Insulin resistance is associated with skeletal muscle protein breakdown in non-diabetic chronic hemodialysis patients

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Deranged protein metabolism is known to complicate uremia. Insulin resistance is evident in chronic hemodialysis (CHD) patients. We hypothesized that the degree of insulin resistance would predict protein catabolism in non-diabetic CHD patients. We examined the relationship between Homeostasis Model Assessment (HOMA) and fasting whole-body and skeletal muscle protein turnover in 18 non-diabetic CHD patients using primed-constant infusions of L-(1-^{13}C) leucine and L-(ring-^{2}H$_5$) phenylalanine. Mean ± s.d. fasting glucose and body mass index were 80.6 ± 9.8 mg/dl and 25.4 ± 4.4 kg/m^2, respectively. Median (interquartile range) HOMA was 1.6 (1.4, 3.9). Mean ± s.e.m. skeletal muscle protein synthesis, breakdown, and net balance were 89.57 ± 11.67, 97.02 ± 13.3, and -7.44 ± 7.14 µg/100 ml/min, respectively. Using linear regression, a positive correlation was observed between HOMA and skeletal muscle protein synthesis ($R^2 = 0.28$; $P = 0.024$), and breakdown ($R^2 = 0.49$; $P = 0.001$). An inverse association between net skeletal muscle protein balance and HOMA was also noted ($R^2 = 0.20$; $P = 0.066$). After adjustment for C-reactive protein, only the relationship between HOMA and skeletal muscle protein breakdown persisted ($R^2 = 0.49$; $P = 0.006$). There were no significant associations between components of whole-body protein turnover and HOMA. This study demonstrates that insulin resistance is evident in non-diabetic dialysis patients, is associated with skeletal muscle protein breakdown, and represents a novel target for intervention in uremic wasting.

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[4]In memoriam

A decline in lean body mass and its precipitant sarcopenia continue to challenge those attempting to avert uremic wasting in patients with end-stage renal disease (ESRD).[1,2] In addition to its epidemiologic association with worsened clinical outcomes,[3–8] recent evidence suggests that deranged protein catabolism itself may directly impair endothelial function,[9] increase oxidative stress burden,[10,11] and weaken the immune response.[6,12] Postulated to underlie this imbalance between protein accretion and breakdown include contributions from metabolic acidosis,[13,14] the dialysis procedure itself,[15–18] chronic inflammation,[19–21] and hormonal imbalances.[22–24]

Recently, our group has reported that poorly controlled diabetes mellitus is a potent-independent predictor for the loss of lean body mass in chronic dialysis patients through increased skeletal muscle protein breakdown.[25,26] Similarly, Chevalier et al.[27] has demonstrated insulin resistance and enhanced whole-body protein catabolism in relatively young healthy obese women compared to lean women during the post-absorptive state. Taken together, these observations suggest resistance to the anti-catabolic effects of insulin as a possible mechanism underlying the muscle wasting seen in several disease states.

In this study, we hypothesized that the extent of insulin resistance in ESRD would be a determinant of the degree of protein breakdown, especially at the muscle metabolism level. In order to test this hypothesis, we examined the relationship between insulin resistance, as measured by Homeostasis Model Assessment (HOMA), and fasting protein turnover (whole body and skeletal muscle) in 18 non-diabetic chronic hemodialysis (CHD) patients, using a primed-constant infusion of L-(1-^{13}C) leucine and L-(ring-^{2}H$_5$) phenylalanine.

RESULTS

Demographics, body composition, and nutritional markers

Table 1 depicts the demographic characteristics, body composition, and baseline fasting nutritional parameters of the 18 patients included in the study. Two-thirds of the population were men and the majority were African-American ($n = 14$; 78%). The most common etiologies of

ESRD were hypertension ($n = 11$; 61%) and focal segmental glomerulosclerosis ($n = 4$; 22%). The mean $\pm$ s.d. age and vintage were 47.7 ± 14.6 and 6.3 ± 4.3 years, respectively.

