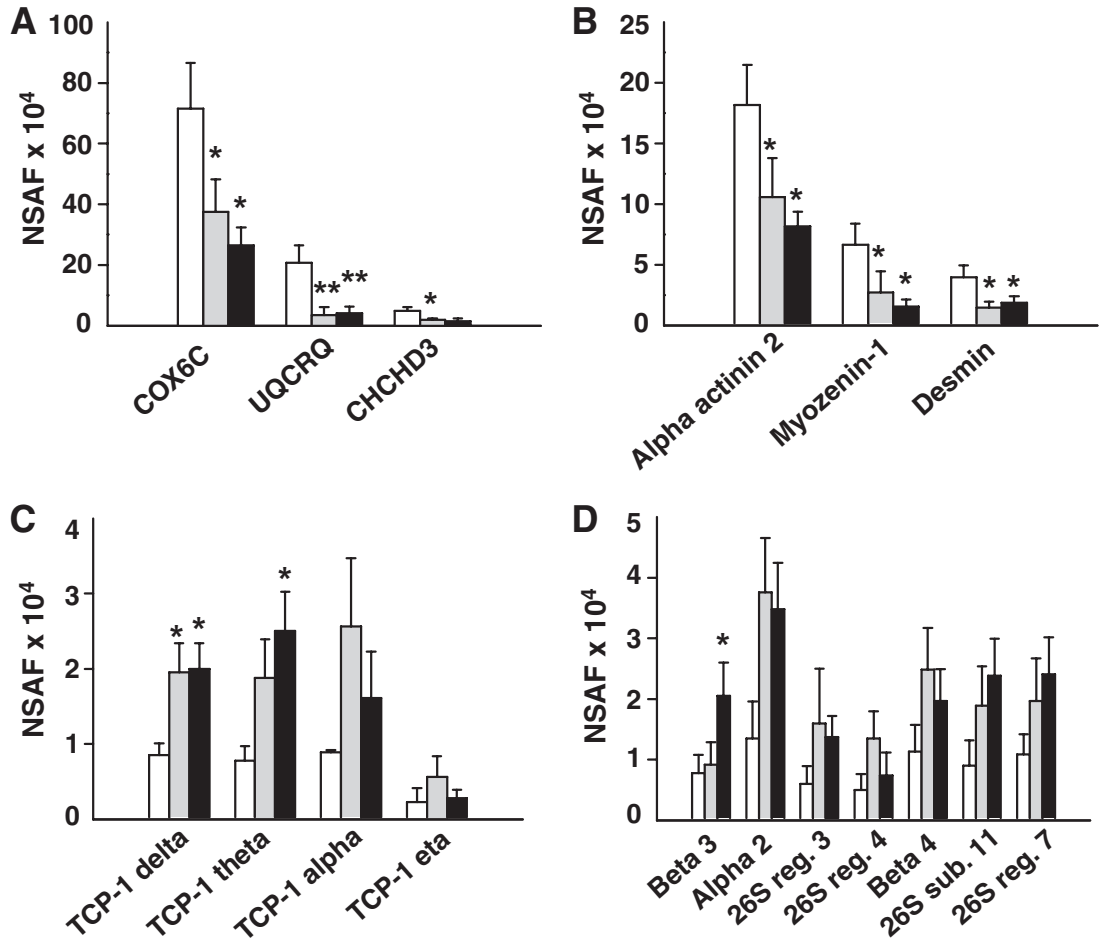

FIG. 4. Relative changes in abundance in mitochondrial proteins (*A*), cytoskeletal and structural proteins (*B*), TCP1 (chaperonin) complex proteins (*C*), and proteins involved in proteolytic process or protein modification leading to degradation (*D*). Data are given as means ± SE NSAF values for lean control (□), obese nondiabetic (▨), and type 2 diabetic participants (■). All NSAF values were multiplied by 10,000 for purposes of presentation. *$P < 0.05$, **$P < 0.01$ vs. lean control subjects (see RESEARCH DESIGN AND METHODS for details). All proteins shown were different among groups by ANOVA (at least $P < 0.05$) or differed by at least a factor of 2 among at least two groups. Numbers in parentheses as follows indicate the number of subjects with that protein assigned (non–zero-NSAF) for lean, obese, and diabetic subjects, respectively: COX6C, cytochrome c oxidase subunit 6c (7, 8, 7); UQCRQ, ubiquinol-cytochrome C reductase complex ubiquinone-binding protein QP-C (6, 2 ,4); CHCHD3, coiled-coil helix coiled-coil helix domain containing protein 3 (8, 7, 4); TCP1, T-complex protein 1 delta (7, 7, 8), theta (7, 7, 8), alpha (6, 6, 7), eta (2, 4, 6); Beta 3, proteasome subunit beta type 3 (4, 4, 6); Alpha 2, proteasome subunit alpha type 2 (4, 8, 7); 26S reg. 3, 26S proteasome regulatory subunit 3 (4, 3, 6); 26S reg. 4, 26S proteasome regulatory subunit 4 (3, 6, 4); Beta 4, proteasome subunit beta type 4 (4, 6, 6); 26S sub. 11, 26S proteasome subunit 11 (4, 6, 7); 26S reg. 7, 26S regulatory subunit 7 (5, 5, 7).

