Regulation of Muscle Protein Phenotype in Humans with Obesity

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PROTOCOL TITLE:

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PIs: Christos S. Katsanos, PhD (ASU PI)
Lori R. Roust, MD (Mayo Clinic PI)

Significance

Obesity is increasing in the developed world, and about 35% of the adults in the US have obesity (1). Skeletal muscle has key role in whole-body metabolism in humans (2), and large body of literature to date already links obesity to impaired glucose and lipid metabolism in skeletal muscle (previously reviewed (3-5)). Recent findings from our laboratory (6-9) as well as finding from other laboratories (10-15), show that obesity impairs the turnover of protein in skeletal muscle. We (6-9) and others (10, 11) have found specifically that the rate of synthesis of overall (i.e., mixed-muscle) and mitochondrial protein is lower in skeletal muscle of humans with obesity. This evidence at the protein turnover level is corroborated by evidence at the proteome level showing that humans with obesity have lower content of mitochondrial proteins in skeletal muscle (16, 17) as well as slow isoform of myosin heavy chain (MHC) protein (i.e., MHC-I) (18). Distorted expression of isoforms of the MHC protein is a hallmark of disrupted proteome homeostasis in muscle of humans with obesity. The overall underlying premise of the proposal is that maintaining proteome homeostasis is important to prevent the development or decrease the impact of obesity-associated metabolic abnormalities, a notion previously reviewed (19-22). However, the biological mechanisms responsible for impaired proteome homeostasis in muscle of humans with obesity have not been identified. Our preliminary evidence shows impaired translation initiation in skeletal muscle of humans with obesity, documented as reduced association of the eukaryotic initiation factor 4G (eIF4G) with eIF4E to form the active eIF4F, which mediates recruitment of ribosomes to mRNA, a rate-limiting step for mRNA translation (23). Detecting impairments in relevant molecular mechanisms allows substantiating current observations showing reduced muscle protein synthesis in vivo in humans with obesity (6-8, 10, 11). Ultimately, knowledge on the underlying molecular mechanisms that impair protein turnover in muscle of humans with obesity is necessary to currently fill a key gap in our understanding of how human obesity impacts remodeling and repair of the muscle proteome.

When muscles are studied ex vivo, an experimental approach that eliminates the direct effects of capillarization on muscle metabolism, substrate metabolism (i.e., glucose uptake) differs between muscles according to their MHC isoform phenotype (24), underlying the importance of biological differences within the muscles in determining the observed differences in substrate metabolism. The content of slow (i.e., MHC-I) versus fast (i.e., MHC-IIa, MHC-IIx) MHC isoforms in human skeletal muscle determines the content of Type-I versus Type-II fibers in muscle (25, 26). Type I fibers have proteome profile characterized not only by the MHC-I isoform, but also by increased abundance of mitochondria, insulin receptors, glucose transporters, hexokinase II, glycogen synthase (25, 27-29), all of which enhance the overall metabolism of glucose in muscle. The content of Type I fibers in skeletal muscle, which correlates directly with the content of MHC-I in muscle (30-33), correlates inversely with insulin resistance in muscle (34). Direct evidence linking MHC isoforms and substrate this regard, insulin-stimulated glucose uptake in muscle fibers varies according to the MHC isoform expressed (35). When compared to muscle fibers containing other MHC isoforms, fibers containing purely the MHC-I isoform not only display the largest capacity for glucose uptake (36), but, importantly, these muscle fibers are resistant to obesity-induced reduction in insulin sensitivity (37, 38). On the other hand, fibers containing fast MHC isoforms are susceptible to obesity-induced insulin resistance (35). Therefore, the most favorable glucose metabolism is observed only in MHC-I-containing/Type I muscle fibers.

Our preliminary evidence shows lower MHC-I isoform content in muscle of humans with obesity, and this is in agreement with previously published evidence (18). In addition, our data at the MHC proteome level are corroborated by substantial amount of evidence over the years at the muscle fiber level, and where numerous reports show lower Type-I/MHC-I-containing fibers in muscle of humans with obesity (29, 34, 39-44). Lower content of Type I fibers in muscle is detrimental for metabolic health in terms of the muscle's capacity to regulate glucose homeostasis. This is because Type I fibers are the type of muscle fibers that have increased glucose handling capacity/insulin sensitivity (28, 45), and as also discussed in the paragraph above, are inherently resistance to developing insulin resistance within the metabolic environment of obesity. These effects are likely mediated not by the MHC-I, per se, but by the overall Type I muscle fiber proteome, including Type I muscle fiber-specific increase in the content of mitochondria (27) as well as unique changes in the stoichiometry of the mitochondrial proteome (46), and with all these proteomic differences being implicated in determining the insulin sensitivity in muscle (47-49)). However, all these effects have been described only in MHC-I containing fibers,

further underlying the notion that expression of MHC-I is prerequisite to have changes in muscle fibers as those seen in relation to muscle mitochondria. The importance of the Type I muscle fiber proteome in health and disease is further shown in experiments where impaired insulin sensitivity/fatty acid oxidation in skeletal muscle is not reversed despite weight loss and when the muscle MHC-I content is not completely restored (18, 50, 51). This evidence suggests that changes in skeletal muscle proteome (i.e., MHC-I, mitochondrial proteins) might be a prerequisite in addition to weight loss to completely restore abnormal substrate metabolism in muscle of humans with obesity. In support of this notion, aerobic exercise, which can favorably modify parameters of the muscle proteome (i.e., MHC-I, mitochondria) (52, 53), is known to provide additional benefits to weight loss intervention and in relation to insulin sensitivity (54). In sum, the evidence discussed herein shows that the MHC proteome homeostasis in muscle is disrupted in humans with obesity. This response is characterized by lower MHC-I content contributing to lower content of Type-I muscle fibers, and, because of that, to metabolic abnormalities observed in the muscle of humans with obesity. Our specific premise is that cannot have increased content of Type I fibers in skeletal muscle, and thus favorable metabolic effects in muscle, without the ability to maintain increased content of MHC-I in skeletal muscle.

Studies with experimental animal models employing gain- and loss-of-function experiments show that determination of the overall metabolic profile in skeletal muscle rests specifically within the MHC-I gene (i.e., MYH7). Expression of MYH7 in skeletal muscle, not only encodes for increased MHC-I protein but also for increased microRNAs that determine the muscle fiber proteome by activating slow and repressing fast myofiber gene programs (55). The MYH7 gene hosts the microRNA(miRNA)-208b, which along with the miRNA-499 that is hosted in the lower abundant MYH7b gene and upregulated in parallel with the MYH7 (56, 57), upregulate MHC-I expression and at the same time downregulate the expression of the fast MHC genes and induce Type-I muscle fibers (55). These miRNAs couple mitochondrial proteome and function to the MHC expression (58-60). In sum, this evidence shows that expression of the gene encoding for MHC-I is key regulator of the overall muscle fiber phenotype, and lower MHC-I gene (i.e., MYH7) expression at the mRNA level will result in reduced capacity to modify the muscle fiber phenotype and metabolic function towards a Type I muscle fiber phenotype. Current evidence, therefore, shows that Type I muscle fiber phenotype depends on the expression of the slow MHC-I gene.