Overall, the study population consisted of non-obese subjects with a mean $\pm$ s.d. body weight and body mass index (BMI) of 73.3 ± 15.3 kg and 25.4 ± 4.4 kg/m^2, respectively. Fat mass percentage as measured by dual-energy X-ray absorptiometry was $30.8 \pm 10.3\%$. Median (interquartile range) normalized protein catabolic rate was 0.8 (0.7, 1.1) g/kg/day within a month of the study protocol and mean serum albumin concentration was 3.6 ± 0.4 g/dl. Median C-reactive protein (CRP) was 5.8 mg/l (2.0, 9.4). As patients with impaired glucose tolerance (fasting blood glucose $\geqslant 100$ mg/dl) were also excluded, the cohort demonstrated good glycemic control with a mean fasting plasma glucose of 80.6 ± 9.8 mg/dl.

Despite exclusion of patients with diabetes mellitus and severe obesity (BMI > 35), the median (interquartile range) HOMA score was 1.6 (1.4, 3.9). Of the limited parameters

Table 1 | Baseline demographic, nutritional, and biochemical characteristics

Demographics	N=18
Gender (% males)	12 (67%)
Race (% AA)	14 (78%)
Age (years)	47.7±14.6
Etiology of ESRD (%)	
Glomerulonephropathy	5 (28%)
FSGS	4 (22%)
IgA nephropathy	1 (6%)
Hypertension	11 (61%)
Polycystic kidney disease	2 (11%)
Duration of hemodialysis (years)	6.3±4.3
Body weight (kg)	73.3±15.3
BMI (kg/m^2)	25.4±4.4
Fat mass by DEXA (%)	30.8±10.3
Blood chemistries	
Serum albumin (g/dl)	3.6±0.4
Serum prealbumin (mg/dl)	30.2±10.2
Serum transferrin (mg/dl)	144.5±52.9
Blood urea nitrogen (mg/dl)	45.7±18.2
Serum creatinine (mg/dl)	10.2±3.5
Hct (%)	37.3 (35.4, 42.2)
Serum CRP (mg/l)	5.8 (2.0, 9.4)
n-PCR (g/kg/day)	0.8 (0.7, 1.1)
Plasma glucose (mg/dl)	80.6±9.8
HOMA	1.6 (1.4, 3.9)
Plasma metabolic hormones	
Insulin (μU/ml)	8.5 (6.0, 18.7)
Glucagon (ng/l)	190.5 (137.2, 259.4)
Growth hormone (ng/ml)	0.6 (0.3, 1.8)
IGF-1 (ng/ml)	161.1±78.2
Cortisol (μg/dl)	12.3±4.3
Epinephrine (pg/ml)	43.6±24.0
Norepinephrine (pg/ml)	641.6±402.2

AA, African-American; BMI, body mass index; CRP, C-reactive protein; DEXA, dual-energy X-ray absorptiometry; ESRD, end-stage renal disease; FSGS, focal segmental glomerulosclerosis; HOMA, homeostasis model assessment; IGF, insulin-like growth factor; IgA, immunoglobulin A; n-PCR, normalized protein-catabolic rate.
Values are presented as mean±s.d. for normally distributed data, median and (IQR) for non-normally distributed data, and percentages as appropriate.

examined in this study (age, CRP, intact parathyroid hormone, and BMI), only BMI was significantly associated with HOMA score on univariate analysis ($\rho = 0.54$, $P = 0.022$).

Forearm muscle protein metabolism

The mean $\pm$ s.e.m. values for skeletal muscle protein synthesis, breakdown, and balance for the study subjects were 89.57 ± 11.67, 97.02 ± 13.37, and -7.44 ± 7.14 μg/100 ml/min, respectively (Table 2). These values were consistent with previously published results from our laboratory.[25] Using simple linear regression analysis, the slope of the regression line between HOMA score and muscle protein synthesis (β) was significantly greater than zero, indicating that muscle protein synthesis tended to increase as HOMA score increased ($\beta = 18.42$; 95% confidence interval = 2.79–34.05; $P = 0.024$; $R^2 = 0.28$; Figure 1a). Compared to synthesis, the slope of the regression line was larger for skeletal muscle breakdown indicating an even stronger association with HOMA score ($\beta = 27.84$; 95% confidence interval = 12.72–42.95; $P = 0.001$; $R^2 = 0.49$; Figure 1b). This resulted in an inverse relationship between HOMA and muscle protein balance, which approached statistical significance ($\beta = -9.42$; 95% confidence interval = -19.53 to 0.69; $P = 0.066$; $R^2 = 0.20$; Figure 1c). After adjusting for CRP, only muscle protein breakdown remained significantly associated with HOMA score ($\beta = 27.88$; 95% confidence interval = 12.24–43.53; $P = 0.006$). Similar results were found after adjustment for fat mass (data not shown).