ANOVA) between obese and lean or diabetic and lean subjects. GO classifications involving mitochondrial compartments or functions, such as electron transport, were significantly lower in abundance in obese and diabetic muscle. GO classifications where protein abundance was greater involved apoptosis, protein phosphorylation, reactive oxygen species metabolism, and microtubule/cytoskeleton function. Results for manually curated sets comprising complexes I–V of the respiratory chain are shown in Fig. 5. Compared with lean control subjects, complex I, III, IV, and V proteins showed a graded decrease from lean to obese nondiabetic subjects to patients with type 2 diabetes. Differences in complexes III and IV achieved statistical significance ($P < 0.05$).

The program GEDI was used to visualize groups of proteins that had similar relative protein abundance differences compared with a global median value (Fig. 6). This analysis showed seven major regions with similar patterns of protein abundance among the three groups. Areas 3 and 5 contain many mitochondrial oxidative phosphorylation proteins (lower in insulin resistance), whereas areas 1 and 2 contain a number of proteins involved in protein degradation (higher in insulin resistance). A list of proteins in the numbered regions is given in supplemental Table 5.

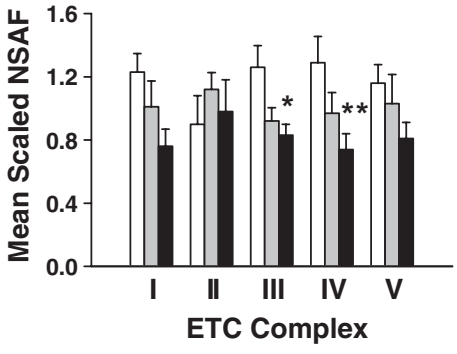

FIG. 5. Results of analysis of manually curated protein sets for complexes I–V of the electron transport chain (ETC). Data are given as means ± SE scaled NSAF values for lean control (□), obese nondiabetic (▨), and type 2 diabetic participants (■). *$P < 0.05$, **$P < 0.01$ vs. lean control subjects (see RESEARCH DESIGN AND METHODS for details).

