Protein metabolism in leg muscle following an endotoxin injection in healthy volunteers

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A B S T R A C T

The human endotoxin model has been used to study the early phase of sepsis. The aim of the present study was to assess leg muscle protein kinetics after an endotoxin challenge given to healthy human volunteers. Six healthy male subjects were studied in the post-absorptive state before and during 4 h following an intravenous endotoxin bolus (4 ng/kg of body weight). Primed continuous infusion of $[^2H_5]$phenylalanine and $[^2H_3]$3-methylhistidine in combination with sampling from the radial artery, femoral vein and muscle tissue were used to assess leg muscle protein kinetics. Both two- and three-compartment models were used to calculate protein kinetics. In addition 26S proteasome activity and protein ubiquitination were assessed. An increase in the net release of phenylalanine from the leg following the endotoxin challenge was observed; however, this phenylalanine originates from the free intracellular pool and not from protein. Net protein balance was unchanged, whereas both protein synthesis and breakdown were decreased. Degradation rates of contractile proteins were not affected by endotoxin, as indicated by an unchanged rate of appearance of 3-methylhistidine from leg muscle. In addition, proteasome activity and protein ubiquitination were unaffected by endotoxaemia. In conclusion, intravenous endotoxin administration to healthy volunteers resulted in an increased release of free phenylalanine from skeletal muscle, whereas protein balance was unaffected. Both protein synthesis and breakdown were decreased to a similar extent.

INTRODUCTION

Both surgical stress and critical illness are characterized by changes in protein metabolism, resulting in a net loss of proteins on a whole-body basis. This loss of proteins originates mainly from skeletal muscle. Critically ill patients with multiple organ failure treated in the ICU (intensive care unit) lose on average 10% of muscle proteins per week during the first couple of weeks of a stay in the ICU [1]. Loss of muscle protein can be the result of decreases in protein synthesis rates, increases in protein degradation rates or combinations thereof. To understand better the mechanisms leading to the dramatic muscle protein wasting in critically ill patients and to be able to evaluate potential interventions it is, therefore, important to measure skeletal muscle protein kinetics.

Previously, we have shown that fractional protein synthesis rates in skeletal muscle of critically ill patients treated in the ICU are on average close to normal and that this appears not to change with ongoing ICU stay [1,2]. Together with the progressive loss of muscle proteins, this indicates that protein degradation rates in skeletal muscle of ICU patients are dramatically increased. However, whether and how protein kinetics

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in skeletal muscle is influenced during the initial phase of disease in these patients is not known.

Most patients treated in the ICU already have a history of illness before arriving to the ICU, hence the early phase is problematic to study. An endotoxin challenge given to healthy volunteers has been used extensively to study the early phase of inflammation [3]. Whole-body protein degradation rates as well as protein synthesis rates have been shown to increase following an endotoxin challenge [4–6]. In addition, the amino acid exchange across the splanchnic area is influenced immediately after endotoxin administration in humans [4]. The net uptake of leucine and phenylalanine in the splanchnic area increases during the first 6 h following endotoxin. We have demonstrated previously an increase in the efflux of most amino acids from leg skeletal muscle 3 h after an endotoxin challenge in healthy volunteers [7]. Whether this increased amino acid efflux is the result of changes in protein synthesis rates, protein degradation rates or both is not known. In the present study we have therefore investigated leg skeletal muscle protein kinetics in human volunteers before and during the 4 h following an endotoxin challenge utilizing stable isotope techniques. Both two- and three-compartment models were used to calculate leg muscle protein kinetics [8,9]. In addition, activity of 26S proteasome and ubiquitination of proteins were analysed as qualitative measures of protein degradation.

MATERIALS AND METHODS

Subjects

Six healthy male subjects were included in the present study. Amino acid results from the same subjects have been reported previously [7]. All subjects were healthy as assessed by standard physical examination and blood analyses. Subjects had a mean age of 27 (range, 22–32) years, mean body weight of 79 (range, 66–88) kg and mean BMI (body mass index) of 24 (range, 23–26) kg/m^2.

All subjects gave their informed consent before the study after detailed information about the study was given both orally and in writing. The study protocol was in agreement with the Helsinki Declaration and approved by the ethical committee of Karolinska Institutet.

