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

Rokhsareh F. Vesali¹, Norbert Cibicek², Towe Jakobsson¹, Maria Klaude¹, Jan Wernerman¹, Olav Rooyackers¹.

¹Department of Anesthesiology & Intensive Care, Karolinska Institutet and University Hospital, Huddinge, Sweden; ²Institute of Clinical Biochemistry and Diagnostics, Charles University in Prague, Faculty of Medicine and University Hospital Hradec Kralove, Czech Republic.

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Correspondence and reprints:

Olav Rooyackers

Dep. of Anesthesiology & Intensive Care

Karolinska Institutet

Karolinska University Hospital

S-141 86 Huddinge, Sweden

Tel: +46 8 58586182

Fax: +46 8 7795424

Email: olav.rooyackers@ki.se

Abstract

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 hours following an intravenous endotoxin bolus (4 ng / kg body weight). Primed continuous infusion of [$^2\text{H}_5$]phenylalanine and [$^2\text{H}_3$]3-methylhistidine in combination with sampling from artery, femoral vein and muscle tissue were used to assess leg muscle protein kinetics. Both 2 and 3-compartment models were used to calculate protein kinetics. In addition 26S proteasome activity and protein ubiquitination were assessed.

Results showed an increased net release of phenylalanine from the leg following the endotoxin challenge. 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. Also proteasome activity and protein ubiquitination were unaffected by endotoxemia.

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 intensive care unit (ICU) lose on average 10% of muscle proteins per week during the first couple of weeks of ICU stay [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 more of 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 seems 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 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 are shown to increase following an endotoxin challenge [4-6]. Also the amino acid exchange across the splanchnic area is influenced immediately after endotoxin administration in humans [4]. The net uptakes of leucine and phenylalanine in the splanchnic area increase during the first six hours following endotoxin. Recently we have demonstrated an increase in the efflux of most amino acids from leg skeletal muscle 3 hours 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. Here we therefore investigate leg skeletal muscle protein kinetics in human volunteers before and during 4 hours following an endotoxin challenge utilizing stable isotope techniques. Both 2 and 3-compartment models were used to calculate leg muscle protein kinetics [8, 9]. In addition, activity of 26S proteasome and ubiquitination of proteins were analyzed as qualitative measures of protein degradation.

Materials and methods

Subjects and ethical approval

Six healthy male subjects were included in the 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 years (range 22-32), mean body weight of 79 kg (range 66-88) and mean BMI of 24 (range 23-26). All subjects gave their informed consent before the study after detailed information of 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 (since 8:00 PM 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 [$^2\text{H}_5$] phenylalanine (prime: 0.5 mg/kg body weight; infusion: 0.5 mg·kg $^{-1}$ ·h $^{-1}$ of a solution containing 4g phenylalanine/L saline; 99% enriched) and [$^2\text{H}_3$]3-methylhistidine (prime: 0.01 mg/kg body weight; infusion: 0.01 mg·kg $^{-1}$ ·h $^{-1}$ of a solution containing 0.1g 3-methylhistidine /L saline; 98% enriched) were started and continued for 8 hours. After 4 hours, subjects received an intravenous bolus of endotoxin (4 ng/kg body weight; Lot EC-6 US Pharmacopeia, Rockville, MD, USA). During the whole experiment heart rate, blood pressure and oxygen saturation was measured (Datex-Engstrom Light Monitor from Datex- engstrom Instrumentarium crp., Finland). Body temperature was measured every 15 minutes.

Blood samples were obtained from the artery at regular intervals during the whole experiment. During one hour before the endotoxin challenge and during the 2nd, 3rd and 4th hour after the endotoxin 4 blood samples per hour 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 10 readings as described in detail previously [7]. Blood samples were immediately put on ice and plasma was obtained by centrifugation (600xg for 15min. at 4°C) within 30 minutes. The plasma samples were immediately frozen and kept at -80°C until analyses. Percutaneous biopsies of the vastus lateralis muscle were obtained at 20 minutes and immediately before the endotoxin challenge as well as 160 and 180 minutes after the endotoxin. The biopsies were obtained with a Bergström needle with local anaesthesia (lidocain) of the skin and the 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 analyzed for both phenylalanine and 3-methylhistidine concentrations and enrichments by HPLC and gas chromatography-mass spectrometry (GC-MS) respectively. For HPLC analysis plasma was deproteinised with 3% 5-sulfosalicylic acid-2-dihydrate (SSA) containing 200 μM norvaline as internal standard. The HPLC analysis has been described in detail before [10]. Sample preparation and GC-MS analysis to measure plasma [$^2\text{H}_5$] phenylalanine and [$^2\text{H}_3$]3-methylhistidine have been described in detail before [11].

