Increased Secretion and Expression of Myostatin in Skeletal Muscle From Extremely Obese Women

Dustin S. Hittel,[1] Jason R. Berggren,[2] Jane Shearer,[1] Kristen Boyle,[2] and Joseph A. Houmard[2]

OBJECTIVE—Obesity is associated with endocrine abnormalities that predict the progression of insulin resistance to type 2 diabetes. Because skeletal muscle has been shown to secrete proteins that could be used as biomarkers, we characterized the secreted protein profile of muscle cells derived from extremely obese (BMI 48.8 ± 14.8 kg/m^2; homeostasis model assessment [HOMA] 3.6 ± 1.0) relative to lean healthy subjects (BMI 25.7 ± 3.2 kg/m^2; HOMA 0.8 ± 0.2).

RESEARCH DESIGN AND METHODS—We hypothesized that skeletal muscle would secrete proteins that predict the severity of obesity. To test this hypothesis, we used a "bottom-up" experimental design using stable isotope labeling by amino acids in culture (SILAC) and liquid chromatography/mass spectometry/mass spectometry (LC-MS/MS) to both identify and quantify proteins secreted from cultured myotubes derived from extremely obese compared with healthy nonobese women.

RESULTS—Using SILAC, we discovered a 2.9-fold increase in the secretion of myostatin from extremely obese human myotubes. The increased secretion and biological activity of myostatin were validated by immunoblot (3.16 ± 0.18, $P < 0.01$) and a myoblast proliferation assay using conditioned growth medium. Myostatin was subsequently shown to increase in skeletal muscle (23%, $P < 0.05$) and plasma (35%, $P < 0.05$) and to correlate ($r^2 = 0.6$, $P < 0.05$) with the severity of insulin resistance.

CONCLUSIONS—Myostatin is a potent antianabolic regulator of muscle mass that may also play a role in energy metabolism. These findings show that increased expression of myostatin in skeletal muscle with obesity and insulin resistance results in elevated circulating myostatin. This may contribute to systemic metabolic deterioration of skeletal muscle with the progression of insulin resistance to type 2 diabetes. ***Diabetes*** **58:30–38, 2009**

Obesity and type 2 diabetes are associated with endocrine abnormalities that are either precipitated by or precede the onset of peripheral insulin resistance (1). These include changes in circulating proteins and peptides that produce endothelial dysfunction, low-grade inflammation, and a prothrombotic state, all of which contribute to increased cardiovascular risk (2–4). Secreted proteins or the "secretome" constitute an important class of biologically active molecules that are released into circulation where they facilitate cross-talk between organ systems. Because secreted proteins are also involved in the progression of cardiovascular disease and cancer, there is significant interest in mining the secretome for novel biological markers (5). Whereas endocrine organs specialize in the secretion of proteins into circulation, there is mounting evidence that adipose tissue and skeletal muscle constitutively or intermittently secrete bioactive proteins (6,7). In this study, we hypothesized that skeletal muscle of extremely obese and insulin-resistant women would secrete proteins into circulation that act as prognostic or diagnostic biomarkers of obesity-associated comorbidities. However, a top-down approach toward identifying protein biomarkers in blood is hampered by an abundant background of serum proteins, wherein a secreted protein of interest may be diluted several orders of magnitude (5,8). To overcome these limitations, we adopted a bottom-up approach for characterizing the skeletal muscle secretome using primary human muscle cells and stable isotope labeling by amino acids in culture (SILAC), which allows for the identification of muscle-specific proteins and for the quantification of proteins between samples (5,8).

Primary human muscle cells are considered a valid model for studying metabolic disorders with obesity and type 2 diabetes because perturbances evident in vivo, such as depressed lipid oxidation and abnormal gene expression, are retained in myotubes raised in culture, suggesting an inherent characteristic (9–12). Herein, we describe our primary finding that myostatin expression and secretion is increased in both cell culture and skeletal muscle of extremely obese human subjects.

RESEARCH DESIGN AND METHODS

To achieve the objectives of this study, we developed a bottom-up workflow for identifying protein biomarkers secreted from obese and insulin-resistant human skeletal muscle. In the first set of experiments, we identified the differential secretion of myostatin from pure cultures of primary human muscle cells using SILAC. The advantage of using this method is that it allowed for the concentration and identification of low-abundance, muscle-derived proteins in conditioned cell culture medium and for the quantification of protein abundance between samples. In the second set of experiments, we confirmed the increased secretion and biological activity of myostatin in conditioned media from extremely obese human myotubes and the increased abundance of myostatin in both skeletal muscle and plasma from extremely obese human subjects. This experimental workflow allowed us to successfully validate our cellular findings at the organ and systemic level.

The clinical characteristics of the four subject populations studied (lean, obese, and extremely obese) are shown in Table 1. Research participants were categorized on the basis of BMI and the classification of overweight and obesity set forth by the National Institutes of Health. BMI criteria for the normal-weight and extremely obese subjects were ≤24.9 and ≥40 kg/m^2, respectively. All subjects were sedentary and not exercising regularly. Skeletal muscle samples were obtained after a 12-h overnight fast from the vastus lateralis as previously described (13,14). With the exception of diabetic patients, no subjects were taking any medications known to affect carbohydrate or lipid metabolism. The experimental protocols were approved by the

From the [1]Human Performance Laboratory, Faculty of Kinesiology, Roger Jackson Center for Health and Wellness, University of Calgary, Calgary, Alberta, Canada; and the [2]Human Performance Laboratory and Department of Exercise and Sport Science, East Carolina University, Greenville, North Carolina.

Corresponding author: Dustin S. Hittel, dhittel@kin.ucalgary.ca.