Whole-body protein metabolism

Table 3 depicts the results for whole-body protein metabolism components expressed as mg/kg fat-free mass/min. The mean $\pm$ s.e.m. for synthesis, breakdown, and net balance were 3.94 ± 0.30, 3.86 ± 0.14, and -0.08 ± 0.26 mg/kg fat-free mass/min, respectively. There were no statistically significant associations between HOMA and the components of whole-body protein turnover before and after adjustment for CRP.

DISCUSSION

Multiple *in vitro* and *in vivo* studies have demonstrated the anabolic effects of insulin beyond simple carbohydrate

Table 2 | Association between HOMA and skeletal muscle protein turnover

Component (μg/100 ml/min)	Mean $\pm$ s.e.m.	β	R^2	95% CI	Significance
Synthesis	89.57±11.67	18.42	0.28	2.79–34.05	$P=0.024$*
Breakdown	97.02±13.37	27.84	0.49	12.72–42.95	$P=0.001$*
Balance	−7.44±7.14	−9.42	0.20	−19.53–0.69	$P=0.066$

CI, confidence interval; HOMA, homeostasis model assessment; s.e.m., standard error of mean.
Skeletal muscle protein components were expressed as mean±s.e.m. Also presented are the β, r^2, and 95% CIs for the simple linear regression performed between components of muscle protein turnover and HOMA score.
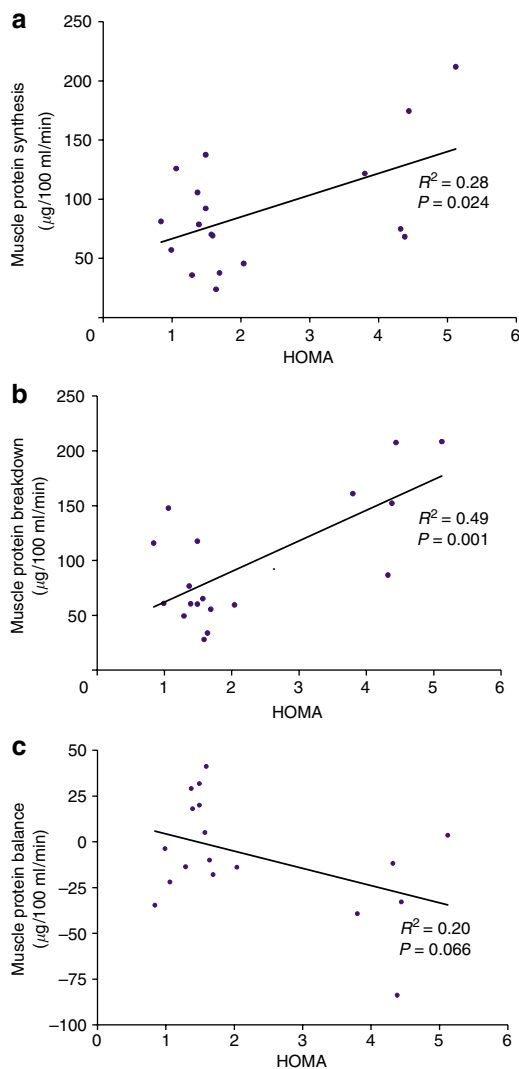*Statistically significant association $P < 0.05$.

Figure 1 | Scatter plots of HOMA versus skeletal muscle protein metabolism components. Simple linear regression revealed a direct relationship between HOMA and (**a**) skeletal muscle protein synthesis ($P = 0.024$) and (**b**) skeletal muscle protein breakdown ($P = 0.001$). (**c**) An inverse relationship between HOMA and net muscle protein balance was observed that approached significance ($P = 0.066$). *Statistical significance $P < 0.05$.

