Proteasome inhibition in skeletal muscle cells unmasks metabolic derangements in type 2 diabetes

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Submitted 7 April 2014; accepted in final form 4 August 2014


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First published August 20, 2014; doi:10.1152/ajpcell.00110.2014.

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inhibition unmasks molecular deficiencies in the context of insulin resistance. Collectively, our findings implicate intrinsic disturbances in cellular protein dynamics in myotubes obtained from type 2 diabetic patients, which may contribute to impairments in whole body metabolic regulation.

MATERIALS AND METHODS

Chemicals and Cell Culture Reagents

Cell culture reagents including DMEM, DMEM F-12, FBS, penicillin/streptomycin, and Fungizone were from GIBCO (Invitrogen, Sweden). The proteasome inhibitor BZ (marketed as Velcade by Millennium Pharmaceuticals) is a highly selective reversible inhibitor of the 26S proteasome used in treatment of multiple myeloma and mantle cell lymphoma (no. S1013; Selleck Chemicals, Houston, TX). The [14C]phenylalanine, [14C]glucose, and [9–10(n)-3H]palmitate were from Perkin-Elmer (Perkin-Elmer Life Sciences, Boston, MA). All other chemicals were from Sigma-Aldrich.

Human Skeletal Muscle Cell Culture

A cohort of age- and body mass index-matched NGT (n = 10) and type 2 diabetic (n = 10) male volunteers were recruited as reported earlier (3). Skeletal muscle biopsies were obtained from vastus lateralis muscle using a Weil-Blakesley conchotome tong. Protocols were approved by the ethical committee at Karolinska Institutet, and informed written consent was received from all participants. Satellite cells were isolated from skeletal muscle biopsies derived from NGT and type 2 diabetic volunteers by trypsin-EDTA digestion and cultured as described previously (2). Experiments were performed on differentiatet myotubes at passages 2–5. Differentiation was initiated at 80% confluency by addition of DMEM-1 g/l glucose, 2% FBS, 1% PeSt, and 1% Fungizone for 6 days.

Myotube Proteome Analysis

Two-dimensional difference gel electrophoresis. For the proteome analysis, primary human skeletal muscle satellite cells were prepared as described previously (2). Differentiated myotubes grown on 150 mm dishes were serum-starved for 24 h with or without 120 nM insulin. Cellular protein was extracted and labeled as described previously (3). Two-dimensional (2-D) PAGE analysis was performed based on the fluorescence difference gel analysis technology (2-D DIGE) (34), with modifications as described previously (3). The nuclease-treated internal standard was labeled with Cy2 fluorescent dye. Cy3 or Cy5 labeling of myotubes was randomly assigned and further analyzed by singlet gels.

First-dimension isoelectric focusing and SDS-PAGE. One Cy3 or Cy5 labeling reaction mix was combined with an equivalent portion of the internal standard reaction mix. Samples were processed and loaded onto 24-cm 3–11NL IPG strips. Insulin-treated and control (basal) myotubes originating from the same individual were loaded onto the same gel. The first-dimension isoelectric focusing was conducted as described previously (3). Before second-dimension SDS-PAGE, IPG strips were equilibrated, rinsed, and positioned onto 10–15% gradient acrylamide gels using the ExQuest robot equipped with a 1.5-mm punch tool (Bio-Rad). Gel plug extraction, digestion, and LC/MS analysis were described in detail previously (3).

MS/MS database searching and pathway analysis. Database searching was performed by Mascot Daemon (v. 2.2) and MS/MS Ion Search software (Matrix Sciences, London, UK), searching human gene catalogue sequences (gc_human; AstraZeneca). Search settings allowed one missed cleavage with the trypsin enzyme selected, one fixed modification (carbamidomethylation of cysteine), a variable modification (oxidation of methionine), mass tolerance: ±0.1 ppm, and fragment mass tolerance: ±0.3 Da. Proteins matched in Mascot Daemon searches were assigned as identity if a minimum of two individual ion scores were above the threshold stated by Mascot software, consistent with a significance level <5% (P < 0.05). The false discovery rate (FDR; decoy database) is <5% (q < 0.05) for all identified proteins reported. Masses repeatedly observed in the MS/MS spectra and other known contaminants were considered as background signals and excluded. Proteins considered to be differentially abundant after insulin exposure vs. the basal condition in myotubes derived from type 2 diabetic patients were matched against canonical pathways using Ingenuity Pathway Analysis (IPA) software.

Following insulin treatment, proteins showing differential abundance in myotubes derived from both NGT and type 2 diabetic donors were isolated and identified using 2-D DIGE technology and LC-MS/MS. A total of 105 insulin-responsive spots were discovered using the PDQuest image analysis and Qlucore Omics Explorer 2 statistical software. From these 105 spots, 166 differentially abundant proteins were identified in myotubes in response to insulin exposure, using FDR criteria <5% (q < 0.05; Supplemental Table S1; Supplemental Material for this article is available online at the Journal website). Right-tailed Fisher’s exact test was used to calculate a P value, which determined the probability that each biological function assigned to a particular data set was due to chance alone. Only 45 of the 166 proteins were identified in single hit protein spots, while 17 highly scored proteins were categorized as multiple hit protein spots, denoted as MIAD for “multiple identification assignments with one dominating protein.” For the identified proteins shown in Supplemental Table S1, changes following insulin exposure were equivalent between myotubes derived from NGT individuals and type 2 diabetic patients. This list of differentially expressed proteins identified with the 2-D DIGE system was processed for pathway and functional analyses using the IPA software (Table 1).