Study protocol

The subjects reported to the research unit in the morning of the study day after an overnight fast (from 20:00 hours the day before). Catheters were placed in the radial artery and in the femoral and cubital veins. Baseline blood samples were taken from the artery and the femoral vein. Subsequently, primed continuous infusions of [^{2}H$_5$]phenylalanine (prime, 0.5 mg/kg of body weight; infusion, 0.5 mg · kg^{-1} of body weight · h^{-1} of a solution containing 4 g of phenylalanine/litre of saline; 99 % enriched) and [^{2}H$_3$]3-methylhistidine (prime, 0.01 mg/kg of body weight; infusion, 0.01 mg · kg^{-1} of

body weight · h^{-1} of a solution containing 0.1 g of 3-methylhistidine/litre of saline; 98 % enriched) were started and continued for 8 h. After 4 h, subjects received an intravenous bolus of endotoxin (4 ng/kg of body weight; Lot EC-6; US Pharmacopeia). During the whole experiment heart rate, blood pressure and oxygen saturation were measured (Datex-Engstrom Light Monitor). Body temperature was measured every 15 min.

Blood samples were obtained from the artery at regular intervals during the whole experiment. During 1 h before the endotoxin challenge and during the 2–4 h after the endotoxin, four blood samples/h were obtained simultaneously from the artery and the femoral vein. Immediately after each sample, blood flow to the leg was measured by venous occlusion plethysmography with a minimum of ten readings, as described in detail previously [7]. Blood samples were immediately put on ice and plasma was obtained by centrifugation (600 g for 15 min at 4°C) within 30 min. The plasma samples were immediately frozen and kept at −80°C until analyses. Percutaneous biopsies of the vastus lateralis muscle were obtained at 20 min and immediately before the endotoxin challenge, as well as 160 and 180 min after the endotoxin. The biopsies were obtained with a Bergström needle with local anaesthesia (lidocain) of the skin and fascia. Samples were immediately cleaned from excess of blood, weighed and frozen in liquid nitrogen. The muscle samples were stored at −80°C until analyses.

Sample analysis

Plasma samples were analysed for both phenylalanine and 3-methylhistidine concentrations and enrichments by HPLC and GC-MS respectively. For HPLC analysis, plasma was deproteinized with 3 % SSA (5-sulfosalisylic acid-2-dihydrate) containing 200 μmol/l norvaline as an internal standard. The HPLC analysis has been described in detail previously [10]. Sample preparation and GC-MS analysis to measure plasma [^{2}H$_5$]phenylalanine and [^{2}H$_3$]3-methylhistidine have been described in detail previously [11].

Analyses of muscle tissue free concentrations of phenylalanine and 3-methylhistidine were performed as described previously [10]. For analysis of [^{2}H$_5$]phenylalanine in muscle, a modification of the method used to analyse plasma [^{2}H$_5$]phenylalanine was utilized. The muscle sample was freeze–dried and pulverized. Fat, connective tissue and blood were carefully removed from the muscle fibres, which were subsequently weighed again. Muscle samples were homogenized in 6 % SSA using a mini-beat beater (Biospec Products). After centrifugation (16 600 g for 10 min at 4°C), the amino acids in the supernatant were purified using ion-exchange chromatography. Furthermore, the samples were derivatized and analysed by GC-MS as described for plasma samples [11].

Proteasome chymotrypsin-like activity was measured in the muscle samples immediately before and 3 h after the endotoxin challenge using a fluorigenic peptide substrate as described in detail previously [12]. The proteolytic activity was expressed as pmol of released AMC (7-amino-4-methylcoumarin) $\cdot \mu g^{-1}$ of protein $\cdot$ min^{-1}. The chymotryptic activity was measured since this is the rate-limiting step in the proteasome pathway.

Total ubiquitination of myofibrillar and sarcoplasmic protein fractions were analysed in the muscle samples immediately before and 3 h after the endotoxin challenge using Western blot analyses. Myofibrillar and sarcoplasmic fractions were obtained by sequential centrifugation as described previously [13]. Muscle biopsies were homogenized in homogenization buffer [50 mmol/l Tris/HCl (pH 7.2), 1 mmol/l EDTA, 100 mmol/l KCl and 5 mmol/l MgCl$_2$] to obtain a 5% (w/v) homogenate, and were then centrifuged at 700 g for 10 min. The resulting supernatant was centrifuged at 15000 g for 10 min. Myofibrillar fractions (700 g pellet) and sarcoplasmic fractions (15000 g supernatant) were kept at $-80\,°$C until analysis. The 700 g pellets were washed three times with the homogenization buffer containing protease inhibitors (1 mmol/l PMSF and 1 μmol/l E64). After the last wash, the pellets were resuspended in the latter buffer.