Analyses of muscle tissue free concentrations of phenylalanine and 3-methylhistidine were performed as described before [10]. For analysis of [$^2\text{H}_5$] phenylalanine in muscle a modification of the method used to analyze plasma [$^2\text{H}_5$]phenylalanine was utilized. The muscle sample was freeze dried and pulverized. Fat, connective tissue and blood were carefully removed from the muscle fibers, which were subsequently weighed again. Muscle samples were homogenized in 6% SSA using a mini beat beater (Biospec Products, Bartlesville, OK, USA). After centrifugation, the amino acids in the supernatant were purified using ion-exchange chromatography. Furthermore, the samples were derivatized and analyzed on the GC-MS as described for plasma samples [11].

Proteasome chymotrypsin-like activity was measured in the muscle samples immediately before and 3 hours after the endotoxin challenge using a fluorogenic peptide substrate as described in detail previously [12]. The proteolytic activity was expressed as pmol of released AMC (7-amino-4-

methylcoumarin) per μg of protein and min. 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 analyzed in the muscle samples immediately before and 3 hours 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 a buffer [50mM Tris (pH 7.2), 1mM EDTA, 100mM KCl, 5mM MgCl_2] to obtain a 5% (w/v) homogenate, and were then centrifuged at 700g for 10 min. The resulting supernatant was centrifuged at 15,000g for 10 min. Myofibrillar fractions (700 g pellet) and sarcoplasmic fractions (15,000 g supernatant) were kept at -80°C until analysis. The 700 g pellets were washed 3 times with the homogenisation buffer containing protease inhibitors (1 mM phenylmethanesulfonyl fluoride -PMSF- and $1\mu\text{M}$ E64). After the last wash the pellets were resuspended in the latter buffer.

Protein concentration was determined using DC protein assay kit (Bio-Rad, Sundbyberg, Sweden). 15 μg of proteins were incubated in 95°C for 5 min in a buffer (v:v) containing Laemmli sample buffer (Bio-Rad) with 5% 2-mercaptoethanol (Bio-Rad). The proteins in the two fractions were separated on a 10 % SDS-PAGE gel (Bio-Rad). Two gels loaded with the same samples were run in parallel. The proteins were transferred to a PVDF (polyvinylidene difluoride) membrane for 120 min at 40 V and thereafter blocked in Tris-buffered saline 0.1 % tween (TBS-T) with 5% non-fat milk for 2 h. The membranes were incubated over night in 4°C with an antibody against polyubiquitinated conjugates (clone FK1, Biomol, Helsingborg, Sweden) diluted 1:500 in TBS-T. The membranes were then washed in TBS-T before incubation for 1 hour with the secondary antibody (sheep anti-mouse IgG-HRP; GE Healthcare, Biosciences AB, Uppsala, Sweden) dilution 1:10000 in TBS-T. After another washing step with TBS-T the membranes were incubated with chemiluminescence detection reagent ECL (GE Healthcare, Biosciences AB, Uppsala, Sweden) and exposed on X-ray film for 5 min. The optical density of the bands was quantified by the analysing software Quantity One (Bio-Rad). Total ubiquitination of all protein bands from the myofibrillar and sarcoplasmic protein fractions are presented. Mean values of the two gels are given as percentage of the ubiquitination before the endotoxin challenge.

Calculations

Mean values for the 4 hourly samples taken during 1 hour before the endotoxin challenge and during the 2nd, 3rd and 4th hour after the endotoxin challenge were used to perform the kinetic calculations. Whole body rate of appearance (R_a) of phenylalanine ($\text{mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) was calculated:

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

In which i is the infusion rate of the tracer; E_i and E_A are the enrichments (atom percent excess) of the infused tracer and plasma phenylalanine respectively.

The net balance (NB) of phenylalanine and 3-methylhistidine ($\text{nmol}\cdot\text{min}^{-1}\cdot 100\text{ml}^{-1}$ 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 ($\text{ml}\cdot\text{min}^{-1}\cdot 100\text{ml}^{-1}$ leg volume).