Table 3 | Association between HOMA and whole-body protein turnover

Component (mg/kg FFM/min)	Mean ± s.e.m.	β	R^2	95% CI	Significance
Synthesis	3.94 ± 0.30	−0.06	0.004	−0.54–0.42	$P = 0.803$
Breakdown	3.86 ± 0.14	0.02	0.002	−0.20–0.23	$P = 0.870$
Balance	0.08 ± 0.26	−0.08	0.010	−0.49–0.33	$P = 0.699$

CI, confidence interval; FFM, fat-free mass; HOMA, homeostasis model assessment; s.e.m., standard error of mean.
Whole-body protein components were expressed as mean ± s.e.m. Also presented are the β, r^2, and 95% CIs for the simple linear regression performed between components of protein turnover and HOMA score.

skeletal muscle protein breakdown and loss of lean body mass compared to matched non-diabetic CHD controls, suggesting that the above findings apply not only to insulin-deficient states, but insulin-resistant ones as well.[25,26] Consistent with these observations, Chevalier et al.[27] recently demonstrated a greater degree of fasting whole-body protein breakdown and a suppressed anabolic response to insulin in relatively healthy obese women compared to their lean counterparts undergoing hyperinsulinemic, euglycemic, isoaminoacidemic clamp studies. Furthermore, the metabolic response to insulin in carbohydrate metabolism has been shown to be impaired with varying degrees of uremia.[36–39] Although the etiology of this impairment has not been fully elucidated, our limited analysis indicates that BMI is an important factor, as has been shown in the general population. Based on these observations, we hypothesized that insulin resistance significantly contributes to the enhanced muscle protein catabolism observed in CHD patients. Our results indicate that in the absence of frank glucose intolerance (fasting blood glucose ≥ 100 mg/dl) or severe obesity (BMI > 35), insulin resistance is evident in the dialysis population and is associated with increased skeletal muscle protein breakdown, a relationship that persists even after adjustment for inflammation.

An interesting observation from this study was that the association between insulin resistance and protein breakdown remained significant only for the muscle data and not for whole-body protein turnover as previously observed in obese subjects with insulin resistance.[27] The lack of a significant association with whole-body protein turnover could be related to several factors, including but not limited to the differences in patient populations. In contrast to Chevalier et al., who studied young and otherwise healthy women with centripetal obesity where visceral fat mass burden may have contributed to their findings, we focused on a relatively non-obese older ESRD cohort where factors including acidosis, inflammation, and dialysis itself are known to influence visceral protein stores. In this setting, it remains possible that the insulin-mediated anabolic actions on protein turnover may be occurring predominately at the skeletal muscle compartment. Furthermore, the use of concomitant substrate administration via dual-clamp procedure in the former study may have influenced measurement of both insulin resistance and protein turnover, whereas our study examined these dynamics exclusively during the post-absorptive state.

metabolism.[28] The earliest observations of the insulinopenic condition in humans (i.e. uncontrolled Type 1 diabetes mellitus) were hallmarked by negative nitrogen balance, lean tissue atrophy, and hyperaminoacidemia.[28,29] The underlying mechanism demonstrated by Mitch et al. appears to be suppression of insulin receptor substrate-1-associated phosphatidylinositol 3-kinase activity resulting in stimulation of the ubiquitin-proteasome proteolytic system via caspase-3.[30,31] Investigations using tracer kinetic models and insulin clamp techniques in healthy individuals have detailed that it remains the blunting of proteolysis rather than enhanced protein synthesis that belies the net protein anabolic effect of insulin in the post-absorptive state.[32–35]

Our laboratory recently reported that the presence of Type 2 diabetes mellitus in CHD patients predicts increased

Our results also indicated a weak correlation between HOMA score and protein synthesis. Studies have shown that in the post-absorptive state, the protein metabolic effects of insulin are chiefly to suppress proteolysis resulting in diminished substrate availability for protein synthesis.[32,33,40] However, provision of amino acids appears to largely reverse this relative depression in amino-acid synthesis, at least at the whole-body level.[15,27] This suggests that both components of protein turnover, synthesis and breakdown, are dynamic and not mutually exclusive. This may reflect an imperfect adaptive mechanism to preserve somatic protein stores in diseases marked by enhanced catabolism and provide a possible explanation of our findings.