Of the total spots matched in position, the intensity of nine spots was significantly altered after insulin stimulation in myotubes from the NGT donors (P < 0.05) but not from the type 2 diabetic patients. LC/MS/MS analysis of the 9 excised spots generated 11 certain identifications, 6 spots of which were considered in this study (5 single hit spots and 1 spot with high score protein; listed as MIAD, see Table 3).
Table 1. Signaling pathways associated with differentially abundant proteins in myotubes treated with insulin from NGT individuals and type 2 diabetic patients

<table>
<thead>
<tr>
<th>Function/Pathways</th>
<th>Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy metabolism</td>
<td></td>
</tr>
<tr>
<td>TCA cycle and oxidative phosphorylation</td>
<td>ETFA ↓, SDHA ▼, DLD ▼, MDH2 ▼, TUFM ▼</td>
</tr>
<tr>
<td>Mitochondrial function</td>
<td>ATP5A1 ▼, TUFM ▼, SDHA ▼</td>
</tr>
<tr>
<td>Carbohydrate metabolism</td>
<td></td>
</tr>
<tr>
<td>Glycolysis/gluconeogenesis</td>
<td>PKM2 ▼, PGK1 ▼, ALDH4A1 ▼</td>
</tr>
<tr>
<td>Pyruvate metabolism</td>
<td>PKM2 ▼, ALDH4A1 ▼, DLD ▼, MDH2 ▼</td>
</tr>
<tr>
<td>Starch and sucrose metabolism</td>
<td>GPI ▼, UGP2 ▼, GANAB ▼</td>
</tr>
<tr>
<td>Pentose metabolism</td>
<td>GPI ▼, UGP2 ▼, KT1 ▼</td>
</tr>
<tr>
<td>Butanote metabolism</td>
<td>SDHA ▼, ALDH4A1 ▼</td>
</tr>
<tr>
<td>Nucleotide sugars metabolism</td>
<td>UGP2 ▼</td>
</tr>
<tr>
<td>Amino acid metabolism</td>
<td>HBCH ▼, ECHS1 ▼, ALDH4A1 ▼</td>
</tr>
<tr>
<td>Fatty acid metabolism</td>
<td>ALDH4A1 ▼, ACA2 ▼, MCC2 ▼, MDH2 ▼</td>
</tr>
<tr>
<td>Cellular stress response</td>
<td></td>
</tr>
<tr>
<td>Oxidative stress</td>
<td>TPM4 ▼</td>
</tr>
<tr>
<td>NRF2-mediated oxidative stress response</td>
<td>ACTB ▼</td>
</tr>
<tr>
<td>Ubiquitin-proteasome degradation pathway</td>
<td>HSPA8 ▼, PSMD11 ▼, PSMD13 ▼, UBE2N ▼, UBE2I ▼</td>
</tr>
<tr>
<td>Calcium-dependent ubiquitination</td>
<td>CACYBP ▼</td>
</tr>
<tr>
<td>and proteosomal degradation</td>
<td></td>
</tr>
<tr>
<td>Aryl hydrocarbon receptor signaling</td>
<td>ALDH4A1 ▼</td>
</tr>
<tr>
<td>Association to apoptosis, NRF2-mediated oxidative stress response</td>
<td>PTPI ▼, ANXA5 ▼, IFI35 ▼, LGALS3 ▼, LAMA1 ▼, PAFAH1B1 ▼, SH3GLB1 ▼</td>
</tr>
<tr>
<td>Protein dynamics</td>
<td></td>
</tr>
<tr>
<td>Chaperone, protein folding/unfolding</td>
<td>C506A ▼, CCT8 ▼, PFDN2 ▼</td>
</tr>
<tr>
<td>Autophagy-lysosomal pathway</td>
<td>SHG1B1 ▼, CTSB ▼</td>
</tr>
<tr>
<td>mRNA transcription, processing, splicing, and translation</td>
<td>EEF1B2 ▼, EEF1D ▼, EEF1G ▼, EEF2 ▼, EEF1AX ▼, EEF4E ▼, PCBP1 ▼, TUFM ▼, SBDS ▼, CSDE1 ▼, RIPA ▼</td>
</tr>
<tr>
<td>Cellular traffic and movement</td>
<td></td>
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<tr>
<td>Cytoskeletal regulatory function</td>
<td>DYNCL2 ▼, PFN2 ▼, PM1 ▼, TPM2 ▼, TPM3 ▼, TPM4 ▼</td>
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<tr>
<td>Clathrin-mediated endocytosis signaling</td>
<td>HSPA8 ▼, ACTR3 ▼, ACTB ▼, SH3GLB1 ▼</td>
</tr>
<tr>
<td>Cytoskeletal organization</td>
<td>PAFAH1B1 ▼, VIM ▼, CAPZA1 ▼, CKN5 ▼, LASP1 ▼</td>
</tr>
<tr>
<td>Regulation of actin-based motility by rho</td>
<td>ACTR3 ▼, ACTB ▼, PFN2 ▼</td>
</tr>
<tr>
<td>Endosome to lysosome transport</td>
<td>HOOK3 ▼</td>
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<tr>
<td>Intracellular signal transduction</td>
<td></td>
</tr>
<tr>
<td>Regulatory function</td>
<td>PAFAH1B1 ▼</td>
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<tr>
<td>Eif2 signaling, regulation of Eif4 and p70S6K signaling</td>
<td>EEF1AX ▼, EEF4E ▼, RPSA ▼</td>
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<tr>
<td>Calcium signaling</td>
<td>TPM1 ▼, TPM2 ▼, TPM3 ▼, TPM4 ▼</td>
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<tr>
<td>G-protein signaling</td>
<td>LANC1 ▼, PKM2 ▼</td>
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<tr>
<td>Gene regulation</td>
<td>UBE2N ▼</td>
</tr>
<tr>
<td>DNA repair</td>
<td></td>
</tr>
<tr>
<td>Nuclear stability</td>
<td>LMNA ▼</td>
</tr>
</tbody>
</table>

The Ingenuity Pathway Analysis software (IPA) was used to match the proteomic 2-D DIGE analysis results to canonical pathways. The proteins presented in this table are obtained from Supplemental Table S1 (the MIAD proteins are shown in italics). Arrows represent reduced protein content (▼) or increased protein content (▲) in myotubes derived from NGT and T2D donors that were exposed for 24 h to insulin (120 nM) vs. baseline. TCA, tricarboxylic acid cycle.