Based on the evidence discussed in above, lower MHC-I gene expression, including MHC-I protein expression, in skeletal muscle results in lower content of Type I fibers in skeletal muscle, and as observed in humans with obesity. We propose to understand biological mechanisms that sustain lower MHC-I expression in muscle of humans with obesity by employing experimental tools that are known to stimulate MHC-I gene expression. In non-obese, healthy humans aerobic exercise increases MHC-I, while decreases MHC-IIx, isoform content in skeletal muscle (52, 61), and there is good evidence showing that exercise can reverse dysregulated metabolism seen in muscle of humans with obesity (50, 62). However, other evidence shows that humans with obesity can be “exercise resistant” (63, 64). Our preliminary evidence shows that, although synthesis of MHC-I fails to increase, mRNA of MHC-I increases immediately after aerobic exercise in subjects with obesity. Therefore, acute exercise can be used as an experimental tool to experimentally manipulate mRNA expression of MHC isoforms, and specifically the slow MHC gene/MYH7 mRNA that is responsible for determining the overall muscle fiber phenotype. Failure to increase the synthesis of MHC-I despite increase in MHC-I mRNA expression after exercise in our preliminary studies provides evidence for “translational resistance” with respect of MHC-I expression. These data, therefore, point to reduced rate of MHC-I translation, as cause, at least in part, for reduced MHC-I synthesis, and thus lower content of MHC-I-containing fibers in muscle of humans with obesity. Increasing the plasma amino acid supply to skeletal muscle stimulates acutely the process of overall translation in muscle (65-68), and by enhancing assembly of the active eIF4F complex (69, 70). We have previously shown that increasing the plasma amino acid concentration alone, increases overall protein synthesis in muscle of humans with obesity (8). We propose to couple the response of increased MHC-I mRNA expression after exercise in subjects with obesity to increased plasma amino acids, and which we will employ as a second experimental tool to experimentally upregulate the overall translation process in skeletal muscle. That way we will discover on whether impaired global translation in muscle contributes to the lower synthesis and thus MHC-I expression in muscle of humans with obesity. We expect increased synthesis rate of MHC-I in the presence of increased plasma amino acids after exercise, suggesting that it is impaired global translation in muscle that

contributes to lower synthesis and expression of MHC-I. On the other hand, failure to increase synthesis rate of MHC-I under these conditions, will suggest a defect that is specific to the MHC-I translation. Our studies will, therefore, pinpoint specific biological defects that sustain unfavorable muscle fiber phenotype in humans with obesity, and which is key in any effort to address metabolic abnormalities (i.e., impaired muscle glucose uptake) originating from an unfavorable muscle fiber phenotype in these humans.

Specific Aims

Aim 1. To determine the turnover rate of MHC isoforms and overall muscle protein in skeletal muscle of subjects with obesity and lean controls in the basal state. We hypothesize lower synthesis rate of MHC-I (due at least in part to lower MHC-I mRNA) along with lower overall muscle protein synthesis in skeletal muscle of humans with obesity, and concomitant with lower formation of the eukaryotic initiation factor 4F (eIF4F) in muscle (key protein-complex signaling overall translation/synthesis). We hypothesize that muscle MHC-IIx synthesis is not reduced in obesity, and despite lower eIF4F complex formation in muscle, because the presence of higher MHC-IIx mRNA expression in muscle of humans with obesity.

Aim 2. To determine stimulation of the turnover rate of MHC isoforms in skeletal muscle of subjects with obesity and lean controls by plasma amino acids and with and without prior exercise. We hypothesize that increasing the plasma amino acid concentrations alone increases the basal rate of synthesis across all MHC isoforms in both lean and obese subjects, revealing a response largely dependent upon their respective mRNA expressions. This is because plasma amino acids enhance the overall protein translation in muscle (i.e., increase muscle eIF4F complex formation). We hypothesize that acute aerobic exercise alone does not increase the basal-state MHC-I synthesis rate in either lean or obese subjects, and despite greater increase in MHC-I mRNA expression in muscle of subjects with obesity. Thus, we also hypothesize that coupling the plasma amino acid stimulus to the exercise stimulus will increase the synthesis rate of the MHC-I in the subjects with obesity, revealing that impaired overall muscle protein translation is also responsible (and in addition to lower basal-state MHC-I mRNA, Aim#1) for lower MHC-I expression in muscle of humans with obesity.

Approach

Screening and Enrollment of Human Subjects

The protocol and consent form will be approved by the Institutional Review Board at Mayo Clinic. Physically inactive subjects with obesity and lean controls who meet the inclusion criteria below, will be invited to participate in the studies. Subjects will first be screened over the phone. Those who meet the initial criteria will be scheduled for further screening. Those subjects that do not sign the consent form will have all their personal health information erased. Those that sign the consent form will proceed with complete screening. Complete screening will take place at the Clinical Studies Infusion Unit (CSIU) in 2A-aAmbulatory Infusion Center (AIC) at Mayo Clinic in Arizona. Each participant will provide written informed consent prior to the initiation of any experimental procedures and after the purpose, procedures, requirements, and risks of the study are described to the subject. Participation in the studies will be determined based on the following inclusion/exclusion criteria:

Inclusion criteria: age, 18-45 years old at the time of enrollment; ability to sign informed consent form; body mass index (BMI), 18.0-26.0 kg/m^2 (lean subjects), 32.0-50.0 kg/m^2 (subjects with obesity). Although obesity is defined as BMI > 30 kg/m^2, our laboratory data over the years show greater decrease as well as more homogenous responses in mixed-muscle protein synthesis in subjects with obesity that have higher BMI values. Therefore, studying subjects with BMI > 32.0 kg/m^2, *enhances our study design* to test our hypotheses, and given also the inconsistent effects of obesity on metabolism resulting from classifying obesity based on BMI alone (71). We will not study humans with BMI >50 kg/m^2 to minimize variance in the measured responses in the obese subjects group, as well as for practical considerations related to obtaining muscle biopsies from subjects with extreme obesity (we have previously successfully obtained muscle biopsies from subjects with obesity at the BMI range proposed herein (18)). For subjects with BMI value of more than ~45 kg/m^2 the ability to obtain muscle biopsy depends largely on body fat pattern/distribution. That is, it is feasible to obtain muscle biopsy from subjects with low fat on thigh (i.e., site of muscle biopsy) and despite a high BMI value. So, subjects with BMI more than ~ 45 kg/m^2 will be asked to come to our CSIU to first evaluate muscle biopsy feasibility and prior to initiating any screening procedures. As part of the screening procedure we will calculate the Matsuda Insulin Sensitivity Index (ISI) from the plasma glucose and insulin responses to an Oral Glucose Tolerance Test (OGTT) (72). Although, overall, we expect subjects with obesity to be less insulin sensitive, this response can vary greatly within subjects with obesity (73) and lean subjects (74). The Matsuda ISI will be used to control in final analyses of the study data for a possible role of the *biological variable of insulin sensitivity* on affecting muscle protein metabolism (74). *Exclusion criteria*: evidence of diabetes (subjects will be excluded based on hyperglycemia-defined criteria and according to the American Diabetes Association criteria for the diagnosis of diabetes (75) that include fasting plasma glucose > 125 mg/dl, 2-h plasma glucose ≥200 mg/dl during OGTT, or A1C ≥6.5%; however, subjects with A1C 5.7–6.4%, impaired fasting glucose, or impaired glucose tolerance (75), indicative of insulin resistance, will be included, because the focus of the proposed studies is on humans with insulin resistance); presence of acute illness; history of liver disease; uncontrolled metabolic disease, including renal disease; heart disease related to atrial fibrillation, history of syncope, limiting or unstable angina, congestive heart failure or ECG documented abnormalities such as >0.2 mV horizontal or downsloping ST-segment depression, or frequent arrhythmias (>10 premature ventricular contractions/min); low hemoglobin or hematocrit; use of anabolic steroids or corticosteroids (within 3 months); not classified as inactive/sedentary based on the Stanford Brief Activity Survey (76) and accelerometry data (77-79); current participation in a weight-loss regimen; extreme dietary practices (i.e., vegan, vegetarian); smoking (cannot abstain as described below); pregnancy; gastro-intestinal surgery; any medication, intake of supplements, other condition or event considered exclusionary by the PI and the study physicians.

The screening visit at the CSIU will include: (i) physical examination and medical history; (ii) electrocardiogram (ECG); (iii) blood tests including complete cell count and liver function, thyroid hormone test, blood glucose, triglycerides, cholesterol and electrolytes; (iv) urinalysis; (v) urine pregnancy test (women); and (vi) 2-hour oral glucose tolerance test (OGTT), which will be used to exclude individuals with diabetes (i.e., glucose >200 mg/dl) and estimate insulin sensitivity using the Matsuda ISI (72). If the subject qualifies, they will be invited on a separate date to first undergo determination of body composition using Dual-energy X-ray Absorptiometry (DEXA), followed by a cycle ergometer test to exhaustion to determine peak oxygen uptake

(VO_2peak; ml/kg FFM/min), to characterize the body composition and physical fitness, respectively, of the study participants. VO_2peak will be measured using an incremental cycle-ergometer test to exhaustion, similar to procedures we have used in the past (8). This test will also serve as a screening tool to determine whether it is safe for the subject to perform the exercise associated with the proposed studies, and also estimate the exercise workload (corresponding to 65% VO_2peak) for the exercise session during the experiments.

Dietary intake will be evaluated from subject dietary information collected using the Automated Self-Administered 24-Hour (ASA24®) Dietary Assessment Tool. This is a software supplied by the National Institutes of Health for research purposes [redacted] Besides using it as descriptive characteristic, dietary intake information will be utilized to exclude subjects with extreme dietary practices (i.e. amino acid/protein intake, intake of specific fatty acids (80), vegan/vegetarian diet (81)). Habitual physical activity will be evaluated using the Stanford Brief Activity Survey (SBAS) (76). Although the SBAS provides valid measures for assessing habitual physical activity when compared to accelerometry (79), we will use accelerometry as an additional control, and to objectively determine physically activity and as previously described (77-79). Inactive/sedentary behavior will be defined as those with < 1.7 METs, based on the accelerometry/ActivPal output data. Diet and physical activity will be evaluated as part of the screening. Diet and physical activity are powerful modulators of protein turnover in skeletal muscle, and excluding subjects based on these variables provides a *robust experimental design* and will *increase the reproducibility* of our findings. To improve the study control prior to and between study visits, subjects will be asked to avoid alcohol as well as any form of exercise (beyond normal daily physical activities) during the 3-day period leading to each study visit. Also, subjects will be asked to record their diet during the day prior to their first infusion study visit, and to consume the same diet the day prior to their second infusion study visit.

Experiments

To test our hypotheses, we propose to study both subjects with obesity and lean controls. Each subject will take part in a single infusion trial. The infusion trial will consist of a Basal study period and an Experimental study period (**Fig. 1**). The Experimental study period will include either an acute session of aerobic exercise following by amino acid infusion (EX+AA trial) or amino acid infusion alone (AA trial). The exercise stimulus is designed to primarily target the MHC-I mRNA expression and synthesis of MHC-I in muscle ((our preliminary evidence for MHC-I mRNA and (63, 64, 82)). The amino acid infusion is designed to specifically upregulate the overall protein translation in muscle (65, 66), and we have shown to increase global (i.e., mixed muscle) protein synthesis in both lean humans as well as humans with obesity (8). Our preliminary evidence does not show increase in the synthesis rates of either mixed-muscle, mitochondrial (data not shown), or MHC-I protein following exercise alone. However, we propose to expand this preliminary evidence in lean humans and humans with obesity. Therefore, we will test the effects of exercise alone (EX trial) in groups of subjects that are separate from the lean and obese subject groups indicated above, and by following same experimental procedures as in the EX+AA trial but without the infusion of amino acids. We will study subjects with obesity (N=9) and lean controls (N=9) to test the hypothesis that exercise alone does not stimulate the synthesis of MHC-I, and despite increase in MHC-I mRNA expression by exercise. With the exception of the exercise session, subjects will be resting in bed for the duration of each trial.

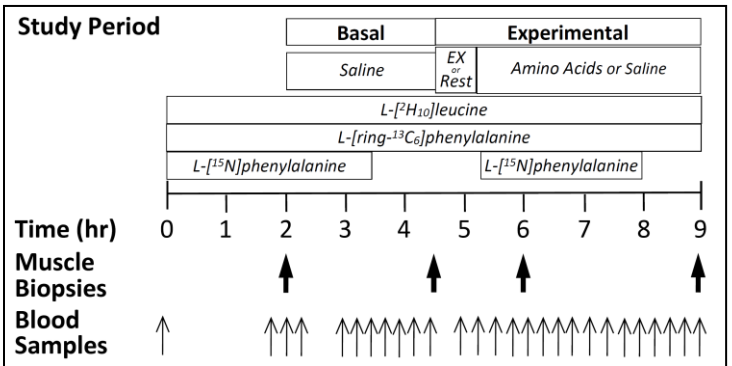

Figure 1. Schematic representation of the infusion and data collection protocol for the Amino Acids trial (rest followed by Amino Acid infusion), Exercise+Amino Acids trial (exercise followed by Amino Acid infusion) and Exercise Trial (exercise followed by Saline infusion) (*EX*, aerobic exercise at 65% VO_2peak).