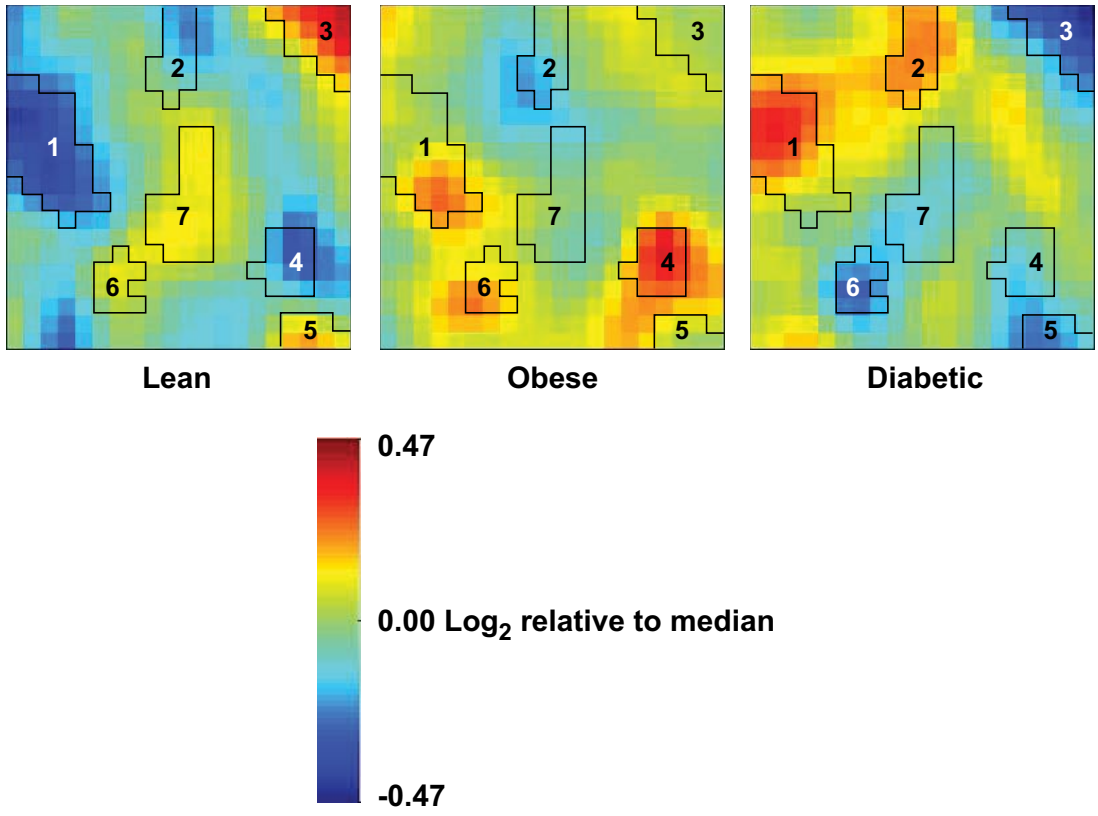

FIG. 6. Heat maps clustering proteins into groups with similar abundance relative to a global median, obtained using the program GEDI. A total of 400 proteins with spectra obtained in at least 12 of 24 subjects were analyzed. Proteins assigned by the GEDI algorithm to areas 1–7 are given in supplemental Table 5. Proteins were grouped based on their pattern of changes relative to a global median, where shades of blue indicate proteins decreased relative to overall median, green indicates little change, and shades of yellow, orange, and red indicate proteins with increased abundance relative to the global median. GEDI maps cluster proteins into "neighborhoods" with other proteins that display similar patterns of changes among the groups; axes are unit-less. (A high-quality color digital representation of this figure is available in the online issue.)

Proteins differing in abundance between diabetic and obese nondiabetic subjects. To gain insight into alterations in protein abundance due to diabetic status, we examined proteins that differed by a factor of 2 between the diabetic and obese groups. Proteins involved in mitochondrial function in general were lower in the diabetic group than in the obese nondiabetic subjects. HSP90 co-chaperone CDC37 and N^G,N^G-dimethylarginine dimethylaminohydrolase-1 were lower in diabetic versus obese subjects, but still greater in abundance relative to lean control individuals.

Immunoblot analysis of desmin and alpha actinin-2. We performed immunoblots for desmin and alpha actinin-2 protein abundance using the remaining lysate from seven of the eight subjects in each of the three groups of lean, obese, and diabetic volunteers. Results from densitometry analysis of these proteins were expressed relative to the 78-kDa protein PDLIM6, which was found not to differ among the groups in abundance in the mass spectrometry analysis ($P = 0.44$, protein assigned in all subjects in all three groups; supplemental Table 1) or immunoblot analysis. The results from quantification of these blots mirrored the results using our NSAF method and statistical analysis (Fig. 7). The correlation coefficients between NSAF values and blot values were 0.80 ($P < 0.0001$) and 0.69 ($P < 0.001$) for alpha actinin-2 and desmin, respectively.