Protein concentration was determined using DC protein assay kit (Bio-Rad Laboratories). A total of 15 μg of protein was incubated at 95 °C for 5 min in a buffer containing Laemmli sample buffer (Bio-Rad Laboratories) with 5% (v/v) 2-mercaptoethanol (Bio-Rad Laboratories). The proteins in the two fractions were separated by SDS/PAGE (10% gel) (Bio-Rad Laboratories). Two gels loaded with the same samples were run in parallel. The proteins were transferred on to a PVDF membrane for 120 min at 40 V and thereafter blocked in TBS-T {Tris-buffered saline [20 mmol/l Tris/HCl (pH 7.4) and 500 mmol/l NaCl] containing 0.1% Tween 20} with 5% non-fat milk for 2 h. The membranes were incubated overnight in 4 °C with an antibody against polyubiquitinylated conjugates (clone FK1; Biomol) diluted 1:500 in TBS-T. The membranes were then washed in TBS-T before incubation for 1 h with the secondary antibody (horseradish-peroxidase-conjugated sheep anti-mouse IgG; GE Healthcare) diluted 1:10000 in TBS-T. After another washing step with TBS-T, the membranes were incubated with chemiluminescence detection reagent ECL$^®$ (GE Healthcare) and exposed on X-ray film for 5 min. The absorbance of the bands was quantified by the analysing software Quantity One (Bio-Rad Laboratories). Total ubiquitination of all protein bands from the myofibrillar and sarcoplasmic protein fractions are presented. Mean values of the two gels are given as a percentage of the ubiquitination before the endotoxin challenge.

Calculations

Mean values for the 4 h samples taken during 1 h before the endotoxin challenge and during the 2–4 h after the endotoxin challenge were used to perform the kinetic calculations.

Whole-body R_a (rate of appearance) of phenylalanine (mg $\cdot$ kg^{-1} of body weight $\cdot$ min^{-1}) was calculated:

$$R_a = \frac{i \times E_i}{E_A} - i$$

in which i is the infusion rate of the tracer, and E_i and E_A are the enrichments (atom percentage excess) of the infused tracer and plasma phenylalanine respectively.

The NB (net balance) of phenylalanine and 3-methylhistidine (nmol $\cdot$ min^{-1} $\cdot$ 100 ml^{-1} of leg volume) over the leg was calculated:

$$NB = (C_A - C_V) \times F$$

in which C_A and C_V are the arterial and venous concentrations respectively (nmol/ml), and F is the plasma flow (ml $\cdot$ min^{-1} $\cdot$ 100 ml^{-1} of leg volume).

R_a and R_d (rate of disappearance) of phenylalanine (nmol $\cdot$ min^{-1} $\cdot$ 100 ml^{-1} of leg volume) over the leg were calculated utilizing the two-compartment model [11]:

$$R_a = C_V \times [1 - (E_V/E_A)] \times F$$
$$R_d = NB + R_a$$

in which E_V is enrichment of the plasma phenylalanine tracer in the vein (atom percentage excess). Leg skeletal muscle PS (protein synthesis) and PB (protein breakdown) (nmol $\cdot$ min^{-1} $\cdot$ 100 ml^{-1} leg volume) rates were calculated utilizing a three-compartment model for phenylalanine [8,9]:

$$PS = (C_A \times E_A - C_V \times E_V) \times F/E_M$$
$$PB = PS - NB$$

in which E_M is the enrichment of the free phenylalanine tracer in muscle (atom percentage excess). The mean enrichment from the two biopsies taken 20 min apart was used for the calculation.

The R_a of 3-methylhistidine over the leg was calculated utilizing a two-compartment model [11]:

$$R_a = C_A \times [(E_A/E_V) - 1] \times F$$

After initial analyses, it was obvious that the intracellular phenylalanine concentrations decreased in skeletal muscle during the endotoxin period. Although the change was not statistically significant, on average the levels decreased 13%. As changes in the intracellular concentration affect the kinetic calculations, we therefore also calculated the kinetics when corrected for this change [14]. In this, the NB is correcting for the changes in intracellular concentrations. For this it was assumed that the fall measured during the first 3 h after the endotoxin was linear

Table 1 Phenylalanine concentrations and kinetics 1 h (basal) before and during 2, 3 and 4 h (Endo 2, 3 and 4) after an endotoxin challenge in healthy volunteers

*Significantly different from basal, as determined by Fisher post-hoc analyses. †P value from ANOVA for repeated measures when four groups were analysed, and Student's t test for paired samples when two groups were analysed. ‡Values are corrected for changes in intracellular phenylalanine concentrations (see text for calculations).