Subjects will arrive at the CSIU at ~ 6:30 AM, after an overnight (10-hour) fast. An intravenous (IV) line will be inserted into an antecubital vein for infusions, whereas a second one will be inserted in a retrograde fashion into a vein of the opposite hand for arterialized blood sampling, and where the hand will be placed into a heated box (i.e., heated-hand technique (83)). Prior to initiation of any infusions, blood samples will be obtained for the measurement of background isotopic enrichment and fasting blood chemistry parameters. At ~ 7:30 AM (time 0; ***Fig. 1***) infusions of L-[2,3,3,4,5,5,5,6,6,6-$^{2}H_{10}$]leucine (d_{10}-leucine; prime: 9.0 umol·kg FFM^{-1}; rate: 0.15 umol·kg $FFM^{-1}\cdot min^{-1}$), L-[ring-$^{13}C_6$]phenylalanine and L-[^{15}N]phenylalanine (for both, prime: 3.0 umol·kg FFM^{-1}; rate 0.06 umol·kg $FFM^{-1}\cdot min^{-1}$) (Cambridge Isotope Laboratories, Inc., Andover, MA) will be started to enrich the muscle pool of intracellular amino acids with amino acid tracers for the determination of the rates of synthesis and breakdown of muscle proteins. The infusions of d_{10}-leucine and L-[ring-$^{13}C_6$] phenylalanine will be continued for the duration of the study, whereas that of the [^{15}N]phenylalanine tracer will be stopped 3.5 hrs after its initiation, and as shown in ***Fig. 1***, for the determination of the fractional breakdown rate of mixed-muscle protein in the Basal state. Alternatively, the stable isotopes of leucine and phenylalanine may be ingested instead of infused. In case of ingestion, the stable isotopes of these amino acids will be provided as small boluses dissolved in water (~10-20ml) and ingested every 10-15 minutes, and at a rate similar to that indicated for infusion. Rate of mixed-muscle protein synthesis in the Basal state will be simultaneously determined with that of mixed-muscle protein breakdown from the L-[ring-$^{13}C_6$] phenylalanine tracer incorporation into mixed-muscle protein. It is noted that combined fractional synthesis and breakdown of mixed-muscle protein will be evaluated only in a subset of subjects, and because of that, the two phenylalanine tracers, which are used together to simultaneously determine synthesis and breakdown of mixed-muscle protein, will not be administered in all subjects. . The d_{10}-leucine tracer will be used for the determination of the rate of synthesis of the MHC isoforms as well as mixed-muscle protein, and administered in all subjects. For the measurements of protein turnover in the Basal state biopsies of vastus lateralis muscle will be collected at 2 (1st biopsy) and 4.5 (2nd biopsy) hours after the start of the amino acid tracer infusions. Blood samples specifically for the determination of mixed-muscle protein breakdown will be collected similar to what has been previously described (84-86). Following data collection in the Basal state, the Experimental Study Period will be initiated immediately after the 2nd biopsy. During the Experimental Study Period in the EX+AA trial, subjects will first perform cycle-ergometer exercise at 65% VO_2peak for 45 min. Following exercise, subjects will immediately receive infusion of an amino acid solution (prime, 82 mg·kg FFM^{-1}; infusion rate, 4 mg·kg $FFM^{-1}\cdot min^{-1}$; 15% Clinisol; Baxter Healthcare Corporation, Deerfield, IL) for the remainder of the study trial. This is a balanced mixture of amino acids. It contains both essential (all essentials) and non-essential amino acids (for a total of 17 amino acids) and increases these amino acids in plasma. The amino acid mixture was chosen because we have previously shown to upregulate protein synthesis in skeletal muscle (8, 87). Increase in the essential amino acids, and specifically leucine, is important to drive upregulation of these processes in muscle (88-90), with the non-essential amino acids supporting the overall protein synthesis machinery. Therefore, this amino acid mixture, which contains sufficient amount of leucine, was chosen specifically based on our own evidence that it induces stimulation of overall/mixed-muscle protein synthesis in both lean humans and humans with obesity (8, 87). Infusion, when compared to ingestion, ensures comparable plasma amino acid concentrations between lean subjects and subjects with obesity (i.e., avoids differences in plasma amino acids as result of differences in gut amino acid absorption), and prevents amino acid-dose response effects seen in heterogenous groups of subjects with orally ingested amino acids (91, 92). In the AA trial, subjects will receive the same infusion of amino acids, but no exercise will be performed prior to the amino acid infusion. We have shown that the proposed dose of amino acids alone is sufficient to stimulate

overall protein synthesis in muscle, a response that is even greater in humans with obesity (8). Thus, this dose of infused amino acids overcomes any potential limitations residing outside the muscle (i.e., delivery of amino acids secondary to capillary involvement) in order to investigate mechanisms regulating protein turnover directly into the muscle of humans. On the other hand, increase in plasma amino acids stimulates translation initiation (65, 66) by enhancing assembly of the active eIF4F complex (69, 70), and which we found to be reduced in the muscle of humans with obesity. Therefore, the proposed experimental manipulations in the EX+AA trial target two separate components of the overall protein synthesis machinery (i.e., MHC-I mRNA expression + translation) to upregulate the synthesis of MHC-I in skeletal muscle. The infusion rates of the amino acid tracers will increase at the initiation of the amino acid infusion for d_{10}-leucine at 0.29 umol·kg FFM^{-1}·min^{-1} (prime: 2.6 umol·kg FFM^{-1}), L-[ring-$^{13}C_6$] phenylalanine at 0.32 umol·kg FFM^{-1}·min^{-1} (prime: 2.1 umol·kg FFM^{-1}), and the L-[^{15}N]phenylalanine infusion will be re-established at 0.32 umol·kg FFM^{-1}·min^{-1} (prime: 16.0 umol·kg FFM^{-1}) to account for the dilution of the amino acid tracers by the corresponding exogenous/infused unlabeled amino acids, and similar to procedures we have used in the past (8, 87). Infusion of L-[^{15}N]phenylalanine will be terminated one hour before the end of the infusion trial for the determination of mixed-muscle protein breakdown in the Experimental study period (***Fig. 1***), and by following procedures as in the Basal study period. A muscle sample (i.e., 3rd biopsy) will be collected 45 mins after the initiation of the amino acid infusion (to coincide with the time that intracellular signaling regulating protein synthesis in muscle is expected to peak (93)), whereas a 4th/last biopsy will be collected at the end of the infusion study.