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TABLE 1
Clinical characteristics of donor subjects for muscle cell culture

	Lean	Obese	Extremely obese
n	6	5	9
Age (years)	41.2 ± 5.0	46.8 ± 4.2	45.8 ± 3.9
BMI (kg/m^2)	25.7 ± 1.3	32.3 ± 1.1*	48.9 ± 4.9*
Glucose (mg/dl)	97.0 ± 6.0	91.1 ± 2.9	97.7 ± 4.4
Insulin (μU/ml)	3.3 ± 0.9	7.2 ± 2.7	14.6 ± 2.7*
HOMA	0.8 ± 0.2	1.3 ± 0.6	3.6 ± 1.0*

Data are means ± SE. *Significant differences at $P < 0.05$, lean vs. obese and extremely obese subjects.

East Carolina University Policy and Review Committee on Human Research, and written informed consent was obtained from all subjects. Plasma glucose was measured with an oxidation reaction using a glucose analyzer (YSI 2300 STAT Plus; YSI, Yellow Springs, OH), and plasma insulin was measured by immunoassay (Access Immunoassay System; Beckman Coulter, Fullerton, CA). The homeostasis model assessment (HOMA) for insulin resistance and β-cell function was calculated from fasting plasma glucose and insulin concentrations (15). Estimates of insulin resistance using the HOMA have been well documented as a reliable estimate of basal insulin resistance and correlate well with the euglycemic-hyperinsulinemic clamp for evaluating insulin sensitivity (15).

Stable isotope labeling and collection of secreted proteins. The advantage of growing cells in stable isotope–labeled media is that all myotube-derived proteins are uniformly labeled with $^{13}C_6$-labeled Lys after three passages, allowing secreted proteins and serum contaminants to be distinguished (5). Primary human muscle cells derived from three lean and extremely obese (based on BMI) subjects were grown in medium with (obese samples) or without (lean samples) ^{13}C-Lys, and then secreted proteins were collected and analyzed. The harvesting and culturing of satellite cells from skeletal muscle biopsy material were conducted as previously described (10,12). For metabolic labeling for quantitative proteomic profiling, cells were incubated in Dulbecco's modified Eagle's medium (DMEM), which was custom made without lysine (Invitrogen, Carlsbad, CA). Labeling media was supplemented with $^{13}C_6$-Lys (Invitrogen) for cells derived from extremely obese donors, whereas unlabeled media were supplemented with regular L-Lys for lean cells and 10% dialyzed fetal bovine serum (FBS) and 50 μg/ml gentamicin/amphotericin B. Cells were subcultured in collagen I–coated 75-cm^2 flasks to passage 3, which has been shown previously to produce fully labeled cells (8). Differentiation into myotubes was initiated at 90% confluence using labeled and unlabeled differentiation medium containing 2% horse serum with media changes every 2 days for a total of 9 days (16). There were no obvious differences in the extent of myotube differentiation between groups. To collect secreted proteins, cells were washed five times with PBS buffer to remove excess equine serum proteins and then incubated in serum-free labeled and unlabeled media for 18 h (8). Secreted proteins were collected for 18 h to reduce the possibility of cell lysis, which would release cytosolic proteins into serum-free medium (5,17). Conditioned media from each of the labeled and unlabeled cultures were pooled in 50-ml tubes (BD Biosciences Falcon, San Jose, CA), centrifuged at 300*g* and then at 1,000*g*, and filtered through a 0.22-μm nylon filter (Millipore, Bedford, MA) before a final spin of 100,000*g* to remove any cell debris. Conditioned media were flash frozen in liquid nitrogen, concentrated 100 times under vacuum, and desalted against 10 mmol/l Tris-HCl, pH 7, using P6 Bio-Spin columns (Bio-Rad, Hercules, CA); and the protein content was determined using the Bio-Rad protein assay reagent following the manufacturer's instructions. Secreted protein fractions were then pooled and separated by SDS-PAGE, stained with Coomassie, cut into six pieces corresponding to separate molecular weight ranges (Fig. 1), and then digested with trypsin (Promega, Madison, WI) as previously described (13).

LC-MS/MS, protein identification, and quantification. The resulting peptide solutions were analyzed by LC-MS/MS at the southern Alberta mass spectrometry center at the University of Calgary (Calgary, AB, Canada). Chromatographic separations of peptides were performed with a C18 analytical column using an Agilent 1100 nanoLC system (Agilent Technologies, Santa Clara, CA). Peptides were loaded onto an enrichment and eluted with 0.2% formic acid and 10% water in acetonitrile over 50 min. The analytical column was connected online to a Qstar XL Hybrid quadrupole time-of-flight (TOF) mass spectrometer fitted with a nanospray ion source (Applied Biosystems, Foster City, CA). TOF MS experiments were performed in positive ion mode over an *m/z* range of 400–1,500. Automated tandem MS