Parameter	Basal	Endo 2	Endo 3	Endo 4	P value†
Whole-body					
R_a (nmol · min^{-1} · kg^{-1} of body weight)	806 ± 178	842 ± 181	$890 \pm 178*$	$895 \pm 169*$	0.001
Leg (two-compartment)					
NB (nmol · min^{-1} · 100 ml^{-1} of leg volume)	-6.1 ± 2.5	-5.0 ± 2.7	-7.1 ± 2.5	$-10.1 \pm 4.8*$	0.048
R_d (nmol · min^{-1} · 100 ml^{-1} of leg volume)	9.5 ± 2.1	7.3 ± 5.2	6.1 ± 2.6	3.9 ± 4.2	0.069
R_a (nmol · min^{-1} · 100 ml^{-1} of leg volume)	15.6 ± 2.7	$12.3 \pm 4.5*$	$13.2 \pm 3.2*$	13.9 ± 2.7	0.038
Net protein balance‡	-6.1 ± 2.5	-0.7 ± 5.2	-2.8 ± 5.7	-5.8 ± 6.3	0.115
R_a from protein	15.6 ± 2.7	$8.0 \pm 6.4*$	$8.9 \pm 6.4*$	$9.6 \pm 6.0*$	0.003
Leg (three-compartment)					
Synthesis (nmol · min^{-1} · 100 ml^{-1} of leg volume)	9.4 ± 2.1		6.0 ± 2.2		0.003
Breakdown (nmol · min^{-1} · 100 ml^{-1} of leg volume)	15.5 ± 2.8		12.3 ± 1.0		0.041
Corrected breakdown†	15.5 ± 2.8		8.0 ± 4.6		0.032
Phenylalanine concentration					
Arterial (μmol/l)	65.1 ± 7.6	66.1 ± 9.7	$59.5 \pm 9.4*$	$55.4 \pm 10.8*$	0.00001
Muscle (μmol/kg of muscle weight)	102 ± 12		89 ± 10		0.07

and of the same magnitude over the full 4 h following the endotoxin challenge. The corrected NB was calculated:

$$\text{Corrected NB} = \text{NB} - [(\text{post-}C_M - \text{pre-}C_M) \times 0.60 \times 100/180)]$$

In which pre- and post-C_M are the intracellular concentrations of phenylalanine before and 3 h after the endotoxin challenge respectively. The factor 0.60 represents the presumed volume of distribution of phenylalanine in the leg [14–16], and the factor 180 is to calculate per min instead of per 3 h. This corrected NB was used to subsequently calculate the corrected protein breakdown.

Statistical analysis

All values are expressed as means ± S.D. ANOVA for repeated measures followed by Fisher post-hoc test was used for statistical analysis between the basal period and 2, 3 and 4 h after the endotoxin challenge. Student's t test for paired samples was used to compare the basal period with 3 h after endotoxin when the three-compartment model was used and for the proteasome and ubiquitination measurements, as these were only measured at two time points.

RESULTS

Whole-body R_a of phenylalanine, reflecting whole-body protein breakdown, was increased approx. 10 % during 2–4 h after the endotoxin challenge ($P = 0.001$; Table 1).

Arterial plasma concentrations of phenylalanine were significantly decreased during 3 and 4 h after endotoxin ($P < 0.001$). The tissue free muscle concentration of phenylalanine was decreased 13 % at 3 h following the endotoxin ($P = 0.07$; Table 1).

The NB of phenylalanine across the leg became more negative following the endotoxin challenge ($P = 0.048$; Table 1). As calculated by the two-compartment model, R_d was not changed ($P = 0.069$), whereas R_a was significantly decreased during 2 and 3 h after endotoxin ($P = 0.038$; Table 1), but was not different from baseline thereafter. However, when corrected for the decrease in intracellular phenylalanine concentrations, the NB did not change following the endotoxin challenge ($P = 0.115$; Table 1) and R_a was significantly decreased ($P = 0.003$; Table 1) at 2, 3 and 4 h following endotoxin.