Variables Determined in Muscle and Blood Samples

Blood samples will be collected for the determinations of leucine and phenylalanine stable isotope enrichments, as well as plasma chemistry parameters (i.e., glucose, insulin, amino acids, glucagon). Skeletal muscle samples (~150 mg/biopsy) will be analyzed for amino acid enrichment of muscle proteins for the determination of rates of protein turnover (i.e., synthesis, breakdown) of groups of proteins (i.e., mixed-muscle, mixed-mitochondrial protein) as well as individual proteins (i.e., MHC-I, MHC-IIa, MHC-IIx) in the Basal (1st and 2nd biopsies) and Experimental (3rd and 4th biopsies) study periods. Protein turnover measurements related to the experimental manipulations will be performed in the later part of the Experimental period (3rd and 4th biopsies), given that protein synthesis decreases during exercise, but it increases immediately after the cessation of the exercise (94). We will isolate muscle fibers in the collected skeletal muscle samples from either the 1st or 2nd biopsies (i.e., Basal state only) to characterize their MHC isoform content (i.e., MHC-I, MHCII-a, MHC-IIx). We will utilize these muscle fibers to also evaluate the metabolome, transcriptome, and proteome of the muscle fibers to better characterize the overall protein phenotype in skeletal muscle of humans with obesity, and together with the measurements of the turnover rate of the MHC isoforms discussed herein.

Basal-state responses will be compared in samples from the 2nd muscle biopsy, whereas responses induced by the experimental manipulations will be evaluated in samples from the 3rd and 4th muscle biopsies. Muscle samples will be analyzed for mRNA and protein expression of MHC-I, MHCII-a, and MHC-IIx. We will quantify muscle miRNA-208b and miRNA-499 because of their role in regulating the overall muscle fiber phenotype programming. We will determine relevant molecular mechanisms regulating signaling for protein synthesis in muscle, importantly the eIF4G·eIF4E and 4E-BP1·eIF4E complexes, given that our preliminary evidence shows dysregulation of the eIF4G·eIF4E complex formation in muscle of humans with obesity. We will evaluate canonical signaling regulating protein synthesis in muscle by evaluating phosphorylation (and total protein) of relative proteins, such as AKT, mTOR, 4E-BP1, S6K1, S6K1. GAPDH will be employed as loading control and to normalize western blot signal intensities. We will evaluate well-established molecular markers relevant to protein breakdown to complement our main findings on muscle protein breakdown measured in vivo. We propose to focus on muscle specific ubiquitin ligases, RING finger-1 (MuRF-1) and muscle atrophy F-box (MAFbx), which are important in the regulation of the ubiquitin-proteasome system, and which appear to increase in obesity (95), and are also regulated by acute aerobic exercise (96-101). Additional potential measurements include evaluation of their transcriptional regulation by measuring FOXO transcription factors, which are in turn regulated by Akt (102, 103), and whose mRNA levels also appear to increase following aerobic exercise (97, 99, 100, 104). Other candidate markers include those related to autophagy and could be evaluated by blotting for ULK1 phosphorylation, processed LC3, and p62 expression who appear to be modulated by obesity, exercise, and/or insulin (104-107). Dr. Scot Kimball, a Co-Investigator in this project, has several decades of experience on

muscle protein metabolism and an established research program focusing on the intracellular mechanisms that regulate protein metabolism (108, 109). He will direct all the aspects of the proposed research related to molecular mechanisms explaining the observed protein turnover responses in vivo.

Analyses of Samples and Calculations

Stable isotope enrichment and MHC quantification. Muscle for the determination of mixed-muscle protein turnover will be processed following procedures we have previously described (8, 87, 89, 91). Mitochondria will be isolated from muscle using procedures we have also previously described (17, 110, 111). Mixed-muscle protein, mixed-mitochondrial protein, and blood samples will be analyzed for determination of amino acid enrichment (i.e., d_9-leucine, $^{13}C_6$-and ^{15}N-phenylalanine) using LC-MS/MS (87). Enrichment of the MHC isoforms with d_9-leucine will be based on the determination of the incorporation of d_9-leucine on selected peptides of the MHC isoforms, following procedures similar to those we have previously described (112). Peptides studied will correspond to MHC-I [NTQAILK(+2), SALAHALQSSR (+3)], MHC-IIa [SLGTELFK(+2), IEDEQALGIQLQK(+2)], and MHC-IIx [SVNDLTSQR(+2), DSLLVIQWNIR(+2)]. MS1 data (when compared to MS2) data offer the advantage of determining enrichment by using peptides that have more than one amino acid in their sequence that can be labeled. This is because for a peptide with, for example, 2 possible positions for d_9-leucine, each one of those 2 amino acids has equal probability, that is 50%, to be labeled with d_9-leucine. Therefore, by targeting unlabeled and single-labeled peptides, the resulting true enrichment of that peptide with labeled amino acids will be (labeled AUC÷2)/unlabeled AUC. We have determined that having peptides that can be labeled in 2 amino acid (i.e., leucine) residues enhances the signal of the labeled peptide, and improves the reproducibility of the measured enrichment. MHC isoform enrichment will be determined by calculating an average enrichment from the enrichments measured for its corresponding targeted peptides. Standard proteomics analyses will be performed for the determination of MHC isoforms abundance, following procedures similar to those we have used previously (17, 113). Determination of the abundance of MHC isoforms in muscle samples, which will be used to determine the fractional change in the MHC isoform concentrations over time, will be performed by spiking the muscle samples using stable isotope-labeled full-length proteins corresponding to the MHC isoforms, and which will be commercially obtained (OriGene Technologies, Inc., Rockville, MD). Characterization of the MHC content of isolated muscle fibers will be done using gel electrophoresis, and as described here (119). The proteome of individual muscle fibers will be evaluated using laboratory procedures described here (120), while their transcriptome and metabolome will be evaluated using experimental techniques currently under development in our laboratory.

Calculations. Fractional synthetic rate of mixed-muscle and mixed-mitochondrial proteins will be determined using the precursor-product method based on the phenylalanine amino acid enrichments measured in the blood amino acid and muscle protein pools, following procedures we have recently described (8, 9, 87). Fractional breakdown rate of mixed-muscle protein will be determined directly in muscle (i.e., *not by using the arterio-venous technique*) following procedures previously described (84-86). We have previously validated a method to determine application of the d_{10}-leucine tracer to measure fractional synthetic rate of protein in muscle (87). Fractional synthetic rate of individual MHC isoforms will be calculated from d_9-leucine enrichments measured in the blood and the muscle MHC isoforms (i.e., MHC-I, MHC-IIa, MHC-IIx). We have previously developed (112), and successfully applied (9) a methodology to measure the fractional synthesis rate of an individual protein in skeletal muscle, and we will use similar procedures with respect to the fractional synthesis rates of the MHC isoforms. Fractional breakdown rate of the individuals MHC isoforms will be calculated from the measurement of the fractional synthesis rate of each MHC isoform in combination with the fractional change in that MHC isoform's concentration over a given time period, and as previously described (114, 115).