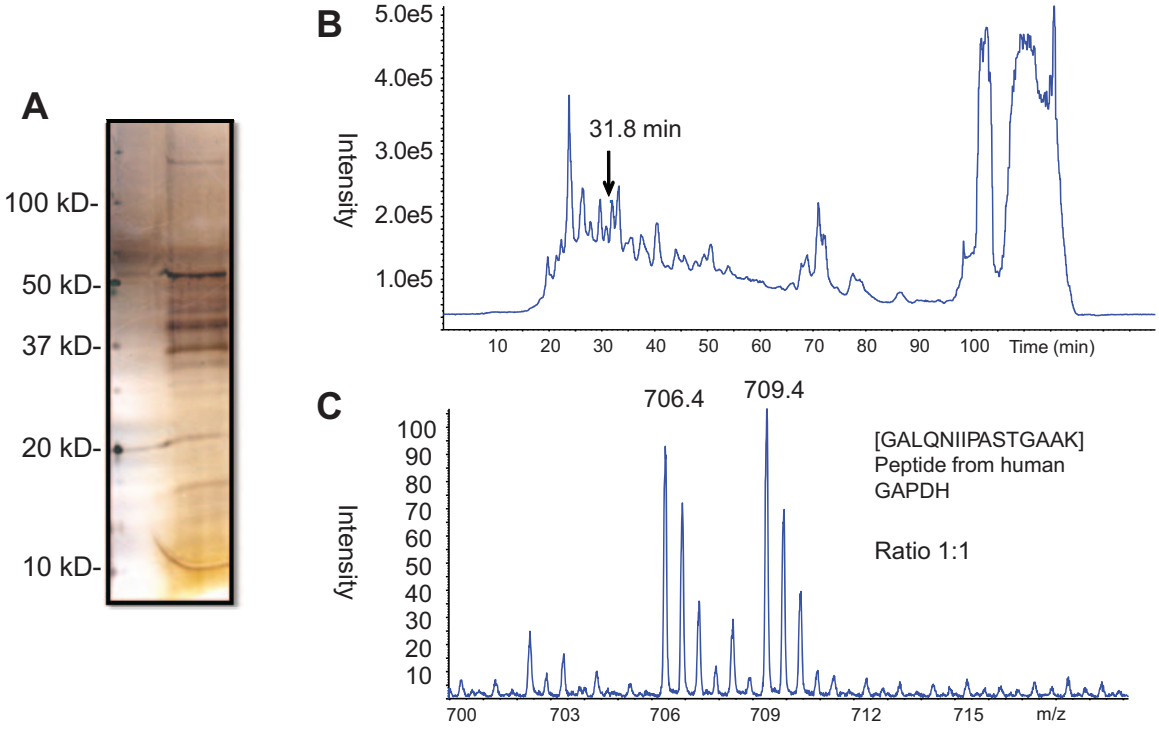
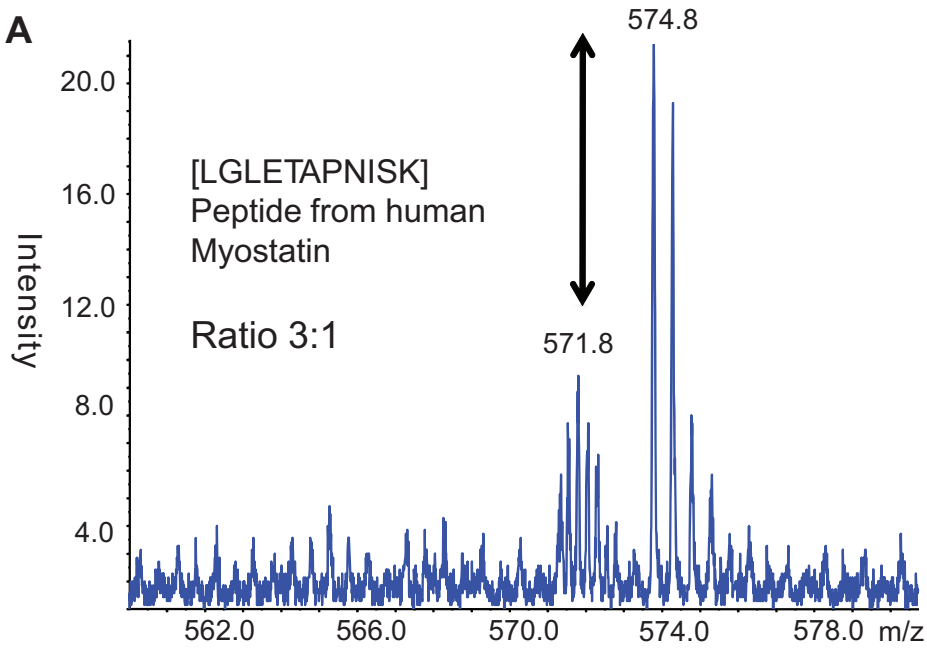


FIG. 1. LC-MS/MS identification and quantification of $^{13}C_6$-Lys and unlabeled secreted proteins from lean and extremely obese primary human myotubes. *A*: Proteins from 18-h conditioned, serum-free media from unlabeled (lean) and $^{13}C_6$-Lys (extremely obese) myotubes were combined 1:1 and resolved by 4–16% SDS-PAGE. *B*: The base peak chromatograph showing overall peak intensities and retention time of all the peptides extracted from a gel slice between 37 and 15 kDa. *C*: A peptide eluting at 31.8 min as a doublet at *m/z* 706.4 and 709.4, corresponding to the unlabeled and $^{13}C_6$-Lys peptide from human GAPDH. The peptide sequence shown on top of the spectrum was obtained by MS/MS analysis. The labeled and unlabeled peptides are 3 Da apart in a 1:1 ratio and agree with one $^{13}C_6$-Lys residue in the doubly charged peptide. (Please see http://dx.doi.org/10.2337/db08-0943 for a high-quality digital representation of this figure.)



B

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1   MQKLQLCVYI YLFMLIVAGP VDLNENSEQK ENVEKEGLCN ACTWRQNTKS
51  SRIEAIKIQI LSK LGLETAP NISK DVIRQL LPKAPPLREL IDQYDVQRDD
101 SSDGSLEDDD YHATTETIIT MPTESDFLMQ VDGKPKCCFF KFSSKIQYNK
151 VVXAQLWIYL RPVETPTTVF VQILRLIKPM KDGTRYTGIR  SLK LDMNPGT
201 GIWQSIDVKT VLQNWLKQPE SNLGIEIKAL DENGHDLAVT FPGPGEDGLN
251 PFLEVKVTDT PKRSRRDFGL DCDEHSTESR CCRYPLTVDF EAFGWDWIIA
301 PKRYKANYCS GECEFVFLQK YPHTHLVHQA NPRGSAGPCC TPTK MSPINM
351 LYFNGK EQII YGKIPAMVVD RCGCS
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FIG. 2. LC-MS/MS identification and quantification of secreted myostatin protein. *A*: The top spectrum shows a peptide doublet at *m/z* 571.8 and 574.8 corresponding to unlabeled and $^{13}C_6$-Lys peptides from human myostatin. The labeled and unlabeled peptides are 3 Da apart in a 3:1 ratio and agree with one $^{13}C_6$-Lys residue in the doubly charged peptide. The peptide sequence shown on top of the spectrum was obtained by MS/MS analysis of the fragmented peptide. *B*: Shown in bold an underlined are the relative positions of the other myostatin peptides identified in the primary sequence of human myostatin.