Although intriguing, these results should be interpreted with the following caveats. First, the cross-sectional nature of the design reveals only an association between insulin resistance and skeletal muscle protein homeostasis. Tests of causality addressing the effect of modifiers of insulin resistance as well as the hormone itself on skeletal muscle protein dynamics should be pursued. Towards that end, Wang et al.[41] have recently elegantly demonstrated in an animal model that the enhanced muscle proteolysis associated with insulin resistance appears to be reversible with the administration of the peroxisome proliferator-activated receptor-γ agonist Rosiglitazone.[41] These results need to be extended to human studies. The demographics of our study population also reflect a disproportionately high percentage of African-American subjects, which may limit the applicability of our findings to the general dialysis population. Finally, we recognize the limitations of sample size, specifically with respect to our ability to adjust for multiple potential confounders. Although the association between HOMA and protein breakdown persisted both before and after adjustment for CRP, the potential for over-fitting within our model of 18 patients exists. However, the extensive nature of the protocol precludes recruitment of subjects on a large scale and we believe this study to be among the largest of its kind.

In conclusion, it has been observed that CHD patients are at risk for a progressive loss in lean body mass not entirely accounted for by inadequate nutrient intake alone.[31,42,43] Given the high prevalence of protein cachexia and its unequivocal association with adverse clinical outcomes, the identification of modifiable contributors is of paramount importance. Moreover, the direct effects of dysregulated protein catabolism have been recently shown to impair endothelial function, indicating that the adverse effects of uremic wasting extend beyond its nutritional aspects alone.[9] As such, the results of this study indicate that insulin resistance in ESRD may be an important contributor to protein wasting that represents a novel target for intervention.

MATERIALS AND METHODS
Patients
Patients were recruited from the Vanderbilt University Outpatient Dialysis Unit. Inclusion criteria for the study were patients who have been on CHD therapy for at least 6 months, with less than 100 ml/

min of urine output, using a biocompatible hemodialysis membrane (Fresenius F80, Fresenius USA, Lexington, MA, USA), receiving an adequate dose of dialysis (double pool $K_t/V \geqslant 1.4$), on a thrice-weekly CHD program. Patients with a known history of diabetes mellitus or impaired glucose tolerance defined as a fasting blood glucose $\geqslant 100$ mg/dl, BMI > 35, active infectious or inflammatory disease (i.e. vascular access infections and overt periodontal disease), hospitalization within the last 3 months before the study, recirculation in the vascular access, and/or vascular access blood flow less than 750 ml/min detected on the arteriovenous (AV) shunt, and those receiving steroids and/or immunosuppressive agents were excluded from the study. The Institutional Review Board of Vanderbilt University approved the study protocol and written, informed consent was obtained from patients before the study. Patient characteristics are shown in Table 1.

Design
After reviewing the inclusion and exclusion criteria, 18 CHD patients were included who underwent the study procedure. Estimation of insulin resistance was performed by the HOMA model, as follows: (plasma insulin (μU/ml) $\times$ plasma glucose (mmol/l))/22.5, which has been shown to correlate closely with insulin sensitivity index as measured by hyperinsulinemic euglycemic clamp in patients with varying degrees of renal failure.[44] Within a week before each study, dual-energy X-ray absorptiometry was performed on a non-dialysis day to measure lean and fat body masses. Studies performing dual-energy X-ray absorptiometry before and after dialysis suggest that there are minimal effects of body-water changes on fat mass and bone measurements.[45,46] Resting metabolic rate was measured via indirect calorimetry to establish energy requirements before experimentation. Patients were admitted to the General Clinical Research Center at approximately 1900 hours the prior evening, received a balanced meal from the General Clinical Research Center bionutrition service, and remained fasting after that.