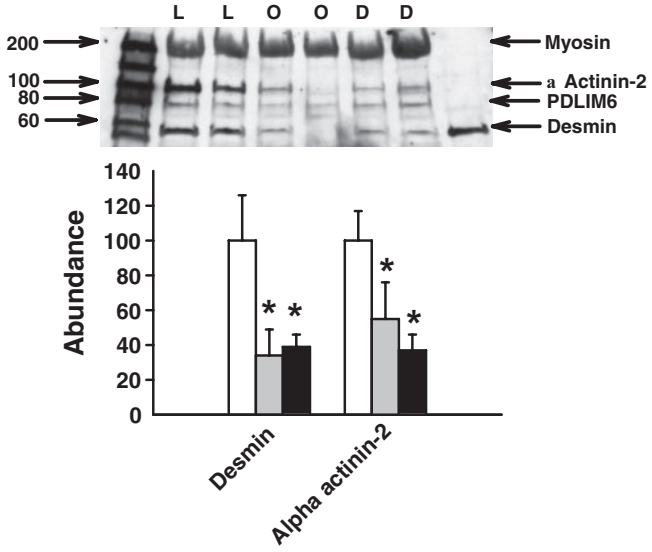

FIG. 7. Immunoblot analysis comparing abundance of desmin (*A*) and alpha actinin-2 (*B*) content in skeletal muscle biopsies from lean (□), obese nondiabetic (▨), and type 2 diabetic (■) ($n = 7$ each). Density values from blots were expressed relative to PDLIM6 protein determined on the same blots and were normalized for each protein to the mean value of the lean controls, expressed as 100%. Representative blots from lean, obese, and diabetic subjects (two of each) are shown in the inset for both proteins. Data are shown as means ± SE. *$P < 0.05$ vs. lean control values.

DISCUSSION

A wide array of abnormalities is associated with skeletal muscle insulin resistance (3–6,8,9). Because investigations of these effects often have focused on a particular protein or biochemical pathway, it has been problematic to discern larger patterns of differences between insulin-resistant and healthy muscle. A more global approach can be useful for identifying novel patterns of changes and lead to new focused hypotheses. Global analyses of mRNA expression in insulin-resistant muscle by microarray analysis revealed a pattern of reduced expression of genes involved in mitochondrial function and metabolism (7–9). These results helped to define mitochondrial dysfunction as a characteristic of insulin resistance. However, mRNA expression levels may not reflect protein abundance, providing a compelling reason for a global analysis of protein abundance changes in insulin-resistant muscle. Therefore, the present study was undertaken to provide data that would serve to generate new hypotheses regarding insulin resistance. To accomplish this, we used one-dimensional SDS-PAGE gels as the first dimension of separation in a multi-dimension, mass spectrometry–based process for protein assignment, with quantification using the NSAF method (21–23).

Previous studies of mRNA expression levels revealed a pattern of decreased expression of nuclear-encoded genes coding for proteins involved in mitochondrial function in insulin-resistant muscle (7–9). The present results extend this to show that the abundance of proteins involved in many mitochondrial functions also is reduced. Lending further evidence for a generalized decrease in mitochondrial protein abundance were results of analysis of protein sets grouped by GO classification. Of the 15 GO sets that were significantly reduced in insulin-resistant subjects, 13 represented mitochondrial proteins. When electron transport chain complex components were curated into individual sets representing subunits of complexes I–V, all but complex II proteins were reduced in insulin-resistant subjects, with complexes III and IV achieving statistical significance ($P < 0.05$) and complex I being marginally significant ($P = 0.066$). These results provide confirmation of the reports of decreased number or size of mitochondria in skeletal muscle from insulin-resistant subjects (29,30). Based on the current findings, those results can be extended to conclude that there is a widespread decrease in mitochondrial proteins, including components of the respiratory chain and protein import machinery.

Results from the present studies also show changes in abundance of several proteins involved in structure or function of the cytoskeleton. A significantly lower abundance was found of the actin capping and Z-disk component protein alpha actinin-2. Myozenin-1, which binds to alpha actinin (31), also was lower in abundance, as was a major intermediate filament component, desmin. On the other hand, a set of microtubule-related proteins, including tubulins and kinesins, was significantly increased in insulin resistance. Previous results have shown the essential nature of interactions of mitochondria with the cytoskeleton for normal mitochondrial function (32–34). It is tempting to speculate that a relationship exists between altered mitochondrial function and protein abundance and abnormalities in the abundance of cytoskeletal elements, particularly intermediate filament and Z-disk components, in insulin-resistant muscle. Intermediate filaments in skeletal muscle integrate force transduction between the sarcomere and extracellular matrix, through proteins such as integrins and the dystroglycan complex (35,36). Moreover, intermediate filaments organize organelles in skeletal muscle, including mitochondria, and are involved in regulating their function (35). We previously showed inflammatory-like extracellular matrix changes in insulin-resistant muscle (37–39). The present results provide the basis for a new hypothesis regarding the mechanisms of insulin resistance in muscle, namely, that changes in the extracellular matrix might lead to altered mitochondrial function by means of altered mechano-signal transduction produced by changes in intermediate filament protein abundance. A scenario connecting the extracellular matrix and mitochondrial function has been described for Bethlem muscular dystrophy, a collagen 6 mutation in humans (40). This hypothesis must be tested in further studies.