Quantitative Real-Time PCR

mRNA from differentiated myotubes was extracted with RNeasy Mini Kit (Qiagen). cDNA was synthesized using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). mRNA expression of proteins identified from the proteome analysis [BAT1, HLA-B associated transcript 1 (DDX39B), heat shock 27-kDa protein 1 (HSBP1), vacuolar protein sorting 29 isoform 1 (VPS29), and the proteosome subunits (PSMA1, PSMA6, and PSMB2)] was measured. Probes for all genes were from ABI (Applied Biosystem, Stockholm, Sweden). Relative target transcript abundance was calculated from duplicate samples using the CT comparative method after normalization against a housekeeping gene (18S, GAPDH, beta-2 microglobulin, and beta-actin). Beta-actin and beta-2 microglobulin were chosen as the most stably expressed reference gene.

siRNA Transfection

PKCδ or proteasome PSMA1, PSMA6, and PSMB2 on-target plus smart pool siRNA oligonucleotides were utilized to induce gene silencing in vitro (Dharmacon). Differentiated myotubes were transfected using Lipofectamine 2000 (Invitrogen) as described previously (2) and treated with scramble control sequence (SCR) or siRNAs against PKCδ or PSM subunits (PSMA1, PSMA6, and PSMB2) at an 80-nM final concentration.

Metabolic Assays

Myotubes were treated with or without inhibitor and/or stimuli (10 nM BZ or 120 nM insulin) in serum-free DMEM for 24 h before assay or direct harvest. Treatment with BZ or the protein kinase C (PKC) specific inhibitor GFX (500 nM; GF-109203X) occurred for 30 min before insulin stimulation. Serum-free media were refreshed after 18 h, and all experiments were terminated after 6 h. DMSO (0.01%) was used as a vehicle. Glucose incorporation into glycogen (glycogen synthesis) was determined in duplicate, as described previously (2). Proteosome activity was monitored in cell extracts using the Proteasome Activity Fluorometric Assay Kit (Biovision, Milpitas, CA) according to the manufacturer’s instructions. The amount of carboxyl formation in protein was assessed using an OxiSelect protein carbonyl ELISA (Cell Biolabs, San Diego, CA). Lactate was determined using a colorimetric L-Lactate Assay (catalog no. A-108; Biomedical Research Center, Buffalo, NY). Free fatty acid oxidation was assessed in duplicate (27) with modifications, including the inclusion of nonradioactive palmitate to measure specific activity (33), as described previously (3). Amino acid incorporation into protein (protein synthesis) was assessed as described previously (3), using phenylalanine as a tracer.

Cell Viability

Cell viability following BZ treatment was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay. Cells were seeded in 96-well plates and treated for 24 h with different concentration of BZ. Two days after transfection, media were removed and 100 μl of serum-free media containing MTT (0.5 mg/ml) were added to each well and incubated for 2 h at 37°C. Solutions were removed, the water-insoluble formazan was dissolved in 100 μl dimethylsulfoxide, and absorbance was measured at 550 nm.

Western Blot Analysis

Myotubes were harvested and processed for Western blot analysis as described previously (2) after the various treatments as specified in the figures. Total and phospho-specific antibodies against AKTSer473, AKTThr308, GSK3α/βSer21/9, GSεSer41, mTORSer2448, S6K1Thr389, pp65Ser235/236, pERK1/2Thr202/Tyr204, p38MAPKThr180/Tyr182, and pJNKThr183/Tyr185 (Cell Signaling Technology); NRF2Ser40 (Epitomics,
Nordic BioSite); and pPKCθ<sup>Thr505</sup> (BD Transduction Laboratories) were used. The total abundance of IRS1, caspase 3, and HSP90A (Cell Signaling Technology); PSM20S (anti-proteasome 20S; Mybiosource); SOD1 (Abcam); PSM26S (anti-proteasome 26S; Mybiosource); and pPKCθ/H9251 (anti-proteasome 20S alpha1-subunit: PSMA1; Pierce Biotechnology); PSM20S-α6 (anti-proteasome 20S alpha6-subunit: PSMA6); PSM20S-β2 (anti-proteasome 20S beta2-subunit: PSMB2); and 26S-5α (anti-proteasome 26S regulatory subunit S5a; Enzo Life Sciences) was measured. A GAPDH antibody (Santa Cruz Biotechnology) was used to confirm equal loading of proteins, if not otherwise stated.

Statistical Analysis

Statistical analysis of the proteomic quantitative data was performed using the Qlucore Omics Explorer 2 statistical software, which is specialized for multivariate data sets. To determine significant differences in protein content at baseline or after insulin treatment, samples from type 2 diabetic and/or NGT donors were analyzed using a General Linear Model controlling for bias from the various CyDyes and gel batches. Analyte concentrations were log<sub>2</sub>-transformed and normalized to the mean for each analyte with a −1 to +1 variance. To adjust for multiple testing and reduce the risk of false discoveries, differentially abundant proteins in response to insulin in myotubes derived from NGT donors, but not in myotubes from type 2 diabetic patients, were defined as insulin-resistant candidate proteins if the FDR was <5 (q < 0.05). Although a large proportion of the detected proteins was differentially expressed, the small sample size (n = 10 donors per group) may have limited the statistical power and hampered discovery of additional insulin-responsive proteins.