A schematic diagram of the metabolic study day protocol is depicted in Figure 2. Each metabolic study consisted of a 2 h equilibration phase followed by a 30 min basal sampling phase. A dialysis catheter was placed at the venous site of the AV shunt of the forearm at 0600 hours to collect a baseline blood sample (to assess baseline biochemical nutritional markers and isotopic backgrounds) and then to initiate the isotope infusion. The arterial side of the AV shunt was the site of choice used for sampling arterial blood. The only occasion that would affect the arterial purity of the samples would be if there were stenoses in the AV shunt causing the venous blood to mix with arterial blood (recirculation). Therefore, recirculation of the AV shunt as well as vascular access blood flow to assess stenoses in the AV shunts was checked in every patient before each study utilizing the ultrasound dilution technique (Transonic Systems Inc., Ithaca, NY, USA). The venous site of the AV shunt was used to infuse the isotopes. Another catheter was placed in a deep vein (on a retrograde insertion) of the contralateral forearm to sample blood draining from the forearm muscle bed.

At the start of the experiment, subjects received a bolus injection of $NaH^{13}CO_3$ (0.12 mg/kg), L-(1-^{13}C) leucine (7.2 μmol/kg), and L-(ring-$^2H_5$) phenylalanine (7.2 μmol/kg) to prime the CO_2, leucine, and phenylalanine pools, respectively. A continuous infusion of leucine (0.12 μmol/kg/min) and phenylalanine (0.12 μmol/kg/min) isotopes was then started and continued throughout the remainder of the study.
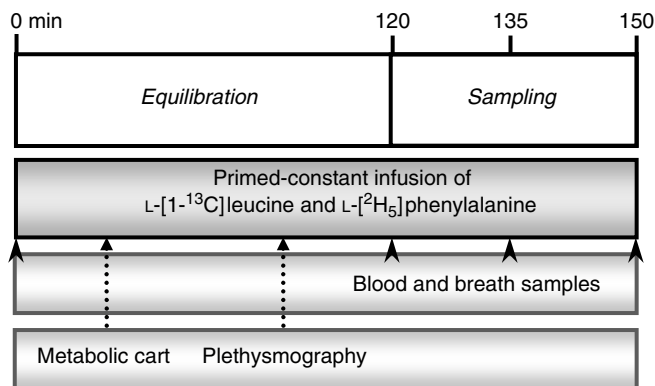
Figure 2 | Schematic diagram of the metabolic study day protocol. ⋏ Denotes time points for blood draws and breath sample collections. Metabolic cart and plethysmography measurements were performed once during the equilibration phase. A primed-constant infusion of L-(1-^{13}C) leucine and L-(ring-^{2}H$_5$) phenylalanine was maintained throughout the entire study (150 min).

Simultaneously with each blood sampling, breath samples were collected from the subjects via a Douglas bag with duplicate 20 ml samples placed into non-siliconized glass vacutainer tubes for measurement of breath $^{13}CO_2$ enrichment. Subjects were asked to breathe through a mask for several breath cycles, each time blood was collected. Forearm blood flow was estimated using capacitance plethysmography (Model 2560 with URI/CP software version 3.0, Moro Bay, CA, USA) during the equilibration phase. Specifically, two blood pressure cuffs were placed on the subjects forearm contralateral to the forearm with the AV shunt: one cuff 3 cm above the antecubital fossa and another cuff on the wrist. The technique involves abrupt interruption of venous outflow with a proximal cuff inflated to above venous pressure, but below arterial pressure (approximately 50 mmHg), which causes the volume of the limb to increase owing to arterial inflow. A mercury strain gauge plethysmograph is used to measure the volume change in the limb. The rate of change of the volume, in percent per minute, is the arterial flow rate at the moment of venous occlusion. This procedure was repeated for at least five times or until five similar measurements were obtained.