analyses were carried out using a standard data-dependent configuration, in which the three most intense peptides (2+ or 3+ charge states) in an MS scan were automatically selected for sequencing. Protein searches were performed against the human NCBInr and SwissProt databases using the Mascot distiller (Matrix Science, London) using the Mascot search algorithm. The mass tolerance for the precursor peptide ion was set at 200 parts per million, and the mass tolerance for the MS/MS fragment ions was set to 0.5 Da. Quantitation was performed manually according to the manufacturer's instructions (Invitrogen). Briefly, we used the Mascot peptide summary list generated for each LC-MS/MS run and Analyst QS 1.1 Software (Applied Biosystems) to analyze selected ion chromatograph data from the raw WIFF file generated by the LC-MS/MS analysis. The retention time and protein identification for each peptide were then confirmed by the *y* ions from each MS/MS and the relative abundance of selected peptide pairs (Figs. 1 and 2) calculated as previously described (8). The search parameters allowed for variable modifications, including amidomethylation of cysteine, oxidation of methionine, and presence of $^{13}C_6$-Lys. Secreted proteins had to have a minimum of one $^{13}C_6$-Lys peptide identified with high confidence and had to be verified by manual inspection for having a consecutive series of *y* ions (8).

Western blot analysis of conditioned media, skeletal muscle, and plasma. Myostatin protein levels in conditioned media, muscle cells, whole skeletal muscle, and plasma were verified using Western immunoblot. To collect secreted proteins for quantifying myostatin levels, cells (9-day myotubes) were washed five times with PBS and incubated with serum-free Optimem (Invitrogen) for an additional 24 h as described previously (18,19). Optimem medium contains growth factors that promote cell survival with extended incubations and is specifically designed for the characterization of secreted proteins. Conditioned medium was decanted into 50-ml tubes (BD Biosciences Falcon), centrifuged at 300*g* and then at 1,000*g*, and filtered through a 0.22-μm nylon filter (Millipore) before a final spin of 100,000*g* to remove any remaining cell debris. Conditioned media were then flash frozen in liquid nitrogen and lyophilized under vacuum and then precipitated with 10% trichloroacetic acid followed by several washes with −20°C acetone as described previously (18). In all, 50 μg protein was collected per sample, separated by SDS-PAGE, and transferred to Immobilon-P PVDF Membrane (Millipore).

For Western blot analysis of myostatin levels in muscle, nitrogen-pulverized skeletal muscle or myotubes were extracted using digitonin 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 40 min (20). This was then centrifuged at 8,000*g* for 20 min to remove insoluble cellular debris. Protein concentration was determined using the Bio-Rad protein assay dye reagent, following the manufacturer's instructions (Bio-Rad, Hercules, CA). Twenty micrograms cellular protein (19,21) was then separated by SDS-PAGE and transferred to Immobilon-P PVDF Membrane (Millipore). Membranes were stained with Ponceau S to confirm equal loading and transfer efficiency.

To analyze plasma proteins for Western blot analysis, 2 μl plasma was diluted 1:10 in PBS, and 100 μg protein was loaded on precast 4–12% gradient SDS-PAGE gels (Invitrogen) and transferred to Immobilon-P PVDF Membrane (Millipore). The primary antibody used in this study was a polyclonal anti-myostatin antibody (R&D Systems, Minneapolis, MN) raised against *Escherichia coli*–derived, recombinant whole-mouse myostatin in goats. We chose this specific antibody both for its ability to reliable detect human myostatin and to neutralize myostatin activity in a biological assay. The primary antibody used to detect glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was a mouse monoclonal antibody (Abcam, Cambridge, MA). Membranes were incubated in 1/1,000 in primary antibody in Tris-buffered

saline with Tween for 20 h at 4°C. After washing, primary antibodies were detected using horseradish peroxidase–conjugated anti-goat secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1/5,000 and a SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Images were acquired and quantified using a ChemiGenius Bioimaging System (Syngene, Frederick, MD).

Myoblast proliferation assay. To assess the biological activity of secreted myostatin protein in conditioned media, we used a myoblast proliferation assay as previously described, which is based on the ability of myostatin to inhibit progression of myoblasts from the G_1- to S-phase of the cell cycle (18,22). Briefly, cells were seeded at 1,000 cells/well in 96-well plates (Nunc Nalgene, Rochester, NY) in pooled 48-h conditioned growth medium (DMEM plus 10% FBS) from human muscle cells derived from three lean and extremely obese subjects. Forty-eight hours has been shown to be sufficient time for the accumulation of secreted myostatin in conditioned media (18,22). As a control, cells were also proliferated in conditioned media that were preincubated with 20 μg/ml anti-myostatin antibody or in unconditioned growth medium. This concentration of anti-myostatin antibody has been shown by the manufacturer (R&D Systems) to neutralize up to 30 ng/ml active myostatin protein. At daily intervals over a 4-day period, proliferating myoblasts were trypsinized, and cell densities were determined (in quadruplet) using a hemocytometer (Hausser Scientific, Horsham, PA).

Statistical analysis. For comparisons of myostatin abundance in muscle and plasma, we used an independent *t* test with two-tailed distribution with the significance level set to $P < 0.05$. For comparison of myostatin protein abundance in conditioned medium and cells and myoblast proliferation assays, we used a Kruskal-Wallis One Way ANOVA using SigmaStat (Systat, San Jose, CA) and a Tukey's post hoc test for multiple comparisons with $P < 0.05$ or 0.01 indicating significance. Linear regression analysis were conducted using the least squares method (SigmaPlot) with $P < 0.05$ as the probability of being wrong in concluding that there is a true association.