For mRNA expression and metabolic analysis, significant differences between baseline and/or treatment were analyzed using Student’s t-test or ANOVA and a Bonferroni post hoc test. Significance was <5% (P < 0.05). Analyses are presented as means ± SE and were performed using Graph Prism (5.0).
RESULTS

Protein Profile After Insulin Exposure

To interrogate the existence of an insulin-responsive proteomic signature, protein content profiling was performed after exposure of myotubes to insulin for 24 h. Proteins showing differential abundance in myotubes derived from NGT and type 2 diabetic donors were identified using 2-D DIGE technology and LC-MS/MS (Supplemental Table S1). The IPA software was used to analyze the list of differentially abundant proteins. The insulin-responsive proteins qualified as “network eligible molecules” were overlaid with molecular networks based on the IPA Knowledge Base. The pathway analysis revealed a coordinated elevation of proteins involved in energy metabolism, such as carbohydrate and lipid metabolism, tricarboxylic acid cycle, and amino acid metabolism after insulin exposure, but also proteins associated with protein homeostasis maintenance (Fig. 1A and Table 1). Several of the identified insulin-responsive proteins have not been previously associated with type 2 diabetes or insulin action (Supplemental Table S1 and Table 2), whereas others have a well-characterized role in metabolism, gene regulation, cell traffic, and stress response (Table 1). These findings reflect the necessity for a balance of cellular protein homeostasis following insulin exposure.

Protein Homeostasis Maintenance System Alterations Reflect an Intrinsic Insulin Resistance

We next examined proteins that were differentially abundant in response to insulin between myotubes derived from type 2 diabetic patients and NGT individuals. These proteins were associated with four groups of the protein homeostasis maintenance system (Fig. 1B and Table 3) and are involved in protein synthesis (translation-ribosomal protein BAT 1), in protein folding and unfolding (chaperone/heat shock protein HSPB1), in the autophagy-lysosomal pathway (vacuolar protein sorting VPS29), and in the UPS (proteasome subunits PSMA1, PSMA6, and PSMB2). With the exception of BAT1, the abundance of these proteins was elevated after insulin exposure in myotubes derived from NGT but not type 2 diabetic donors. This finding marks the

Table 3. Proteins identified as an insulin-resistant signature in myotubes from type 2 diabetic patients

<table>
<thead>
<tr>
<th>Spot ID</th>
<th>Symbol</th>
<th>UniProtKB/Swiss-Prot</th>
<th>Entrez Gene Name</th>
<th>Mascot Score</th>
<th>Fold Change</th>
<th>Multiple Hit</th>
<th>Function</th>
<th>Association to Insulin action/T2D</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 102</td>
<td>BAT1</td>
<td>Q13838</td>
<td>HLA-B-associated transcript 1</td>
<td>1370</td>
<td>−1.09 MIAD</td>
<td></td>
<td>Purine metabolism, spliceome, nuclear mRNA splicing and protein synthesis regulation</td>
<td>N.K., N.K.</td>
</tr>
<tr>
<td>2 155</td>
<td>DHR54</td>
<td>Q9BTZ2</td>
<td>Dehydrogenase/reductase (SDR family) member 4</td>
<td>276</td>
<td>1.06 MIAD</td>
<td></td>
<td>Carbonyl reductase (NADPH) activity, oxidoreductase activity, and oxidation reduction metabolism</td>
<td>N.K., N.K.</td>
</tr>
<tr>
<td>3 84</td>
<td>HNRPL</td>
<td>P14866</td>
<td>Heterogeneous nuclear ribonucleoprotein L</td>
<td>107</td>
<td>−1.05 MIAD</td>
<td></td>
<td>Nuclear mRNA splicing, via spliceosome and formation, packaging, processing, and function of mRNA</td>
<td>N.K., N.K.</td>
</tr>
<tr>
<td>4 169</td>
<td>HSPB1</td>
<td>P04792</td>
<td>Heat shock 27-kDa protein 1</td>
<td>3315</td>
<td>1.18</td>
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<td>Chaperone, actin interaction, oxidative stress, endoplasmic reticulum stress response and activation of proteasome</td>
<td>(19α), N.K.</td>
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<tr>
<td>5 151</td>
<td>PSMA1</td>
<td>P25786</td>
<td>Proteasome (prosome, macropain) subunit, alpha type, 1</td>
<td>552</td>
<td>1.06</td>
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<td>Proteasome degradation, protein ubiquitination process, ubiquitin-dependent protein catabolic process in a nonlysosomal pathway</td>
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<tr>
<td>6 155</td>
<td>PSMA4</td>
<td>P25789</td>
<td>Proteasome (prosome, macropain) subunit, alpha type, 4</td>
<td>421</td>
<td>1.06 MIAD</td>
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<td>Proteasome degradation, protein ubiquitination process, ubiquitin-dependent protein catabolic process in a nonlysosomal pathway</td>
<td>(25α), N.K.</td>
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<td>7 164</td>
<td>PSMA6</td>
<td>P60900</td>
<td>Proteasome (prosome, macropain) subunit, alpha type, 6</td>
<td>998</td>
<td>1.06</td>
<td></td>
<td>Proteasome degradation, protein ubiquitination process, ubiquitin-dependent protein catabolic process in a nonlysosomal pathway</td>
<td>(25α), N.K.</td>
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<tr>
<td>8 180</td>
<td>PSMB2</td>
<td>P49721</td>
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<td>242</td>
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<td>Proteasome degradation, protein ubiquitination process, ubiquitin-dependent protein catabolic process in a nonlysosomal pathway</td>
<td>(25α), N.K.</td>
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<tr>
<td>9 109</td>
<td>PSMC5</td>
<td>P62195</td>
<td>Proteasome (prosome, macropain) 26S subunit, ATPase, 5</td>
<td>1745</td>
<td>1.08 MIAD</td>
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<td>Proteasome degradation, protein ubiquitination process, ubiquitin-dependent protein catabolic process in a nonlysosomal pathway</td>
<td>(25α), N.K.</td>
</tr>
<tr>
<td>10 181</td>
<td>VPS29</td>
<td>Q9UBQ0</td>
<td>Vacuolar protein sorting 29 homolog (S. cerevisiae)</td>
<td>367</td>
<td>1.07</td>
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<td>Cellular vesicle traffic: retrograde transport of proteins from endosomes to the trans-Golgi network</td>
<td>(25α), N.K.</td>
</tr>
<tr>
<td>11 84</td>
<td>WDR1</td>
<td>O75083</td>
<td>WD repeat domain 1</td>
<td>134</td>
<td>−1.05 MIAD</td>
<td></td>
<td>Actin filaments disassembly and protein to protein interaction</td>
<td>N.K., N.K.</td>
</tr>
</tbody>
</table>

The protein content profile changes after 24 h insulin treatment (120 nM) in myotubes obtained from NGT individuals (n = 10 NGT) vs. type 2 diabetic patients. Data are shown as fold change above baseline. Negative fold change value indicates a lower protein content following insulin treatment vs. baseline; n = 10 subjects group. Statistical significance was set at P < 0.05 and q < 0.01 (q value indicates the FDR). N.K.: the role of the protein is Not Known for a role in insulin action or type 2 diabetes (italicized). Reference nos. are shown in parentheses.
existence of an insulin-resistant signature retained by myotubes derived from type 2 diabetic patients.