Analytical procedures

Blood samples were collected into Venoject tubes containing 15 mg Na$_2$-ethylenediaminetetraacetic acid (Terumo Medical Corp, Elkton, MD, USA). A 3-ml aliquot of blood was transferred to a tube containing ethylenediaminetetraacetic acid and reduced glutathione with the plasma stored at −80°C for later measurement of plasma epinephrine and norepinephrine concentrations by high-performance liquid chromatography.[47] The remaining blood was spun in a refrigerated (4°C) centrifuge (Beckman Instruments, Fullerton, CA, USA) at 3000 r.p.m. for 10 min and plasma was extracted and stored at −80°C for later analysis. Plasma glucose concentrations were determined by the glucose oxidase method (Model II Glucose Analyzer, Beckman Instruments, Fullerton, CA, USA).

Nutritional biochemical parameters were carried out at a specialized ESRD clinical and special chemistry laboratory (RenaLab, Richland, MS, USA). Serum albumin was analyzed using bromcresol green technique. Serum prealbumin was analyzed by an antigen–antibody complex assay and serum transferrin was analyzed by turbidimetric reading (Hitachi 717, Boehringer Mannheim,

Indianapolis, IN, USA). CRP was measured using nephelometric analysis at the Vanderbilt University Medical Center Clinical Chemistry laboratory.

Plasma c-peptide and insulin were measured by a double-antibody radioimmunoassay (Linco research Inc., St Charles, MO, USA). Immunoreactive insulin was determined in plasma with a double-antibody system. Plasma aliquots for glucagon determination were placed in tubes containing 25 kallikrein-inhibitor units of aprotinin (Trasylol, FBA Pharmaceutical, New York, NY, USA) and were later measured by established radioimmunoassay with a double-antibody system modified from the method of Morgan and Lazarow[48] for insulin. Insulin and glucagon antisera and standards, as well as (^{125}I) labeled hormones, were obtained from RL Gingerich (Linco Research, St Louis, MO, USA). Clinical Assays Gammacoat Radioimmunoassay kit (Travenol-GenTech, Cambridge, MA, USA) was used to measure plasma cortisol concentrations. Plasma insulin-like growth factor-I concentrations were determined by a radioimmunoassay acid-extraction procedure (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA). Plasma amino-acid concentrations were determined by reversed-phase high-performance liquid chromatography after derivatization with phenyliosthiocynate.[49]

Plasma enrichments of (^{13}C) leucine, (^{13}C) ketoisocaproate (KIC), and (ring-^{2}H$_5$) phenylalanine were determined using gas chromatography/mass spectrometry (GC/MS-Hewlett-Packard 5890a GC and 5970 MS, San Fernando, CA, USA). Plasma was deproteinized with 4% perchloric acid, and the supernatant was passed over a cation exchange resin to separate the keto and amino acids. The keto acids were further extracted with methylene chloride and 0.5 M ammonium hydroxide.[50] After drying under nitrogen gas, keto and amino acids fractions were derivatized[51] with N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide containing 1% t-butyl-dimethylchlorosilane (MtBSTFA + 1% t-BDMCS, Regis Technologies Inc., Morton Grove, IL, USA). The derivatized samples were then analyzed with GC/MS for plasma leucine, phenylalanine, and KIC enrichments using selected ion monitoring. The major fragments analyzed for the tBDMCS derivative of KIC and ^{13}C-KIC were the (M-57) ion fragments 301 and 302 *m/z*, respectively. The enrichment was quantified in plasma as the ratio of ^{13}C-KIC:KIC (ion abundance of 301/302 *m/z*). Enrichment measurements were made in duplicate, and duplicates had a coefficient of variation <3%. Breath $^{13}CO_2$ was measured by isotope ratio mass spectrometry (Metabolic Solutions, Nashua, NH, USA).[52]