RESULTS

Analysis of secreted proteins. The 42 proteins identified in 18-h conditioned, serum-free media from primary cultured human myotubes are listed in Table 2. Of these, 28 have been previously shown to be secreted. The remaining 14 are metabolic enzymes, cytoskeletal, or contractile proteins. The presence of intracellular proteins in conditioned culture media has been described in numerous surveys of protein secretion (8,17,23). Several of the proteins secreted by primary human myotubes have been described previously in studies of differentiating mouse myotubes (17) and human adipose (23) and retinal pigment epithelial cells (8). Indeed, two of these proteins, Type I Collagen and Osteonectin, are secreted by all three (Table 2), suggesting that they are essential secreted components of many cell types. It is also worth noting that 13 of the 28 secreted proteins were predicted using in silico modeling of the human skeletal muscle secretome (24).

In Fig. 1, we show a representative LC-MS chromatograph (Fig. 1*B*) and the identification of a peptide doublet from Human GAPDH (Fig. 1*C*) for unlabeled and labeled peptide pairs at *m/z* 706.4 and 709.4, respectively. The difference in the mass between these doubly charged ions is 3 Da, indicating that the peptide should contain one lysine residue. According to the ratio of the monoisotopic intensities, GAPDH was secreted in an ~1:1 ratio from lean relative to extremely obese myotubes. GAPDH has also been shown to be secreted into the growth media of a number of cell lines (25). GAPDH was therefore chosen as a loading control for subsequent Western immunoblot validation of myostatin secretion. In Fig. 2, we show a mass spectrum obtained for a peptide unique to myostatin protein with peaks for unlabeled and labeled peptide pairs at *m/z* 571.8 and 574.8, respectively. The difference in the mass between these doubly charged ions is 3 Da, indicating that the peptide should contain one lysine residue. The sequence of the peptide was confirmed by the MS/MS analysis of the peptides and comparison of the *y* and *b* ions as described previously (8). According to the ratio of the peptide intensities, the secreted ratio of myostatin was calculated as 3:1 from extremely obese versus lean myotubes. The other labeled peptides identified from myostatin (Fig. 2*B*) did not appear to have unlabeled companion peptides, indicating significantly higher abundance in conditioned media from extremely obese cells.

Western blot validation of myostatin secretion. Because myostatin was identified as the most robustly differentially secreted protein in conditioned medium (Fig. 2), it became the main focus for validation. As shown in Fig. 3, there were significant differences in the secretion of the 26-kDa mature form of myostatin into conditioned medium from extremely obese (3.16 ± 0.18, $P < 0.01$) myotubes relative to those derived from healthy nonobese subjects. This relative difference approximates the 3:1 ratio calculated using SILAC (Fig. 2). Unlike other studies (18), we did not detect the secretion of the 52-kDa precursor form of myostatin. This may be because of our choice of antibody or the exceptional care we took to minimize cell lysis. Finally, human GAPDH protein was detected using SILAC as being secreted in a ~1:1 ratio (Fig. 1*C*) and was selected as a loading control for secreted proteins. As shown in Fig. 2*A*, GAPDH was detected in similar amounts in conditioned media from lean, obese, and extremely obese cells when compared against differences in myostatin abundance.

Myostatin expression in cultured myotubes, muscle, and plasma. Because of the increased myostatin secretion into conditioned media from extremely obese relative to lean cells (Fig. 3), we also determined the expression of myostatin protein in cultured myotubes, skeletal muscle, and plasma. In Fig. 4, we show that expression of the 52-kDa myostatin precursor protein was significantly elevated (2.0 ± 0.12, $P < 0.01$) in cells cultured from extremely obese compared with lean nonobese donors. The 40-kDa myostatin propeptide was similarly elevated in extremely obese myotubes. Although the mature 26-kDa myostatin peptide was clearly increased in extremely obese cells, it was only sporadically detected (three of eight samples). The processing of the 52-kDa myostatin precursor protein produces an NH_2-terminal, 40-kDa latency-associated peptide (LAP) and the biologically active 26-kDa COOH-terminal dimer (19). These findings are consistent with those of previous studies: that myostatin is rapidly processed and secreted from cultured muscle cells compared with mature muscle (19,22). Because both the expression and secretion of myostatin was increased in cells derived from extremely obese human donors, we also studied its expression in skeletal muscle and plasma in a separate patient cohort.

In Fig. 5, we show a 23% ($P < 0.05$) increase in myostatin 52-kDa precursor protein and a 35% increase in the 26-kDa dimer, respectively ($P < 0.05$), in extremely obese muscle and plasma relative to lean samples. Unlike myotubes, the 52-kDa precursor protein has been shown to be the most abundant immunoreactive species in mature skeletal muscle (19,21). Linear regression analysis of myostatin protein levels relative to HOMA (Fig. 6*A*) revealed a significant correlation ($r^2 = 0.6$, $P = 0.03$), although it seems to correlate better with body mass (Fig. 6*B*) ($r^2 = 0.737$, $P < 0.01$), which is not entirely surprising given the extreme differences in BMI between these groups.

Myoblast proliferation bioassay for myostatin. Myostatin reduces muscle mass in vivo and by inhibiting the

TABLE 2
List of proteins identified in conditioned media of primary human myotubes