**Altered mRNA and Protein Abundance of Proteasome Subunit After Insulin Exposure**

To validate the findings of the proteomic analysis, the expression of selected proteins associated with homeostasis maintenance was measured in myotubes derived from NGT and type 2 diabetic donors studied at baseline or after insulin exposure for 24 h. The mRNA and protein levels of PSMA1, PSMA6, and PSMB2 were elevated after insulin exposure in myotubes obtained from NGT but not type 2 diabetic donors ($P < 0.05$, NGT-baseline vs. insulin; Fig. 2, A–C). Insulin also increased the mRNA expression of HSPB1 in myotubes derived from NGT but not type 2 diabetic donors (Fig. 2D). Insulin was without effect on mRNA expression of BAT1 or VPS29 in either myotubes obtained from NGT or type 2 diabetic donors (data not shown).

**Combined siRNA Silencing of Proteasome Subunits Reduces Glucose Metabolism**

Given the essential role of the proteasome on cellular protein dynamics, we further investigated whether a direct link exists between the skeletal muscle proteasome and glucose metabolism. siRNA mediated-gene silencing was utilized to disturb the UPS by disrupting PSMA1, PSMA6, and PSMB2, individually or in combination, in myotubes obtained from NGT donors. Silencing of PSMA1 and PSMB2 increased mRNA expression of other PSM subunits, indicative of compensatory regulation by the proteasome (Fig. 3A). Silencing of the three proteasome 20S subunits in combination (siCOMB) significantly decreased the total protein abundance of PSMA1, PSMA6, PSMB2, but not proteasome 26S (Fig. 3, B–E). Proteasome subunit abundance was not altered by the insulin treatment. Moreover, siRNA silencing of proteasome subunits was similar between basal and insulin-treated myotubes. The effect on basal and insulin-stimulated glucose metabolism was explored by assessing glucose incorporation to glycogen following proteasome subunits siRNA transfection (Fig. 4A). Basal and insulin-stimulated glucose incorporation into glycogen was reduced 24 and 38%, respectively, after combined proteasome subunits siRNA targeting ($P < 0.05$, vs. SCR). Basal glucose incorporation into glycogen was unaltered by siRNA transfection of individual proteasome subunits. However, glucose incorporation into glycogen was reduced following PSMB2 siRNA transfection only after insulin stimulation ($P < 0.05$, vs. SCR). Combined silencing of proteasome subunits did not alter AktThr308 or AktSer473 phosphorylation (Fig. 4, B and C) but moderately reduced proteasome activity (Fig. 4D). Together, these results demonstrate that targeted

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Fig. 2. Proteasome subunit expression in primary myotubes. Protein abundance and mRNA expression of the proteasome subunits after insulin exposure in myotubes derived from NGT and type 2 diabetic donors. PSMA1 (A), PSMA6 (B), PSMB2 (C), and HSPB1 (D) measured after 24-h insulin (120 nM) exposure. B, baseline; I, insulin. Results are means ± SE; $n = 5$ NGT and $n = 5$ type 2 diabetic subjects. *$P < 0.05$, baseline vs. insulin; #$P < 0.05$, insulin-stimulated myotubes from NGT vs. type 2 diabetic donors.
disruption of the proteasome partially impairs glucose metabolism in human myotubes.

Effect of BZ Treatment on Glycogen Synthesis and Proteasome Protein Content

Due to the compensatory mechanisms of different proteasome subunits and a nonsignificant reduction on proteasome activity, as evident from the siRNA silencing experiments, a direct and more complete inhibition of the whole proteasomal degradation system may be required to uncover a link and/or reveal unknown connections between the proteasome and glucose metabolism. Direct proteasome inhibition was induced using the chemotherapeutic agent BZ, a reversible inhibitor of the 26S proteasome approved for the treatment of multiple myeloma and mantle cell lymphoma. To determine the optimal concentration of BZ, the effect of proteasome inhibition on glucose metabolism was determined in myotubes obtained from NGT donors. Results are means ± SE. SCR, scramble. Effect of combined silencing of proteasome subunits (PSMA1/PSMA6/PSMB2: siCOMB) on proteasome subunits expression PSMA1 (B), PSMA6 (C), PSMB2 (D), and proteasome 26S (E). Protein content of proteasome subunits was normalized by GAPDH. Membranes probed with antibodies against PSMA1 or PSMA6 were reprobed with 26Sa5 and PSMB2, respectively, after striping. Protein abundance of PSMB2 and 26Sa5 was normalized by GAPDH as shown in C and B, respectively. *P < 0.05; SCR vs. siPSMA1 or siCOMB; #P < 0.05, SCR vs. siPSMA6; †P < 0.05, SCR vs. siPSMB2; n = 5.

To confirm the inhibitory influence of BZ on the proteasome, total cellular proteasome abundance was determined following BZ exposure. The total proteasome content of 20S and 26S was reduced at baseline after BZ treatment, while content of 20S after insulin stimulation was also reduced by BZ (Fig. 6, A and B). The protein level of proteasome subunits was also evaluated in isolated myotubes from NGT or type 2 diabetic patients incubated in the absence or presence of BZ and/or insulin. BZ treatment abolished the insulin-mediated increase of PSMA1 and PSMA6 in myotubes derived from NGT donors and reduced the abundance of PSMA6 and PSMB2 only in myotubes derived from type 2 diabetic patients (Fig. 6, C–E).