The R_a and rate of disappearance (R_d) of phenylalanine ($\text{nmol}\cdot\text{min}^{-1}\cdot 100\text{ml}^{-1}$ leg volume) over the leg were calculated utilizing the 2-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 percent excess). Leg skeletal muscle protein synthesis (PS) and protein breakdown (PB) ($\text{nmol}\cdot\text{min}^{-1}\cdot 100\text{ml}^{-1}$ leg volume) rates were calculated utilizing a 3-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 enrichment of the free phenylalanine tracer in muscle (atom percent excess). The mean

enrichment from the 2 biopsies taken 20 minutes apart was used for calculation.

The R_a of 3-methylhistidine over the leg was calculated utilizing a 2-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 by 13%. Since 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 drop measured during the first 3 hours after the endotoxin was linear and of the same magnitude over the full 4 hours following the endotoxin challenge. The corrected NB was calculated:

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

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

Statistics

All data are expressed as means \pm SD. ANOVA for repeated measures followed by Fisher post-hoc test was used for statistical analysis between the basal period and hours 2, 3 and 4 after the endotoxin challenge. Student's t-test for paired samples was used to compare the basal period with hour 3 after endotoxin when the 3-compartment model was used and for the proteasome and ubiquitination measurements since these were only measured at 2 time points.

Results

Whole body R_a of phenylalanine, reflecting whole body protein breakdown, was increased by approximately 10% during 2-4 hours after the endotoxin challenge ($p=0.001$; Table 1).

Arterial plasma concentrations of phenylalanine were significantly decreased during the 3rd and 4th hour after endotoxin ($p<0.001$). Tissue free muscle concentration of phenylalanine showed a decrease by 13% at 3 hours following the endotoxin ($P=0.07$) (Table 1).

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

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

3-Methylhistidine net balance 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 in 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· μ g protein⁻¹·min⁻¹ at basal versus 0.158 ± 0.032 3 hours after the endotoxin). Also 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.

Discussion

An endotoxin challenge given to human volunteers resulted in an increase in whole body rate of appearance of phenylalanine. In leg muscle, the endotoxin challenge increased the net release of phenylalanine and decreased the rate of appearance of phenylalanine. In addition, both protein synthesis and protein breakdown as calculated using the 3-compartment model were decreased. However, there was no effect of the endotoxin on the net protein balance when the net balance was corrected for the intracellular concentration changes of phenylalanine.

The increased whole body rate of appearance of phenylalanine immediately following an endotoxin challenge to humans indicates increased protein degradation – a finding that has been reported before [4-6]. From an earlier 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. Rates of appearance and disappearance of phenylalanine were assessed using a 2-compartment approach frequently used before [17, 18]. Results from this model suggested a trend towards decreased rate of disappearance indicating a possible decrease in protein synthesis following the endotoxin challenge ($p=0.069$). The rate of appearance of phenylalanine showed a decrease initially, but did not change later after the challenge, indicating an unchanged or maybe slightly decreased protein breakdown. The 3-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 decreased protein synthesis and protein breakdown 3 hours after the endotoxin challenge. The third model used was the measurement of the rate of appearance 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 indicate that endotoxin may decrease protein synthesis during the first few hours. The measures 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, while the contractile proteins were not influenced. The intramuscular free phenylalanine concentration decreased by 13% during the first 3 hours after the endotoxin. This decrease in phenylalanine tissue concentration invalidates the calculation of the rate of appearance and the protein breakdown rates in the 2- and 3-compartment models respectively because the models require a steady state. Since the intramuscular 3-methylhistidine concentrations did not change, the calculation of contractile protein breakdown rates was valid. The net balance 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 phenylalanine net balance over the leg is 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 subject were in the post absorptive state.

Also the increased whole body rate of appearance might be partly explained by an increased efflux of free phenylalanine from muscle tissue and it is not sure that this increased rate of appearance is actually representing an increased whole body protein breakdown in the endotoxin model. Assuming that the free phenylalanine pool decreases to the same extend in all skeletal muscles in the body and that 50% of the human body consists of skeletal muscle its contribution to the increased rate of appearance can be estimated. Doing this about half of the increased rate of appearance can be attributed to the efflux of free phenylalanine. The remaining 5 % increase, which is still statistically significant, is then due to an increased breakdown of protein in other tissues than skeletal muscle. Despite this increased appearance of phenylalanine into the plasma pool, the plasma phenylalanine