	SwissProt	Peptides	Secreted	Evidence
Immune response/complement				
Complement component 4B	P0C0L5	7	Yes	8,24
Complement factor H-related 5	Q9BXR6	2	Yes	8
Growth factors/chemokines				
Epidermal growth factor	P01133	2	Yes	Swiss-Prot
Fibroblast growth factor 17	O60258	2	Yes	Swiss-Prot
Insulin-like growth factor 1	Q59GC5	1	Yes	24
Interferon α2/β1	Q14608	1	Yes	Swiss-Prot
Lymphotactin	P47992	1	Yes	Swiss-Prot
Monocyte chemotactic protein 4, MCP-4	Q99616	2	Yes	Swiss-Prot
Myostatin	Q8HY52	3	Yes	Swiss-Prot
Cell adhesion/extracellular matrix				
Collagen, type III, α1	P02461	2	Yes	17
Collagen, type IV, α2	P08572	4	Yes	17,24
Collagen, type IV, α3	Q01955	2	Yes	17,24
Collagen, type I, α1	P02452	5	Yes	8,17,23,24
Extracellular matrix protein 2	O94769	1	Yes	23,24
Laminin, α2	P24043	4	Yes	24
Laminin, α3	Q16787	3	Yes	24
Tenascin C	P24821	5	Yes	24
Tenascin XB	Q5JNX3	2	Yes	24
Aggrecan core protein	P16112	2	Yes	Swiss-Prot
S100 calcium-binding protein A6/calcyclin	P06703	3	Yes	Swiss-Prot
Osteonectin/SPARC	P09486	18	Yes	17,23,24
Vimentin	P08670	18	Yes	17
Galectin-1	P09382	6	Yes	17
α-Crystallin	P02511	2	Yes	Swiss-Prot
Protease and protease inhibitors				
Plasma protease (C1) inhibitor precursor	P05155	2	Yes	23
Metalloproteinase inhibitor 1 precursor	P01033	12	Yes	8,24
Metalloproteinase inhibitor 2 precursor	P16035	12	Yes	24
Bone morphogenetic protein 1	P13497	2	Yes	Swiss-Prot
Ubiquitin	P62988	4	Yes	17
Intracellular, contractile, and shed proteins				
Dystonin	Q5TBT2	12	No	
Fetal myosin heavy chain	Q9Y623	4	No	
Glyceraldehyde-3-phosphate dehydrogenase	P04406	3	Yes	25
Integrin, α6	P23229	2	No	
Myosin binding protein C, fast type	Q14324	3	No	
Phosphofructokinase, muscle	P08237	5	No	
Titin	Q8WZ42	32	No	
Triosephosphate isomerase 1	P60174	6	No	
Vinculin	P18206	6	No	
Tropomyosin β	P07951	15	No	
SH3 binding glutamic acid–rich protein	Q5T122	2	No	
Desmin	Q549R9	3	No	
Myosin light chain 1	P14649	3	No	
β-Coat protein	P53618	2	No	
Stathmin	P16949	4	No	
Triosephosphate isomerase	P60174	15	No	
Ubiquitin	P62988	4	No	
Phosphoglycerate mutase 1	P18669	4	No	
α-Crystallin	P02511	2	No	
Nebulin	EAX11499	6	No	
Myoglobin	Q8WVH6	3	No	

Proteins identified using SILAC are shown with their accession numbers, number of peptides identified, and literature evidence for being secreted proteins.

proliferation of myoblasts (18,19,22). We cultured mouse C_2C_{12} myoblasts in the presence of 48-h conditioned growth media from lean and extremely obese cultured myotubes (Fig. 7). To differentiate the effects of myostatin from other secreted molecules, we preincubated media with neutralizing concentrations of anti-myostatin antibody. In Fig. 7, we show a significant inhibition in the proliferation of myoblasts grown in conditioned medium from the extremely obese myotubes. These observations are consistent with previous studies of the growth-inhibiting effects of myostatin on myoblast proliferation (18,19,22). After 2 days, these cells were proliferating

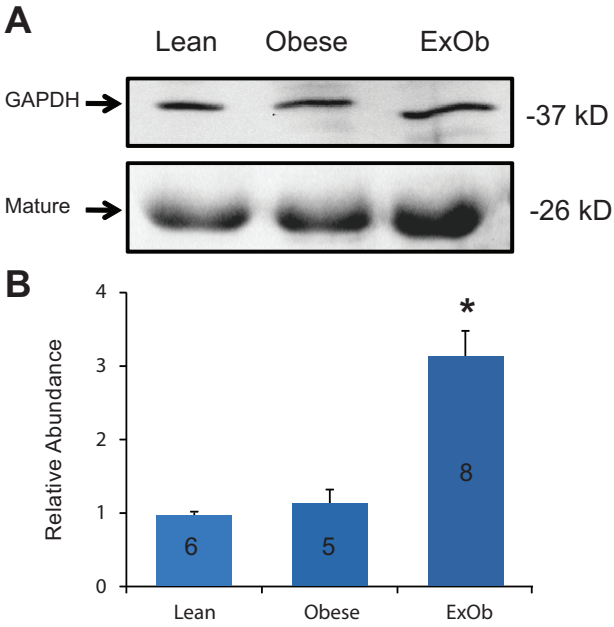

FIG. 3. Myostatin secretion is increased from extremely obese primary human myotubes. Equal amounts of protein from 24-h–conditioned, serum-free media resolved by 15% SDS-PAGE. Myostatin (*A*) and GAPDH (*B*) protein were detected with polyclonal goat anti-myostatin and monoclonal mouse anti-GAPDH antibodies, respectively. The mature form of myostatin is indicated in a representative blot, and the molecular masses of the detected bands are indicated. Number of replicated are indicated for each sample, and the asterisk indicates significant differences ($P < 0.05$). Data are normalized to average lean expression level ± SE. ExOb, exobese. (Please see http://dx.doi.org/10.2337/db08-0943 for a high-quality digital representation of this figure.)