Effect of BZ Treatment on Protein Carbonylation and Glucose And Lipid Metabolism

Primary skeletal muscle myotubes derived from type 2 diabetic patients exhibit an altered proteomic profile after insulin treatment, with marked deficiencies in the insulin responsiveness of the protein homeostasis maintenance system. To further explore the mechanism by which this system is disrupted and evaluate the role of the proteasome, protein carbonyl content, a measure of oxidative stress, was determined. Protein carbonyl levels at baseline were unaltered

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**Fig. 3.** Effect of siRNA gene silencing of proteasome subunits in cultured myotubes. A: mRNA expression of proteasome subunits following siRNA transfection with a single proteasome PSMA1 (siA1), PSMA6 (siA6), PSMB2 (siB2) subunit, or PSMA1/PSMA6/PSMB2 in combination (siCOMB) in myotubes obtained from NGT donors. Results are means ± SE. SCR, scramble. Effect of combined silencing of proteasome subunits (PSMA1/PSMA6/PSMB2; siCOMB) on proteasome subunits expression PSMA1 (B), PSMA6 (C), PSMB2 (D), and proteasome 26S (E). Protein content of proteasome subunits was normalized by GAPDH. Membranes probed with antibodies against PSMA1 or PSMA6 were reprobed with 26Sa5 and PSMB2, respectively, after striping. Protein abundance of PSMB2 and 26Sa5 was normalized by GAPDH as shown in C and B, respectively. *P < 0.05; SCR vs. siPSMA1 or siCOMB; #P < 0.05, SCR vs. siPSMA6; †P < 0.05, SCR vs. siPSMB2; n = 5.
between myotubes derived from NGT and type 2 diabetic donors. However, in response to insulin and BZ treatment, protein carbonyl content was increased 27 and 73%, respectively, in myotubes from type 2 diabetic patients (Fig. 7A).

Due to the lack of an effect of insulin on proteasome subunits abundance in myotubes derived from type 2 diabetic patients, we determined whether reduced proteasome function alters substrate metabolism. To further probe the effect of BZ

**Fig. 4.** Effect of proteasome subunits silencing on glycogen synthesis, insulin signaling, and proteasome activity. A: glycogen synthesis was assessed after siRNA silencing of a single proteasome PSMA1, PSMA6, PSMB2 subunit, or PSMA1/PSMA6/PSMB2 in combination (siCOMB) in myotubes. Effect of combined proteasome subunits silencing on pAkt<sup>Thr308</sup> (B), pAkt<sup>Ser473</sup> (C), and proteasome activity (D). Abundance of pAkt<sup>Ser473</sup> and pAkt<sup>Thr308</sup> was normalized by GAPDH as shown in Fig. 3, B and C, respectively. *P < 0.05, SCR-insulin vs. siRNA-Insulin; €P < 0.05 SCR-baseline vs. siCOMB-baseline; **P < 0.05, basal vs. insulin.

**Fig. 5.** Effects of bortezomib (BZ) treatment on glucose metabolism, cell viability, and proteasome activity. Myotubes from NGT donors were treated with DMSO or BZ (1, 10, and 100 nM and 1 μM BZ). Glucose incorporation into glycogen was assessed in myotubes obtained from NGT individuals after 24-h incubation with DMSO (Baseline), or 1, 10, and 100 nM and 1 μM BZ (A); Western blot of caspase 3, indicating cleavage events with presence of 17- and 19-kDa bands, n = 6 (B); BZ dose-dependent reduction of cell viability (C); and proteasome activity (D). Results are means ± SE. *P < 0.05, BZ vs. baseline; **P < 0.05, BZ + I vs. BZ; n = 5.
treatment on metabolic responses, glucose and lipid metabolism was determined in myotubes derived from type 2 diabetic patients or NGT individuals. Insulin treatment (120 nM) for 24 h increased glucose incorporation into glycogen (22%; \( P < 0.05 \)) only in myotubes derived from NGT donors, further signifying an intrinsic insulin resistance in type 2 diabetes. BZ exposure reduced glucose incorporation into glycogen in myotubes derived from NGT and type 2 diabetic donors under basal and insulin-stimulated conditions (Fig. 7B). Lactate production was increased in response to insulin in myotubes derived from NGT and type 2 diabetic donors. BZ exposure enhanced lactate production 45% (\( P < 0.05 \)) only in myotubes derived from type 2 diabetic patients (Fig. 7C), although a trend was noted in myotubes derived from NGT donors.

The effect of BZ treatment on basal and insulin-stimulated palmitate oxidation was also determined. Basal palmitate oxidation was reduced 33% in myotubes from type 2 diabetic vs. NGT donors, while BZ-induced palmitate oxidation was reduced 45% only in myotubes derived from NGT individuals (\( P < 0.05 \); Fig. 7D). BZ treatment reduced protein synthesis in myotubes derived from type 2 diabetic patients (Fig. 7E) but not NGT individuals.

**Effect of BZ Treatment on Insulin Signaling**

Since BZ treatment reduces glycogen synthesis in myotubes obtained from NGT donors, the potential link between the proteasome and the canonical insulin signaling cascade was examined. In contrast to the suppression of glucose incorporation into glycogen, BZ exposure (10 nM) enhanced insulin-stimulated phosphorylation of Akt\( ^{Ser473} \) and Akt\( ^{Thr308} \) (Fig. 8, A and B) but did not alter total IRS1 protein abundance (Fig. 8C). Similarly, BZ exposure increased basal and insulin-stimulated phosphorylation of glycogen synthase kinase-3\( ^{Ser9} \) (GSK3\( ^{Ser9} \)). This effect was neutralized when adjusted to the total abundance of GSK3\( ^{Ser9} \), although a significant elevation was still observed in myotubes exposed to both BZ and insulin (Fig. 8D). Similar results were observed for glycogen synthase phosphorylation (G\( ^{Ser41} \); Fig. 8E).