Immunoprecipitation and immunoblotting. We will follow procedures similar to those we and Dr. Scot Kimball have used in the past (7, 8, 116-118). With respect to the 4E-BP1·eIF4E and eIF4G·eIF4E complexes (which are key measurements in this proposal), these will be quantified after eIF4E is immunoprecipitated (IP) from aliquots of supernatants using an anti-eIF4E monoclonal antibody (generated in Dr. Scot Kimball's laboratory). Antibodies not generated in Dr. Kimball's laboratory will be commercially obtained and authenticated in Dr. Kimball's laboratory. We will also measure in the same muscle samples MOTS-c (mitochondrial open reading frame of the twelve S rRNA type-c). MOTS-c will be measured in muscle samples as well as plasma samples.

MOTS-c is a novel mitochondrial peptide in muscle, and which is also released into the circulation in association with physical activity/exercise. Recent evidence shows that it regulates metabolic processes in muscle that can be linked to protein synthesis.

We will perform the following measurements in our effort to explain differences in protein synthesis in muscle and understand regulation of the MHC genes in humans with obesity versus the lean controls: plasma and muscle insulin growth factor 1 (IGF-1) and associated hormones (i.e., growth hormone) and binding proteins that determine the IGF-1 function; plasma cytokines, such as TNFalpha, IL-6, IL-8, IL-10 (cytokine measurements will be performed by Dr. Ramon Velazquez's laboratory at ASU); chromatin-associated modification in isolated DNA from the muscle samples (DNA modification measurements will be performed by Dr. Zong Wei's laboratory at Mayo Clinic-Arizona). We will identify mechanisms involved in the responses we observe in vivo (i.e., differences in protein synthesis and MHC isoform expression between muscle from lean subjects and muscle from subjects with obesity) by performing in vitro, cell culture experiments, and where myoblasts isolated from the muscle biopsy samples are propagated to myotubes and associated changes in protein synthesis and MHC in response to changes in the expression of regulatory factors (i.e., IGF-1) can be modified in the absence of any other hormonal or metabolic differences.

Real-time quantitative PCR determination of gene expression. Expression of genes in skeletal muscle will be determined following procedures we have previously described (7, 8). Briefly, isolated mRNA will be purified using the RNeasy MinElute Cleanup Kit and be used to perform cDNA synthesis using the ABI High Capacity cDNA Reverse Transcription kit. Real time PCR will be performed on an Applied Biosystems 7900HT Fast Real-Time PCR System using pre-designed TaqMan® gene expression assays (probe/primer sets from ThermoFisher Scientific), and by following the manufacturer's protocols. The mRNA levels will be normalized to GAPDH, and relative differences in the mRNA levels will be analyzed by the $2^{-\Delta\Delta CT}$ method. miRNA-208b and miRNA-499 in muscle will be quantified following procedures we have previously described (7), and normalized to RNU-6B. Blood chemistry parameters will be quantified using commercially available assays and kits.

Statistical Design and Power

Our main end-points in the proposed studies are the synthesis rates of mixed-muscle protein, mixed-mitochondrial protein, as well as MHC isoforms, particularly MHC-I, whose synthesis rate we found to be lower in subjects with obesity, in our preliminary studies. These preliminary data also show that MHC-I synthesis rate does not increase in response to our experimental manipulation (i.e., exercise stimulus alone) despite an increase in MHC-I mRNA. Therefore, other main end-points are the expression of mRNA of MHC isoforms as well as the formation of the active eIF4F complex, which has key role in translation initiation in skeletal muscle, and which we found in our preliminary studies to be lower in muscle from humans with obesity. Statistical significance will be calculated using 2-way repeated measures ANOVA.

We will study equal number of males and females. We have detected a difference of 1.1 SD with respect to eIF4F complex formation in the Basal state between subjects with obesity and lean controls. Therefore, a sample of 17 subjects with obesity and 17 lean subjects will have sufficient power (80%) to detect 1.1 SD difference ($\alpha = 0.05$) in eIF4F complex formation, and also will have >95% power ($\alpha = 0.05$) to detect a difference of 2.0 SD and 3.5 SD with respect to the rates of mixed-muscle protein synthesis and breakdown, respectively. Also, in the Basal state, we have detected a difference of 2.9 SD for MHC-I synthesis between groups, suggesting that 17 subjects/group will have >95% power to detect this difference ($\alpha = 0.05$). With respect to mRNA responses in the Basal state, we have detected a difference of 1.26 SD for MHC-IIx expression suggesting that a sample of 17 subjects/group will have >80% power to detect significant difference in this variable between groups ($\alpha = 0.05$). We have reported a difference of 2.3 SD in increasing mixed-muscle protein synthesis with increased plasma amino acid concentrations alone in humans with obesity (8). Considering a conservative estimate of a difference of at least half of that (i.e., 1.15 SD) in the response of the MHC-I, 17 subjects/group will provide >90% power ($\alpha = 0.05$) to detect statistically significant increase from Basal in the rate of synthesis of MHC-I in response to the increase in plasma amino acid concentrations coupled to the exercise stimulus in the subjects with obesity ($\alpha = 0.05$). We will test another 17 subjects with obesity that will serve as controls (i.e., increase in plasma amino acid concentrations alone), for a total of 34 subjects with obesity participating in the study. The same experiments will be performed in lean subjects, and where 17 subjects will undergo increase in plasma amino acid

concentrations coupled to the exercise stimulus, and another 17 will serve as controls, for a total 34 lean subjects participating in the study. Main outcome in the exercise-alone studies is the increase in MHC-I mRNA expression after aerobic exercise, and which we show to increase only in the subjects with obesity. In our preliminary studies we detected a difference of 0.94 SD for MHC-I mRNA expression between before and after exercise in subjects with obesity. A sample of 9 subjects per group will have sufficient power (80%) to detect 0.94 SD difference ($\alpha = 0.05$) in MHC-I mRNA expression. Therefore, we will study 9 lean subjects and 9 subjects with obesity in the groups that will undergo exercise-alone trial. Sample size estimates and power were calculated using paired t-tests with an overall $\alpha = 0.05$ and $\alpha = 0.0125$ for Bonferroni-adjusted multiple comparisons. We propose to enroll 2 more subjects/group for the amino acid infusion and exercise+amino acid infusion studies (to the overall 34 subjects per group indicated above for those studies), to account for unsuccessful experiments and/or increase the statistical power in pair-wise comparisons with the lowest effect size, for a total of 36 lean subjects and 36 subjects with obesity for the studies related to the amino acid infusion and exercise+amino acid infusion. Therefore, we will enroll a total of 90 subjects.