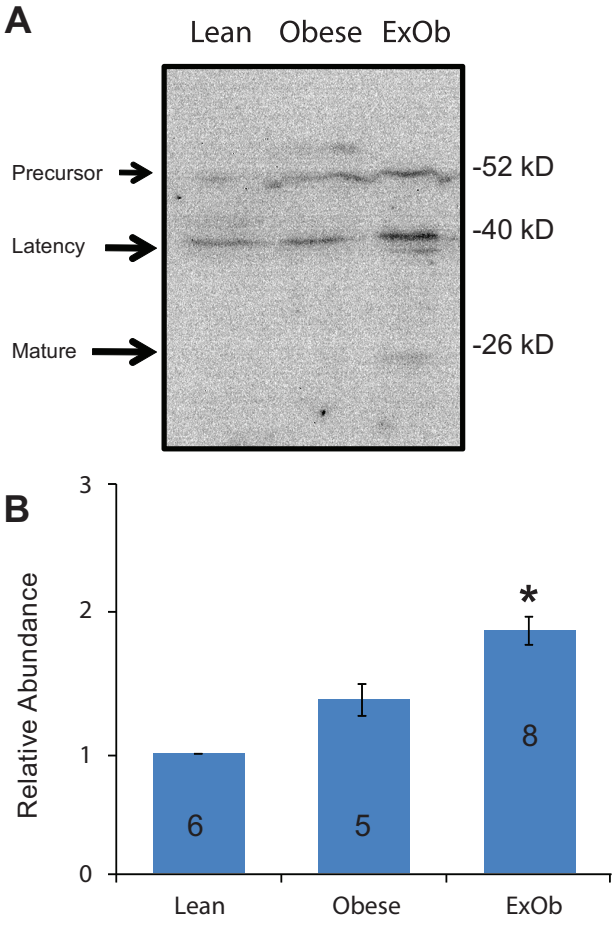

FIG. 4. Myostatin protein is increased in extremely obese primary human myotubes. Equal amounts of total protein from primary human myotubes were resolved by 15% SDS-PAGE. *A*: Myostatin protein was detected with goat polyclonal anti-myostatin antibodies. Precursor, mature, and LAP forms of myostatin are indicated in a representative blot. Molecular masses of the detected bands are indicated. *B*: Only the 52-kDa precursor form of myostatin was quantified; however, the LAP appeared to follow a similar expression profile. Small amounts of the 26-kDa mature myostatin were detected only in extremely obese myotubes. Number of replicated are indicated for each sample, and the asterisk indicates significant differences ($P < 0.05$). Data are normalized to average lean expression level ± SE. (Please see http://dx.doi.org/10.2337/db08-0943 for a high-quality digital representation of this figure.)

significantly more slowly (−2.3 ± 0.11-fold, $P < 0.05$) than control cells. This inhibition of proliferation became more pronounced after 3 days of incubation when there were significant differences in lean compared with obese conditioned medium (2.06 ± 0.11-fold, $P < 0.01$) and between cells grown in anti-myostatin antibody precleared medium (−1.5 ± 0.15-fold, $P < 0.01$). Interestingly, antibody-preincubated cells grown in obese medium grow at the same rate as control cells and faster than lean cells until day 4, suggesting the presence of proliferation-promoting factors in obese conditioned media. Trypsinized cells cultured in lean and obese conditioned medium were routinely stained with Trypan blue and showed no significant differences in the percentage of viable cells.

DISCUSSION

Although skeletal muscle is not considered a secretory organ per se, there is a growing body of evidence that it may constitutively or intermittently secrete biologically active molecules in health and disease (17,24,26,27). The goal of this study was to identify proteins that are secreted into circulation by muscle that could be used as biological markers for the severity of obesity. Here, we report that myostatin is expressed and actively secreted into circulation from cultured muscle cells and mature skeletal muscle from extremely obese relative to healthy nonobese subjects. This is the first known quantitative description of myostatin protein level with obesity and insulin resistance in human tissue. These results confirm and expand on previous studies describing changes in myostatin mRNA expression in a number of experimental models of obesity and weight loss (28–30).

Myostatin is a potent antianabolic inhibitor of muscle growth and development belonging to the transforming growth factor-β (TGF-β) family. As with other TGFs, myostatin is translated as a precursor protein that is cleaved to yield a NH_2-terminal LAP and COOH-terminal peptide dimer that is from the cell (18,19). The mature myostatin dimer is secreted from muscle in a noncovalent association with the LAP, which protects it from degradation and maintains its active conformation. Mature myostatin is released from the LAP by the extracellular matrix–associated bone morphogenetic protein-1/tolloid metalloprotease, which is thought to activate both locally acting (paracrine) and circulating (autocrine) forms of myostatin. The free mature myostatin peptide then binds to the activin type II B receptor through which it exerts its well-characterized antianabolic effects on resident muscle myoblasts by inhibiting their progression from the G1- to S-phase of the cell cycle (18,22). This is why the deletion or inhibition of circulating myostatin produces a hypermuscular phenotype (31,32).

Myostatin was first hypothesized to play a role in metabolism after it was observed that hypermuscular

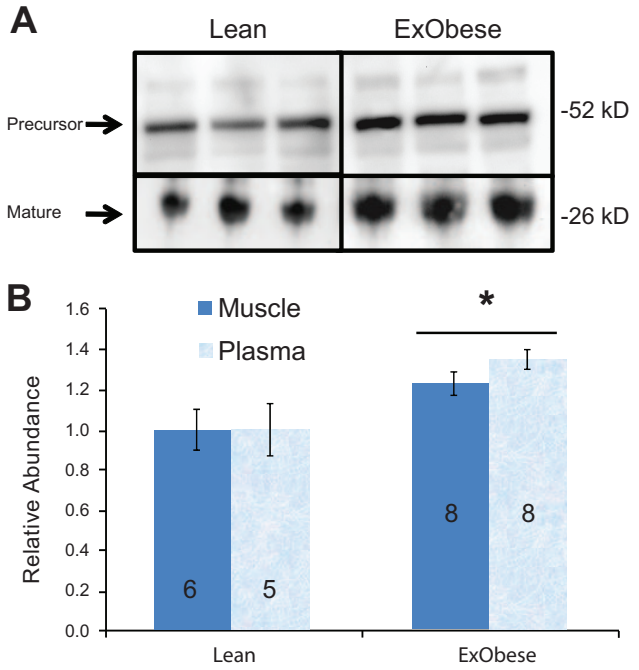

FIG. 5. Myostatin protein is increased in skeletal muscle and plasma from extremely obese subjects. Equal amounts of total protein from human skeletal muscle and plasma were resolved by 15% SDS-PAGE. *A*: Myostatin protein was detected with goat polyclonal anti-myostatin antibodies. Precursor (52 kDa) and mature dimer (26 kDa) forms of myostatin are indicated in representative blots. *B*: Only the 52-kDa precursor form of myostatin was quantified in muscle and the 26-kDa mature form in plasma. Number of replicated are indicated for each sample, and the asterisk indicates significant differences ($P < 0.05$). Data are normalized to average lean expression level ± SE. (Please see http://dx.doi.org/10.2337/db08-0943 for a high-quality digital representation of this figure.)