Another novel change observed in the present study is the increase in abundance of chaperonin proteins, namely T-complex proteins (TCP-1) and HSP90 co-chaperone CDC37. TCP-1 is a mammalian cytosolic chaperonin required for proper folding of proteins involved in cytoskeletal formation and contractile activity (41,42). Eight of the 10 subunits of TCP-1 were assigned in this study. Of these, two (TCP-1 δ and θ) were significantly increased in one or both groups of insulin-resistant subjects, and two others, TCP-1 α and η, were increased by at least twofold in one of the insulin-resistant groups, but were not significant by ANOVA. The expression of TCP-1 subunits θ and α is under coordinated control of extracellular signal-regulated kinase (ERK)-1/2 signaling (41), and a number of TCP-1 subunits have coordinately regulated expression (42). These findings are consistent with reports that MAP kinase/ERK signaling is preserved or increased in insulin-resistant skeletal muscle (3,4). In insulin resistance, plasma insulin concentrations are increased as a compensatory effort to normalize glucose metabolism. Because ERK signaling is preserved or increased in insulin-resistant muscle, in the setting of hyperinsulinemia, ERK signaling is likely to be increased. The present finding of a coordinated increase in TCP-1 abundance is the first reported potential consequence of the increase in ERK signaling in insulin-resistant muscle. The increased abundance of the major cytosolic chaperonin complex TCP-1 may be related mechanistically to the increases in abundance of several cytoskeletal and structural proteins also found in this study.

Another protein that showed an increase in abundance in insulin-resistant muscle was N^G,N^G-dimethylarginine dimethylaminohydrolase-1. The function of this enzyme is to metabolize N^G,N^G-dimethylarginine, also called asymmetric arginine, or ADMA (43). ADMA is a naturally occurring inhibitor of NO synthase (44) and is increased in plasma of individuals with hypercholesterolemia, hypertension, and type 2 diabetes (45–47). The increase in abundance of N^G,N^G-dimethylarginine dimethylaminohydrolase-1 in insulin-resistant muscle suggests that there may be a compensatory response in muscle in an effort to reduce ADMA.

The present findings also show that there are increases in the abundance of a number of proteins involved in proteasome function in insulin-resistant muscle. Although poorly controlled diabetic subjects are characterized by increased rates of proteolysis (48), it is less clear that a general increase in protein turnover characterizes insulin-resistant muscle, although some conditions associated with insulin resistance are also associated with increased

protein degradation, such as aging or type 1 diabetes (49,50). On a molecular level of analysis, Wang et al. (51), using insulin-resistant mice, showed that increased proteasome activity could be attributed to decreased signaling through IRS-1 and Akt, which occurs in insulin-resistant human muscle (3,4).

It should be noted that the diabetic subjects were on average 11 years older than the lean control subjects, although they were not significantly older than the obese nondiabetic subjects. All participants were middle-aged and very sedentary, suggesting that there are no age-related differences in physical activity. Moreover, gene expression studies over the age range of 20 to >80 years show few changes, and those who were observed had a very slow rate of change over age (52). Another major caveat of the results of this study is that not all proteins were assigned in each subject in each group. Given this caveat, and the limitations of statistical analysis of large datasets where some assumptions of statistical tests employed may not be met in every case, it must be stressed that the results from this study should serve as a starting point for additional, more focused studies designed to test hypotheses that might be generated by these findings.

In summary, the current results suggest that there is a wide array of changes in protein abundance in insulin-resistant skeletal muscle. Several potential themes emerge that must be addressed in future studies. These themes include lower abundance of mitochondrial proteins, alterations in abundance of proteins involved in cytoskeletal structure and function, and higher abundance of proteasome subunits and molecular chaperones. These changes provide new information about potential relationships among various pathways that may converge to create insulin resistance.

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