Protection of Human Subjects

Risks to Human Subjects

Human Subjects Involvement, Characteristics, and Design

We will study 90 subjects (18-45 years old at the time of enrollment). All subjects will be studied in association with a day-long infusion study, and while resting in bed. All subjects, and as part of their participation in the study, will undergo physical/cycle ergometry exercise for 45 mins, and which will be performed approximately halfway during the infusion study. Subjects recruited for these studies will be representative of the ethnic and racial makeup of the Greater Phoenix Metropolitan Area in Arizona. Because of the invasive nature of the studies, and because muscle protein metabolism can differ during growth in children that may confound our findings, children will not be included in this project. Subjects will first be screened over the phone, and if they meet the initial criteria, they will be invited to sign a consent form. They will be invited for complete screening at the Clinical Studies Infusion Unit (CSIU) at Mayo Clinic in Arizona. Screening at the CSIU will include the following: (i) physical examination and medical history; (ii) electrocardiogram (ECG); (iii) blood tests including complete cell count and liver function, blood glucose, triglycerides, cholesterol and electrolytes; (iv) urinalysis; (v) urine pregnancy test (women); and (vi) 2-our oral glucose tolerance test (OGTT). If the subject qualifies, they will be invited on a separate date to first undergo determination of body composition, followed by a cycle ergometer test to exhaustion to determine peak oxygen uptake (VO_2peak). Diet and physical activity will be evaluated as part of these screening procedures. The inclusion/exclusion criteria for subject participation include:

Inclusion Criteria:

- age, 18-45 years old
- ability to sign informed consent form
- body mass index (BMI), 18.0 – 26.0 kg/m^2 (lean subjects), 32.0-50.0 kg/m^2 (subjects with obesity)

Exclusion Criteria:

- evidence of diabetes
- presence of acute illness
- history of liver disease
- uncontrolled metabolic disease, including renal disease
- heart disease related to atrial fibrillation, history of syncope, limiting or unstable angina, congestive heart failure or ECG documented abnormalities such as >0.2 mV horizontal or downsloping ST-segment depression, or frequent arrhythmias (>10 premature ventricular contractions/min)
- low hemoglobin or hematocrit
- use of anabolic steroids or corticosteroids (within 3 months)
- not classified as inactive/sedentary based on accelerometry/ActivPal data (i.e., physical activity > 1.7 METs).
- current participation in a weight-loss regimen
- extreme dietary practices (i.e., vegan, vegetarian)
- smoking (including electronic cigarettes and marijuana) subjects will be included as long as they are willing to abstain for 5 days prior to screening and infusion studies.
- pregnancy - women trying to become pregnant will be excluded from the study; women of child-bearing-potential will be able to participate in this study only if they have a negative pregnancy test and agree to use acceptable birth control during the course of the study [surgical sterilization, approved hormonal contraceptives (such as birth control pills, Depo-Provera), barrier methods (such as a condom or diaphragm) used with a spermicide, an intrauterine device (IUD), abstinence].
- gastro-intestinal surgery
- any medication, intake of supplements, other condition, or event considered exclusionary by the PI and the study physician.

Sources of Materials

Research material obtained from subjects will consist of blood, muscle and urine samples and the data resulting from the analysis of said samples. In addition, research material includes data resulting from the

measurements of body composition using Dual-energy X-ray Absorptiometry (DEXA), as well as the measurements of expired gases during exercise. All research material will be used explicitly for the purpose of this research. All data will be stored electronically and/or in hardcopy format and will be available only to members of the research team for the purposes of the research being conducted.

Potential Risks

Potential risks from participation in the study include problems associated with peripheral catheter insertion, infusions of stable isotopes and medications, blood drawing, muscle biopsy, abnormal blood pressure response during exercise. Subjects will be withdrawn from the study if any of the side effects are displeasing to them and they do not wish to continue the study, or they meet any of the previously described subject exclusion criteria (above), or the study physician determines that there is a reason to discontinue participation.

Adequacy of Protection Against Risks

Recruitment and Informed Consent

Research will be initiated only after obtaining approval from the Institutional Review Board (IRB) at Mayo Clinic. We plan on using a variety of recruitment procedures that include advertisement through flyers and online postings on the Arizona State University and the two Mayo Clinic campuses in Arizona, as well as through our subject registry within the Metabolic Program at Mayo Clinic in Arizona. Recruitment of subjects will also be done using physician referrals of participants in our Weight and Wellness Solution program at Mayo Clinic in Arizona.

A member of the study team will explain all experiment-related procedures and risks thoroughly to each subject. Before informed consent is obtained, subjects will be encouraged to thoroughly read the informed consent form and ask questions regarding the outlined procedures and risks. If a subject agrees to participate as a study volunteer and to sign the informed consent form, signature will be obtained in the presence of the principal investigator/co-investigators, postdoctoral fellow, study coordinator, or a CSIU nurse for witness purposes. The signed consent form will be kept on file in the CSIU or the Metabolic Program within the Mayo Clinic Research unit and a copy will be given to the subject.

Protections Against Risk

General Screening: All subjects will have an assessment of their medical history and will undergo a comprehensive physical exam at the time of enrollment. Subjects will be screened with a routine physical examination, blood tests including cell count and liver function, urinalysis, blood glucose, electrolytes, and electrocardiogram. Lori Roust, MD, will examine the screening results and initial to denote approval for enrolling the subject in the study. Records of approval for enrollment will be kept on file in the CSIU or the Metabolic Program.

Study Procedures: All procedures will be performed by appropriately trained and credentialed personnel under the supervision of Lori Roust, MD.

Blood Sampling: Blood samples will be collected solely for the purpose of experimentation. The blood will be used to measure the concentration of various plasma substances such as amino acids, insulin, glucose, as well as leucine and phenylalanine stable isotope enrichments. The amount of blood taken during each infusion study will be no more than approximately 150 mL (~300 ml for both visits), which is well below the amount (~ 500 mL) taken during a typical blood donation. Saline will be given as replacement during the data collection protocol and subjects will have no noticeable effects.