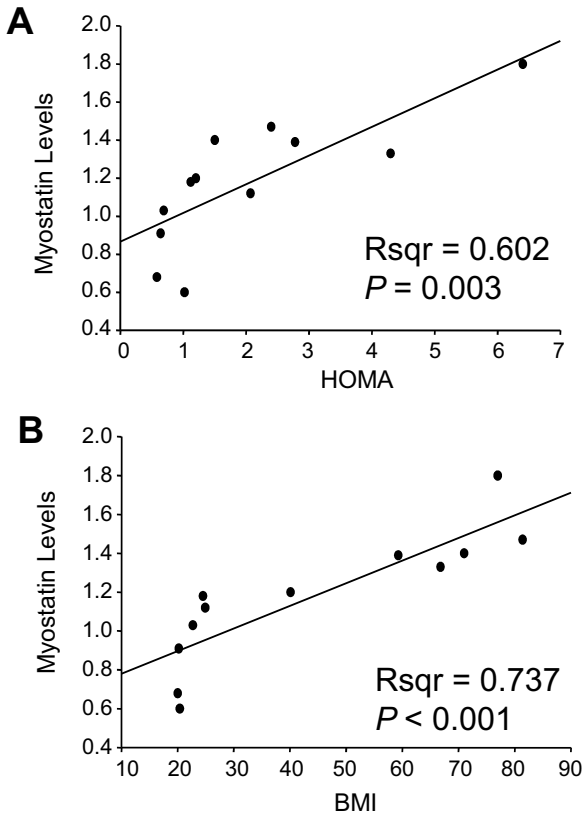

FIG. 6. Linear regression analysis of myostain expression in muscle. Linear regression analyses were conducted using the least squares method to compare muscle myostatin expression levels to HOMA (*A*) and BMI (*B*). Rsqr, r^2.

myostatin null mice have reduced fat mass and are seemingly immune to dietary-induced insulin resistance (33,34). Whether this is due solely to increased lean muscle mass or other metabolic effects of reduced circulating myostatin have yet to be determined (33). Recent studies, however, have shown that myostatin inhibits glucose uptake in placental cell lines, suggesting that it may contribute to systemic insulin resistance with obesity (35). Others have implicated myostatin in a network of genes potentially regulated by insulin with extreme obesity (30). Severe caloric restriction and starvation have also been shown to increase myostatin expression in skeletal muscle, presumably to reduce the metabolic burden of a large muscle mass (36). The evolutionary context for this model is that under starvation conditions, increased circulating myostatin is survivalistic because it preserves brain function, and hence the ability to forage for food, by maintaining blood glucose (37). Considering these important observations, we hypothesize that skeletal muscle insulin resistance induces myostatin expression in response to a cellular state of starvation. Or put another way, the metabolic milieu of obese skeletal muscle may be similar to that of starving muscle because of the insulin-resistant state in which glucose uptake is severely constrained and the oxidative catabolism of lipid is reduced or incomplete; however, this hypothesis has not been tested (10,13,14,38). It has also been shown that myostatin expression in muscle is reduced acutely with endurance and resistance exercise (39). Because physical inactivity and a glycolytic fiber type distribution are common features of obesity, elevated myostatin levels could also be secondary to sedentary lifestyle (40,41). Because insulin resistance is not consistently maintained in primary cultured muscle cells, increased myostatin expression could be an inherent characteristic of the extremely obese (14). Finally, although we have shown correlation between muscle insulin

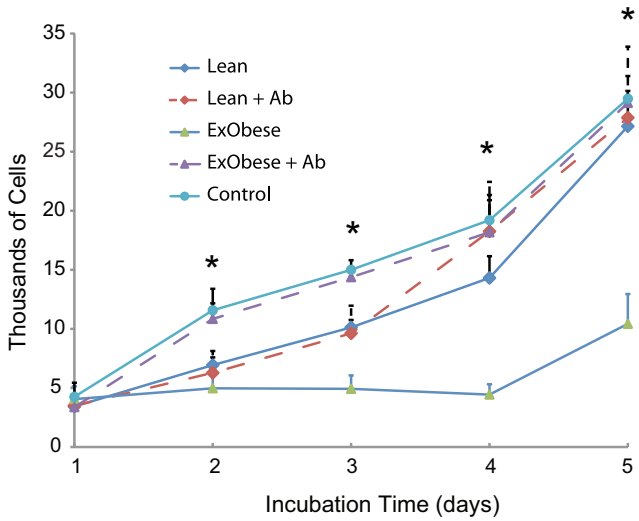

FIG. 7. Proliferation rate of C_2C_{12} mouse myoblasts grown in human myotube-conditioned growth medium. Equal numbers of cells were grown in lean or extremely obese human myotube conditioned growth medium, which was preincubated with or without goat polyclonal anti-myostatin antibodies to neutralize myostatin activity. Plotted values represent the average of three experiments ± SD. Asterisks indicate significant differences ($P < 0.05$). Control cells were grown in unconditioned growth medium.

resistance and myostatin expression in muscle, a causal or mechanistic relationship has not been established.

Although there is clearly a loss of muscle mass with frank type 2 diabetes (42,43), it is unclear whether increased circulating myostatin plays a direct role in the metabolic deterioration of skeletal muscle with obesity and insulin resistance. Supporting a role for myostatin in diabetic muscle atrophy is the high level of myostatin expression in *ob/ob* diabetic mice that have both reduced muscle mass and fiber cross-sectional area (33,44,45).

In summary, we have shown increased expression of myostatin protein with extreme obesity at the cellular, organ, and systemic level. To our knowledge, this is the first identification of a noncancer biomarker using quantitative secreted protein profiling (46–48). Future studies will test our hypothesis that myostatin expression is induced by insulin resistance in muscle.

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