concentration decreased following the endotoxin challenge. This might be explained by an increased uptake and utilisation 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 show 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 seen during the early phase of sepsis [3]. This implies that also early during sepsis an initial decrease in protein turnover might appear not leading to 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 shows very similar changes in human skeletal muscle metabolism during an interleukin-6 infusion using similar techniques as in the present study [21]. This study shows 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 this study no corrections for intracellular concentration changes were made. A small, but not significant decrease in intramuscular phenylalanine is however also shown in this study. This shows that the changes seen in the endotoxin model could well be the effect of increased interleukin-6. The plasma concentrations of interleukin-6 obtained during the infusion [21] are about half of that normally observed in the endotoxin model after 2 to 3 hours [22]. In another study it was shown that TNF α infusion did not change human skeletal muscle protein metabolism [23], suggesting even stronger that interleukin-6 plays an important role.

As described above, the over all protein breakdown in skeletal muscle following the endotoxin challenge was decreased, whereas breakdown of the contractile protein seemed to be unchanged, the latter being indicated by an unchanged 3-methylhistidine production from the muscle tissue. These results point to a decline in breakdowns rates of other proteins in the muscle cell, most likely 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 increased rate of appearance 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 decreased protein turnover. This study also shows that changes in intracellular amino acid concentrations are important to measure and consider when estimating protein turnover rates in vivo using these tracer models.

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

	Basal	Endo 2	Endo 3	Endo 4	P-value [#]
Whole body					
R _a (nmol·min ⁻¹ ·kg ⁻¹)	806±178	842±181	890±178*	895±169*	0.001
Leg (2-comp)					
Net balance (nmol·min ⁻¹ ·100ml ⁻¹)	-6.1±2.5	-5.0±2.7	-7.1±2.5	-10.1±4.8*	0.048
R _d (nmol·min ⁻¹ ·100ml ⁻¹)	9.5±2.1	7.3±5.2	6.1±2.6	3.9±4.2	0.069
R _a (nmol·min ⁻¹ ·100ml ⁻¹)	15.6±2.7	12.3±4.5*	13.2±3.2*	13.9±2.7	0.038
Net protein balance ¹	-6.1±2.5	-0.7±5.2	-2.8±5.7	-5.8±6.3	0.115
R _a from protein ¹	15.6±2.7	8.0±6.4*	8.9±6.4*	9.6±6.0*	0.003
Leg (3-comp)					
Synthesis (nmol·min ⁻¹ ·100ml ⁻¹)	9.4±2.1		6.0±2.2		0.003
Breakdown (nmol·min ⁻¹ ·100ml ⁻¹)	15.5±2.8		12.3±1.0		0.041
Corrected breakdown ¹	15.5±2.8		8.0±4.6		0.032
Concentrations					
[arterial] µmol/L	65.1±7.6	66.1±9.7	59.5±9.4*	55.4±10.8*	0.00001
[muscle] µmol/kg	102±12		89±10		0.07

#: P-value from ANOVA for repeated measures when 4 groups and Student's t-test for paired samples when 2 groups. *: significantly different from basal as by Fisher post-hoc analyses. ¹: values corrected for changes in intracellular phenylalanine concentrations (see text for calculations).

Table 2. 3-Methylhistidine concentrations and kinetics (in $\text{nmol} \cdot \text{min}^{-1} \cdot 100\text{ml}^{-1}$ leg volume) during 1 hour (basal) before and during 2, 3 and 4 hours (Endo 2, 3, 4) after an endotoxin challenge in healthy volunteers

	Basal	Endo 2	Endo 3	Endo 4	P-value [#]
Leg (2-comp)					
Net balance ($\text{nmol} \cdot \text{min}^{-1} \cdot 100\text{ml}^{-1}$)	-2.2±3.9	-1.6±2.8	-1.7±2.6	-1.6±2.4	0.56
R_a ($\text{nmol} \cdot \text{min}^{-1} \cdot 100\text{ml}^{-1}$)	1.3±1.2	1.5±1.2	1.4±1.1	1.8±0.8	0.79
Concentrations					
[arterial] $\mu\text{mol/L}$	9.0±6.3	8.1±5.0	7.2±3.9	6.9±3.4	0.086
[muscle] $\mu\text{mol/kg}$	234±54		213±59		0.37

#: P-value from ANOVA for repeated measures when 4 groups and Student's t-test for paired samples when 2 groups.