The three-compartment model showed a decrease in both muscle protein synthesis (36 %; $P = 0.003$) and breakdown (21 %; $P = 0.041$) during 3 h after endotoxin (Table 1). When using the corrected NB, protein breakdown was decreased to a larger extent (48 %).

3-Methylhistidine NB across the leg ($P = 0.56$) and R_a ($P = 0.79$) from the leg were not significantly changed following the endotoxin challenge (Table 2). Concentrations of 3-methylhistidine in plasma ($P = 0.09$) and muscle ($P = 0.37$) were not significantly changed following the endotoxin challenge (Table 2).

The activity of 26S proteasome was not changed following the endotoxin challenge (0.158 ± 0.029 pmol · μg^{-1} of protein · min^{-1} at basal compared with 0.158 ± 0.032 pmol · μg^{-1} of protein · min^{-1} at 3 h after the endotoxin). In addition, the total ubiquitination of the myofibrillar (95 ± 33 % of basal) and the sarcoplasmic (106 ± 13 % of basal) protein fraction was not changed following the endotoxin challenge.

Table 2   **3-Methylhistidine concentrations and kinetics 1 h (basal) before and during 2, 3 and 4 h (Endo 2, 3 and 4) after an endotoxin challenge in healthy volunteers**

†P value from ANOVA for repeated measures when four groups were analysed, and Student's t test for paired samples when two groups were analysed.

Parameter	Basal	Endo 2	Endo 3	Endo 4	P value†
Leg (two-compartment)					
NB (nmol · min^{-1} · 100 ml^{-1} of leg volume)	-2.2 ± 3.9	-1.6 ± 2.8	-1.7 ± 2.6	-1.6 ± 2.4	0.56
R_a (nmol · min^{-1} · 100 ml^{-1} of leg volume)	1.3 ± 1.2	1.5 ± 1.2	1.4 ± 1.1	1.8 ± 0.8	0.79
3-Methylhistidine concentration					
Arterial (μmol/l)	9.0 ± 6.3	8.1 ± 5.0	7.2 ± 3.9	6.9 ± 3.4	0.086
Muscle (μmol/kg of muscle weight)	234 ± 54		213 ± 59		0.37

DISCUSSION

The findings of the present study have shown that an endotoxin challenge given to human volunteers resulted in an increase in the whole-body R_a of phenylalanine. In leg muscle, the endotoxin challenge increased the net release of phenylalanine and decreased the R_a of phenylalanine. In addition, both protein synthesis and protein breakdown, as calculated using the three-compartment model, were decreased. However, there was no effect of the endotoxin on the net protein balance when the NB was corrected for the intracellular concentration changes in phenylalanine.

The increase in whole-body R_a of phenylalanine immediately following an endotoxin challenge in humans indicates increased protein degradation, a finding that has been reported previously [4–6]. From a previous study, in which whole-body protein kinetics together with splanchnic protein kinetics were measured following an endotoxin challenge in humans, it was concluded that the increased whole-body protein breakdown must be due to increased protein breakdown in muscle [4].

In the present study, three approaches to assess protein kinetics in human leg muscle were utilized to investigate the acute effects of endotoxin. R_a and R_d of phenylalanine were assessed using a two-compartment approach frequently used previously [17,18]. Results from this model suggested a trend towards a decrease in R_d, indicating a possible decrease in protein synthesis following the endotoxin challenge ($P = 0.069$). The R_a of phenylalanine showed a decrease initially, but did not change later after the challenge, indicating an unchanged or maybe slightly decreased protein breakdown. The three-compartment model allows the calculation of protein synthesis and breakdown rates in relation to a measure of the intramuscular precursor pool for protein synthesis. This model also showed a decrease in protein synthesis and protein breakdown 3 h after the endotoxin challenge. The third model used was the measurement of the R_a of 3-methylhistidine, a marker of contractile protein breakdown rates [11]. This model showed that contractile protein breakdown rates were not affected by the endotoxin challenge. The results on protein synthesis indicated that endotoxin may decrease protein synthesis during the first few hours. The measurements of protein breakdown, however, showed a decrease in total protein degradation, but no change in contractile protein breakdown. These results might suggest that degradation of the non-contractile proteins decreased immediately following the endotoxin challenge, whereas the contractile proteins were not influenced. The intramuscular free phenylalanine concentration decreased 13% during the first 3 h after the endotoxin. This decrease in phenylalanine tissue concentration invalidates the calculation of R_a and the protein breakdown rates in the two- and three-compartment models respectively, because the models require a steady state. As the intramuscular 3-methylhistidine concentrations did not change, the calculation of contractile protein breakdown rates was valid. The NB of phenylalanine can, however, be corrected for the changes in intracellular concentrations [14]. This correction will then give the net protein balance that can subsequently be used to calculate the corrected protein breakdown. These corrected calculations show that the whole increase in the NB of phenylalanine over the leg was due to the loss of free phenylalanine from leg muscle, and that the protein balance is not affected by the endotoxin. When corrected, protein breakdown decreased even more than when uncorrected. These corrections clearly show that protein turnover decreased in skeletal muscle following the endotoxin challenge and that the amount of protein loss following the endotoxin challenge is the same as before the challenge when the subjects were in the post-absorptive state.