Muscle Biopsies: Muscle samples will be collected using strict sterile procedures from the lateral portion of the vastus lateralis muscle. This tissue will be used to measure enrichment of muscle free amino acids and muscle proteins with stable isotopes. Prior to taking the muscle biopsy, the skin will be numbed with EMLA cream (2.5% lidocaine and prilocaine 2.5%) for approximately 15 min. The skin will then be cleaned with topical antiseptic (i.e., Betadine, ChloraPrep) and the skin and tissue below injected with local anesthetic (lidocaine) to eliminate any pain. A small incision will be made through the skin through which a 6mm Bergström needle will be advanced into the muscle and suction applied. A piece of muscle (~150 mg/per biopsy) will then be removed with the needle, and the skin will be closed with Steristrips. To minimize the risk of infection and bruising, an antibiotic

ointment and pressure dressing will be applied, respectively. The risks of this procedure involve pain, which will be minimized by the injection of local anesthetic, and the possible formation of a bruise from localized bleeding. As with any medication, allergic reactions are possible; allergic reactions to the numbing medicine could cause problems such as a skin sore, swelling, or hives. Bleeding from the biopsy site is highly unlikely since no major artery or vein is located in the area of the vastus lateralis. The most likely complication of this procedure is infection due to lack of sterility during the procedure. We will minimize the risk of infection by using strict sterile procedures. There is risk of hematoma associated with the muscle biopsy. Bleeding can also be seen as bruising at the place on the leg where the muscle was taken. The swelling or bruising usually goes away with rest within 2-3 days, although sometimes it may take a week. Very rarely some subjects may experience numbness or tingling at the biopsy site. This is usually temporary and goes away in few days. Evidence for hematoma at the muscle biopsy site will be evaluated during the course of the infusion study, and also prior to discharge of the study participant. Direct pressure will be applied after the biopsy to prevent bruising and bleeding. Study participants will be given specific instructions prior to discharge to check for signs of hematoma and contact the research team if there is evidence of hematoma. Patients will be instructed to abstain for a minimum of 7 days prior to the infusion study from anticoagulants, such as aspirin, and will not be allowed to participate in the study if unable to refrain from their use.

Stable Isotopes: Stable isotopes are tested by the manufacturer (Cambridge Isotope Laboratories, Andover, MA) for sterility and pyrogenicity. They are mixed with normal saline prior to infusion. Stable isotope infusates will be prepared by the Pharmacy at Mayo Clinic in Arizona and delivered to the CSIU the morning of the study. Stable isotope tracers are naturally occurring compounds and are not radioactive. Stable isotopes are already present in the body in varying amounts. The stable isotopes will increase the level of naturally occurring isotopes by <7%. By making small elevations in the concentration of these stable isotopes in the blood, and by monitoring their changes over time in blood, as well as measuring their incorporation in muscle proteins, we will be able to determine the metabolism of muscle proteins. Any adverse reactions during the isotope infusion that suggest allergic reaction or infection will be promptly addressed by the physician. Depending on the seriousness of the reaction, the infusion study will be terminated. Thus, the risk of this procedure is no different than the general risk of infused substances.

Amino Acids Solution: Amino acids will be infused to increase the plasma amino acid concentrations. Per the manufacturer, local reactions consisting of a warm sensation, erythema, phlebitis and thrombosis at the infusion site can occur with peripheral intravenous infusion of amino acids. In such cases the infusion site will be changed promptly to another vein. Generalized flushing, fever and nausea also have been reported during peripheral infusions of amino acid solutions, as well as symptoms of hyperammonemia in the case of excessive rates of infusion or in patients with hepatic insufficiency where may result in plasma amino acid imbalances, hyperammonemia, prerenal azotemia, stupor or coma. Infusion of amino acids is routinely used in research in experiments similar in nature to this research and in clinical practice for the nutritional support of infants and young pediatric patients requiring TPN. Any adverse reaction during the amino acids solution infusion that suggests allergic reaction or infection will be promptly addressed by the physician in charge, and depending on the seriousness of the reaction, the infusion study will be terminated. Per the manufacturer, hypersensitivity to one or more amino acids, severe liver disease or hepatic coma, anuria or metabolic disorders involving impaired nitrogen utilization are contraindications. For that reason, subjects with such conditions will be excluded.

Dual-Energy X-ray Absorptiometry (DEXA): This is a common clinical procedure used to estimate body composition and exposes subjects to a very low amount of radiation. This radiation dose is far less than the amount used during normal chest X-ray. The amount of radiation in a DEXA scan is considered safe for adults, but it may cause damage to unborn babies. For that reason, a urine pregnancy test will be performed just prior to a DEXA scan.

Subject Monitoring: A nurse and/or a physician from the research team will monitor subjects during the infusion study, including the exercise session, and will document observations in the subject's chart. There will always be a medical staff by the bedside for the duration of the infusions. Subjects will be monitored during exercise for abnormal blood pressure and/or heart rate responses. In case of an abnormal response, the study physician will determine the seriousness of the response and whether to discontinue the exercise. If that is the case, subject participation in the infusion study will be terminated. A urine pregnancy test will be performed prior

to each infusion study, and the infusion study will not commence if positive. At the conclusion of the infusion study, the peripheral IVs will be removed and pressure applied to the site until bleeding is no longer apparent. Prior to discharge, biopsy sites will be inspected. Subjects will be given contact numbers and told that they should contact our staff if they perceive anything unusual. Problems subsequent to the study will be brought to the attention of the physician. If the physician sees the subject, a procedure note will be entered into the subject's folder.

Subject Confidentiality: All study data will be collected by the research team, reviewed by the PI and Co-Investigators, and stored in secure, locked files and/or databases in order to protect them from inadvertent loss or improper access. All laboratory specimens, evaluation forms, reports, and other records will be identified by coded number only to maintain subject confidentiality. Information gained from this study that can be linked to the subject's identity will not be released to anyone other than the investigators, the subject, and the subject's physician. All the information obtained in connection with these studies will remain confidential as far as possible within state and federal law. The results of these studies will be published in scientific journals without identifying the subjects by name.

Potential Benefits of the Proposed Research to Human Subjects and Others

There will be no direct benefit to the subjects participating in these studies. Subjects will have access to their screening results, which may alert them to potential health problems. However, the knowledge gained from these studies will have a benefit to society in terms of enhancing our understanding of the biological mechanisms that sustain unfavorable protein metabolism in muscle of humans with obesity, and ultimately improve the level of health in these individuals. The risks to the subjects are minimal and are outweighed by the benefit of the information that will be acquired from the studies.

Importance of the Knowledge to be Gained

The metabolism of proteins is impaired in skeletal muscle of humans with obesity. These studies are designed to understand the underlying cellular mechanism(s) that contribute to impaired skeletal muscle protein metabolism in these individuals. Unfavorable content and distribution of specific proteins (i.e., myosin heavy chain isoforms) in the muscle of humans with obesity is associated with unhealthy metabolic profile. Acute exercise and increase in plasma amino acids will be used as investigational tools to modulate the metabolism of muscle proteins, and in order to unravel biological mechanisms that sustain the unfavorable protein metabolism in muscle of humans with obesity. Findings from these studies will provide important knowledge that is necessary in order to favorably modify proteins with important metabolic implications in skeletal muscle of humans with obesity.

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