The abundance of proteins involved in protein synthesis and mTOR signaling was also determined. mTOR\( ^{Ser2448} \) phosphorylation was reduced by BZ (Fig. 8F). Although p70S6K1 was unaltered, phosphorylation of ribosomal protein S6 (rpS6) was enhanced after a 24-h BZ exposure (Fig. 8, G and H). Hence, BZ may directly affect the protein synthesis pathway.
Effect of BZ Treatment on PKC Signaling

To further investigate the mechanism underlying the inhibitory effect of BZ on glucose metabolism, PKCδ was examined as a possible negative regulator of glycogen synthesis. BZ treatment increased PKCδThr505 phosphorylation, although normalization with total protein abundance indicates an overall decrease in phosphorylation/protein (Fig. 9A). The effect of BZ exposure on glucose incorporation into glycogen in the presence or absence of the PKC inhibitor GFX was also determined. GFX treatment for 24 h enhanced baseline glucose incorporation into glycogen 95% and prevented the complete suppression of glycogen synthesis by BZ (Fig. 9B). To address whether PKCδ is directly linked to the BZ inhibition of glucose metabolism, siRNA gene silencing was employed to reduce PKCδ signaling. PKCδ silencing reduced PKCδ protein abundance ~50% in myotubes, independent of BZ treatment (Fig. 9C). PKCδ silencing did not rescue the BZ-induced reduction of glycogen synthesis (Fig. 9D), indicating BZ inhibits glycogen synthesis through a PKCδ-independent mechanism.

Effect of BZ Treatment on Oxidative Stress

Due to the effect of BZ on cell arrest and its possible role on development of oxidative stress, the relationship between the proteasome and cellular stress response via the NRF2 system was investigated. Total protein content of the transcription factor NRF2 was increased by BZ exposure in myotubes derived from NGT donors. NRF2Ser40 phosphorylation was elevated when adjusted to total protein abundance (Fig. 10A). Both insulin and BZ treatment increased abundance of the NRF2-regulated stress-responsive heat shock protein HSP90A (Fig. 10B). However, BZ treatment reduced protein abundance of the NRF2-regulated antioxidant and scavenger enzyme superoxide dismutase (SOD1; Fig. 10C), suggesting an increase in oxidative stress after BZ treatment. As oxidative stress leads to an activation of MAPK, we further investigated the link between BZ treatment and MAPK signaling in myotubes obtained from NGT individuals. Phosphorylation and protein content of ERK and p38 were unchanged by BZ exposure (data not shown). However, JNK phosphorylation, but not total protein content, was increased with BZ treatment (Fig. 10D), suggesting this pathway is activated and may be involved in insulin resistance.

DISCUSSION

The genome-to-proteome system functions in a coordinated manner to maintain cell protein dynamics. Disorders that disrupt this homeostatic balance impair energy metabolism and insulin sensitivity. Several lines of evidence implicate intrinsic abnormalities in the skeletal muscle gene/protein signature in the manifestation of insulin resistance in type 2 diabetes (21, 22, 24, 29). Genomic and proteomic analyses reveal that inherent defects in insulin action, protein dynamics, and energy metabolism in skeletal muscle are linked to insulin resistance and type 2 diabetes through different conduits (6, 10, 11, 14, 16, 23, 36). To further unravel the intrinsic nature of insulin
resistance, we have performed a large-scale 2-D DIGE proteomic analysis of myotubes derived from people with normal glucose tolerance or type 2 diabetes (3). We have shown that the abundance of proteins involved in the basal control of metabolism is increased in myotubes derived from type 2 diabetic patients vs. NGT individuals (3). Here, we report an altered abundance of insulin-responsive proteins involved in carbohydrate and amino acid metabolism and protein synthesis, as well as several novel insulin-responsive targets, detected using 2-D DIGE proteomic analysis (Supplemental Table S1 and Table 2). Additionally, the abundance of several proteins involved in protein homeostasis was reduced in insulin-stimulated myotubes obtained from type 2 diabetic patients vs. NGT individuals. Careful examination of the UPS system revealed a link between proteasome maintenance and insulin signaling, implying that defects in the protein homeostasis system may impact insulin action and force a shift from substrate metabolism to cell development and survival in type 2 diabetes.

In our 2-D DIGE proteomic analysis, proteins identified as insulin-responsive only in NGT-derived myotubes were associated with four categories within the protein homeostasis maintenance system, including the translation-ribosome system (BAT; RNA-dependent ATPases that mediate ATP hydrolysis during pre-mRNA splicing), the chaperone/protein folding family [heat shock 27-kDa (HSPB1)], the autophagosomal/lysosomal pathway (the vacuolar protein sorting 29 isoform1 VPS29), and the ubiquitin-UPS (proteasomes PSMA1, PSMA6, PSMB2). In myotubes derived from individuals with NGT, insulin increases ubiquitin-dependent protein degradation and, conversely, through control of protein degradation, the UPS restricts levels of available signaling molecules for insulin action (7). Thus insulin resistance in type 2 diabetes may disturb the well-orchestrated degradation by the UPS.

We further examined a direct link between type 2 diabetes and the proteasome by exploring molecular alterations in UPS components. Consistent with changes in UPS protein abundance, we found that insulin increased mRNA and protein levels of the proteasome subunits PSMA1, PSMA6, and PSMB2 and HSPB1 in myotubes from NGT but not type 2 diabetic donors. Enhanced PSMA1 and PSMB2 gene expres-
sion has been observed in human skeletal muscle during a hyperinsulinemic clamp (23), implicating the ubiquitin-UPS in insulin signaling (5, 19, 26). However, the role of BAT1, PSMA1, PSMA6, PSMB2, and VPS29 has not been previously associated with insulin resistance or type 2 diabetes. HSPB1 induction may improve insulin sensitivity in type 2 diabetes (20) and control cell growth and stress responses through the proteasome pathway (23). A combined silencing of UPS components reduced basal and insulin-stimulated glucose metabolism in myotubes derived from NGT individuals. Thus altered protein dynamics may contribute to a metabolic disease phenotype, further implicating a role for insulin signaling in the regulation of cellular protein content balance (25). Collectively, our findings suggest that alterations in ubiquitin-UPS components may constitute a molecular underpinning for the pathogenesis of insulin resistance. In addition, intrinsic differences between type 2 diabetes and NGT are detectable in skeletal muscle at the transcript level.