In addition, the increase in whole-body R_a might be partly explained by an increased efflux of free phenylalanine from muscle tissue, and it is not clear whether this increased R_a actually represents an increase in whole-body protein breakdown in the endotoxin model. Assuming that the free phenylalanine pool decreases to the same extent in all skeletal muscles in the body and that 50% of the human body consists of skeletal muscle, its contribution to the increase in R_a can be estimated. Doing this, approximately half of the increase in R_a can be attributed to the efflux of free phenylalanine.

The remaining 5% increase, which is still statistically significant, is then due to an increase in breakdown of protein in tissues other than skeletal muscle. Despite this increased appearance of phenylalanine in the plasma pool, the plasma phenylalanine concentration decreased following the endotoxin challenge. This might be explained by an increase in the uptake and utilization of phenylalanine and other amino acids in the splanchnic tissues [4].

What do these decreased skeletal muscle protein turnover rates following an endotoxin challenge mean? Both critically ill patients with multiple organ failure [2,19] or with severe burns [20] have on average unchanged or even increased protein synthesis rates in skeletal muscle despite the progressive loss of muscle proteins [1]. Similarly, patients undergoing severe surgical stress have protein synthesis rates that are, on average, not different from normal [2]. The human endotoxin model is often considered a good model for acute inflammation since the cytokine patterns are reported similar to those observed during the early phase of sepsis [3]. This also implies that early during sepsis an initial decrease in protein turnover might appear that does not lead to a loss of muscle protein. It is, however, also possible that the metabolic stress in the endotoxin model is less severe as during the initial phase of sepsis.

A recent study has shown very similar changes in human skeletal muscle metabolism during an IL-6 (interleukin-6) infusion using similar techniques to those described in the present study [21]. That study showed both decreased protein synthesis and breakdown in skeletal muscle, with a slightly larger decrease in protein synthesis resulting in a negative protein balance, meaning a net loss of muscle protein. However, in the present study, no corrections for intracellular concentration changes were made. A small, but not significant, decrease in intramuscular phenylalanine was, however, also shown in the present study. This shows that the changes observed in the endotoxin model could well be the effect of increased IL-6 levels. The plasma concentrations of IL-6 obtained during the infusion [21] are approximately half of that normally observed in the endotoxin model after 2–3 h [22]. In another study it was shown that TNFα (tumour necrosis factor α) infusion did not change human skeletal muscle protein metabolism [23], suggesting even more strongly that IL-6 plays an important role.

As described above, the overall protein breakdown in skeletal muscle following the endotoxin challenge was decreased, whereas the breakdown of the contractile protein appeared to be unchanged, the latter being indicated by an unchanged 3-methylhistidine production from the muscle tissue. These results point to a decline in breakdown rates of other proteins in the muscle cell, most probably fast turning-over proteins which will be contributing relatively more to protein turnover

measurements performed over only a few hours. There are no methods available to assess breakdown rates of different protein fractions in vivo and we therefore measured the total ubiquitination of the myofibrillar and sarcoplasmic proteins, together with 26S proteasome activities, as possible surrogate measures. However, no differences in ubiquitination were observed between the two protein fractions, indicating that the method might not be sensitive enough or that changes in ubiquitination and proteasome activity are not involved in the decreased protein breakdown following the endotoxin challenge.

In summary, the increase in R_a of phenylalanine from leg muscle in humans following an endotoxin challenge was due to an increased efflux of free intracellular phenylalanine, rather than from muscle protein. Skeletal muscle protein balance was unaffected by endotoxin, despite a decrease in protein turnover. The present study also shows that it is important to measure and consider changes in intracellular amino acid concentrations when estimating protein turnover rates in vivo using these tracer models.

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