Calculations

Net skeletal muscle protein balance (synthesis–breakdown) was determined by dilution and enrichment of phenylalanine across the forearm as described by Gelfand and Barrett.[34] Because phenylalanine is neither synthesized *de novo*, nor metabolized by skeletal muscle, rate of appearance (Ra) of unlabeled phenylalanine reflects muscle protein breakdown, whereas the rate of disappearance (Rd) of labeled phenylalanine estimates muscle protein synthesis.[34] Phenylalanine Rd was calculated by multiplying the fractional extraction of the labeled phenylalanine (based on plasma arterial and venous phenylalanine enrichments and concentrations) by the arterial phenylalanine concentration and normalized to forearm blood flow measured by plethysmography (expressed as 100 ml/min). Net phenylalanine Ra was calculated by subtracting the net AV balance of phenylalanine across the extremity from the phenylalanine Rd.[34,53] Rates of skeletal muscle protein breakdown and net synthesis were determined from the phenylalanine Rd and Ra,
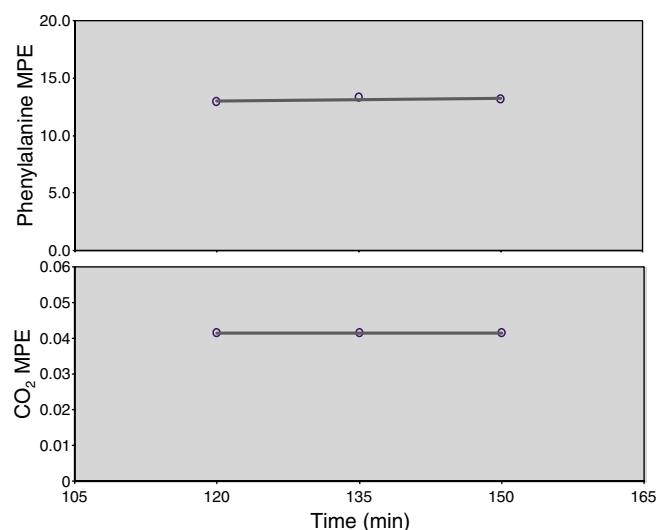
Figure 3 | Plasma mass percent enrichment of expired CO_2 and phenylalanine at study time points.

assuming that 3.8% of skeletal muscle protein is comprised of phenylalanine.

The steady-state rates of total whole-body leucine Ra were calculated by dividing the (^{13}C) leucine infusion rate by the plasma (^{13}C) KIC enrichment.[53] Plasma KIC provides a better estimate of intracellular leucine enrichment than does plasma leucine enrichment, owing to the fact that KIC is derived from intracellular leucine metabolism.[53] Steady-state conditions for phenylalanine and CO_2 (Figure 3) enrichments were achieved as evidenced by slopes within each phase not significantly different than zero. Breath $^{13}CO_2$ production was determined by multiplying the total CO_2 production rate by the breath $^{13}CO_2$ enrichment.[53] The rate of whole-body leucine oxidation was calculated by dividing breath $^{13}CO_2$ production by 0.8 (correction factor for the retention of $^{13}CO_2$ in the bicarbonate pool)[54] and by the plasma KIC enrichment. The leucine Rd, an estimate of whole-body protein synthesis, was determined indirectly by subtracting leucine oxidation from total leucine Rd. Rates of whole-body protein breakdown, amino-acid oxidation, and protein synthesis were calculated from the endogenous leucine Ra, the leucine oxidation rate, and the non-oxidative leucine Rd, respectively, assuming that 7.8% of whole-body protein is comprised of leucine.[55]

Rates of whole-body amino-acid oxidation were determined from indirect calorimetry in combination with the leucine oxidation data. The energy expended owing to amino-acid oxidation was subtracted from the resting energy expenditure, and the net rates of carbohydrate and lipid oxidation were calculated based on the non-protein respiratory quotient.[56] The assumptions and limitations of calculating net substrate oxidation based on indirect calorimetry measurements have been previously reviewed.[56]

Statistical analysis

Variables measured more than once during the study were averaged for statistical analysis. Normally distributed continuous variables are presented in the text and figures are presented as means ± s.d. or s.e.m., whereas non-normally distributed variables are presented as medians and interquartile ranges. Spearman's rank correlation coefficient was used to determine the association between HOMA and the following variables: age, BMI, intact parathyroid hormone, and CRP. Linear regression was used to assess the relationship between HOMA score and the continuous parameters of whole-body and skeletal muscle protein turnover. Residual assumptions were verified for these analyses. If residual analysis did not fulfill the assumptions of normality, sensitivity analysis was performed with transformation of the dependent variable which did not affect the overall significance. The statistical software package SPSS 14.0 (Chicago, IL, USA) was used for analyses and two-sided *P*-values of less than 0.05 were required to achieve statistical significance.

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