To further investigate the molecular underpinnings of insulin resistance and protein homeostasis maintenance, complete interruption of the UPS was induced using BZ, an anti-cancer agent that specifically inhibits proteasome activity. Our aim was to reveal the intrinsic defects underlying skeletal muscle insulin resistance in type 2 diabetes. BZ treatment was more effective than gene silencing of specific proteasome subunits in reducing the proteasome activity. BZ treatment reduced glucose metabolism in myotubes derived from NGT and type 2 diabetic donors. Furthermore, BZ treatment increased lactate production in myotubes from type 2 diabetic donors, with a trend noted in NGT-derived cells. Palmitate oxidation was suppressed by BZ exposure but only in myotubes from NGT donors. This difference may be explained by a lower baseline level of lipid oxidation in type 2 diabetes. We also found protein synthesis measured as phenylalanine incorporation into protein was decreased and protein carbonylation, a form of protein oxidation promoted by reactive oxygen species, was increased in myotubes derived from type 2 diabetic patients after BZ exposure. The lack of an effect of BZ on protein carbonylation in myotubes from NGT donors may reflect a well-balanced oxidative stress response, whereas the metabolic disturbances observed in the type 2 diabetic donors may reflect an exaggerated stress response arising from the diseased state. In sum, proteasome inhibition unveils intrinsic disorders in cellular oxidative stress response and metabolism related to type 2 diabetes.

Our proteomic analysis and proteasome inhibitor studies suggest that proteasomal dysfunction may exacerbate metabolic disorders in type 2 diabetes by imparting an insulin-resistant signature in skeletal muscle. To further understand the molecular mechanisms underlying the UPS system in type 2 diabetes, we investigated the abundance and phosphorylation of proteasome subunits in response to UPS inhibition. Our findings suggest that proteasome inhibition promotes an exasperated stress response arising from the diseased state. In sum, proteasome inhibition unveils intrinsic disorders in cellular oxidative stress response and metabolism related to type 2 diabetes.

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**Fig. 9.** Effects of BZ treatment and PKCδ inhibition on glucose metabolism. A: protein abundance and phosphorylation of pPKCδThr505 was determined in myotubes derived from NGT donors. Results are reported as phosphorylated/total protein. B: glucose incorporation into glycogen was determined after a 24-h exposure of myotubes to BZ (10 nM) or vehicle in the absence or presence GFX (500 nM). C: PKCδ protein abundance was determined after incubation in the absence or presence of BZ and siRNA-mediated PKCδ silencing. D: glycogen synthesis was determined in the absence or presence of BZ and siRNA-mediated PKCδ silencing. Results are means ± SE. GFX, GF-109203X. *P < 0.05, Baseline vs. stimuli; #P < 0.05, BZ vs. BZ and GFX; #P < 0.05 siPKCδ vs. SCR; n = 5–8 NGT subjects.
indicating BZ inhibits glycogen synthesis by a PKCδ-independent mechanism.

Oxidative cellular stress is directly correlated with proteasome action (1). An amplified Nrf2 response to oxidative stress may contribute to the removal of oxidized proteins and metabolic disorders (8). Our study reveals that treatment of human myotubes with BZ impacts the cellular stress response by increasing NRF2 phosphorylation and consequently HSP90 expression. Oxidative stress leads to JNK activation, which may contribute to the impairment in insulin sensitivity observed after proteasome inhibition. BZ may act at the translational and posttranslational level to reduce proteasome abundance, which may subsequently disturb cellular protein homeostasis and enhance the NRF2-mediated oxidative stress response. These findings imply that the induction of a cellular stress response through proteasome inhibition may cause redox homeostasis disruption and metabolic arrest (8, 32).

In conclusion, the 2-DIGE proteome analysis reported herein reveals that insulin-induced protein turnover is impaired in type 2 diabetes due to a dysregulation of proteins associated with the proteasome-degradation system. Pharmacological inhibition of the proteasome using BZ, a reversible inhibitor of the 26S proteasome, negatively impacts glucose metabolism, with concomitant alterations in phosphorylation and abundance of proteins involved in insulin signaling, metabolism, and oxidative stress responses. Our findings may directly link the proteasome degradation system to the maintenance of cellular stress and substrate metabolism and further support the existence of intrinsic insulin resistance in skeletal muscle from type 2 diabetic patients.

ACKNOWLEDGMENTS

We are grateful to Gunilla Elam for the design of the graphical Fig. 1.

GRANTS

This work was supported by grants from the Center for Gender Medicine at Karolinska Institutet, Swedish Research Council, Swedish Society of Medicine, Åke Wiberg Foundation, Magnus Bergvalls Foundation, Fredrik and Ingrid Thuring Foundation, Knut and Alice Wallenberg Foundation (2005.0120), Strategic Research Programme in Diabetes at Karolinska Institutet, and European Research Council Ideas Program (ICEBERG, ERC-2008-AdG23285).

DISCLOSURES

J. Östling, A. C. Nyström, and J. Oscarsson are employees of AstraZeneca Research and Development (Mölndal, Sweden). AstraZeneca is a global biopharmaceutical company specializing in the discovery, development, manufacturing, and marketing of prescription medicine. L. Al-Khalili, T. de Castro Barbosa, J. Massart, P. G. Cuesta, M. E. Osler, M. Katayama, and J. R. Zierath report no potential conflicts of interest relevant to this article.

AUTHOR CONTRIBUTIONS

REFERENCES