Effects *in vivo* of decreased plasma and intracellular muscle glutamine concentration on whole-body and hindquarter protein kinetics in rats

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ABSTRACT

Glutamine is considered to be a 'conditionally' essential amino acid. During situations of severe stress like sepsis or after trauma there is a fall in plasma glutamine levels, enhanced glutamine turnover and intracellular muscle glutamine depletion. Under these conditions, decreased intramuscular glutamine concentration correlates with reduced rates of protein synthesis. It has therefore been hypothesized that intracellular muscle glutamine levels have a regulatory role in muscle protein turnover rates. Administration of the glutamine synthetase inhibitor methionine sulphoximine (MSO) was used to decrease glutamine levels in male Wistar rats. Immediately after the MSO treatment ($t = 0$ h), and at $t = 6$ h and $t = 12$ h, rats received intraperitoneal injections (10 ml/100 g body weight) with glutamine (200 mM) to test whether this attenuated the fall in plasma and intracellular muscle glutamine. Control animals received alanine and saline after MSO treatment, while saline was also given to a group of normal rats. At $t = 18$ h rats received a primed constant infusion of L-[2,6-^{3}H]phenylalanine. A three-pool compartment tracer model was used to measure whole-body protein turnover and muscle protein kinetics. Administration of MSO resulted in a 40% decrease in plasma glutamine and a 60% decrease in intracellular muscle glutamine, both of which were successfully attenuated by glutamine infusions. The decreased intracellular muscle glutamine levels had no effect on whole-body protein turnover or muscle protein kinetics. Also, glutamine supplementation did not alter these parameters. Alanine supplementation increased both hindquarter protein synthesis and breakdown but the net balance of phenylalanine remained unchanged. In conclusion, our results show that decreased plasma and muscle glutamine levels have no effect on whole-body protein turnover or muscle protein kinetics. Therefore, it is unlikely that, *in vivo*, the intracellular muscle concentration of glutamine is a major regulating factor in muscle protein kinetics.

INTRODUCTION

The five-carbon amino acid glutamine has many important metabolic functions, e.g. as a vehicle for the transfer of nitrogen and carbon between tissues, as a fuel for rapidly dividing cells, as a precursor for nucleic acid biosynthesis and as a regulator of acid/base homoeostasis. There is considerable evidence that glutamine should now be re-classified as a conditionally essential amino acid, since its requirement markedly increases in

Key words: amino acid, glutamine, glutamine synthetase, muscle, protein degradation, tracer.
Abbreviations: α-AN, α-amino nitrogen; MSO, methionine sulphoximine; PAH, *para*-aminohippuric acid.
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situations of severe stress such as during sepsis or after trauma. In these situations there is a fall in plasma glutamine levels, enhanced net efflux of glutamine by skeletal muscle (the major glutamine-producing organ) and intramuscular glutamine depletion [1–4]. Under these conditions the decreased intramuscular glutamine concentration correlates with reduced rates of protein synthesis, suggesting a role of glutamine in the regulation of protein synthesis [5]. Evidence for this hypothesis was provided by MacLennan et al. who reported increased protein synthesis [6] and inhibited protein breakdown [7] in perfused rat hindquarter when glutamine was added to the perfusate. These findings and others have resulted in the therapeutic use of glutamine supplementation. Clinical studies have provided evidence that parenteral glutamine supplementation after surgery raises the depleted intramuscular glutamine concentrations and enhances nutritional status by promoting positive nitrogen balance and increasing *in vitro* indices of muscle protein synthesis [8,9]. However, animal studies did not give clear evidence for a stimulating effect of glutamine administration on muscle protein synthesis. Although Ardawi [10] reported improved intramuscular glutamine levels and improved *in vitro* protein synthesis in incubated muscle of septic rats given parenteral glutamine, this was not confirmed by another group [11]. Also, *in vivo* studies using endotoxin [12] and turpentine administration [13,14] as stress models demonstrated raised intracellular muscle glutamine levels after glutamine supplementation but no difference in rates of muscle protein synthesis. An explanation for these inconsistent results could be the confounding influence of additional factors in these stress models, such as insulin or thyroid hormone levels.

In the present study we wanted to test the hypothesis that intracellular muscle glutamine level *per se* has a regulating role in muscle protein turnover. We therefore used a previously developed [15] *in vivo* model of sustained decreased arterial and intracellular muscle glutamine levels by administration of L-methionine sulphoximine (MSO), an effective inhibitor of glutamine synthetase [16,17]. Rats treated with MSO were given glutamine parenterally to test whether this attenuated the fall in plasma and muscle tissue glutamine levels, and consequently whether this improved muscle protein metabolism. Protein kinetics were measured with a three-pool compartment tracer model which enables simultaneous measurement of rates of intracellular protein synthesis and breakdown [18,19].

MATERIALS AND METHODS

Animals

The experiments were performed in 40 male specified pathogen-free Wistar rats, weighing 311 ± 8 g (randomly bred Wistar/CPB, WU/Bor, Winkelmann, Borchen, Germany). Rats were subjected to standard 12-h light–dark cycle periods (07.30 to 19.30 hours) and were individually housed in metabolic cages during an acclimatization period of 4 days and during the experiments. During acclimatization to the cage-environment the rats were fed standard rat chow (SRMA 1210, Hopefarmes, Woerden, The Netherlands) and water *ad libitum*. All animal use procedures were performed by licensed personnel and in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, as applied in our institute [20].

Experimental groups

Four days before the beginning of the experiments, rats ($n = 10$ per group) were assigned randomly to one of four groups: 1, NORM-saline; 2, MSO-saline; 3, MSO-ALA; and 4, MSO-GLN. On the first day (day 0) of the experiment, rats of the MSO groups received an intraperitoneal injection of 25 mg/kg body weight L-MSO (Sigma M 5379, St. Louis, MO, U.S.A.) dissolved in normal saline (0.25 ml/100 g body weight; 150 mM NaCl, Merck, Darmstadt, Germany). Rats of the NORM-saline group received an intraperitoneal injection of an equal volume of normal saline. Immediately after, at 0, 6 and 12 h, rats received subcutaneous injections (10 ml/100 g body weight) of either normal saline (NORM-saline and MSO-saline), alanine (MSO-ALA, 200 mM L-alanine, Sigma A-7627) or glutamine (MSO-GLN, 200 mM L-glutamine, Sigma G-1326). During the experiment the rats were not allowed to eat or drink. Rats were sampled 18 h after the first injections.

Surgical procedure

Under ether (Chempropack BV, Dordrecht, The Netherlands) anaesthesia and at constant, pre-anaesthesia body temperature, a laparotomy was performed. Subsequently, a tertiary branch of the superior mesenteric vein, the right renal vein, the portal vein, the inferior caval vein, the right carotid artery and the abdominal aorta (just above the bifurcation) were cannulated as described in detail previously [21,22]. The complete procedure took approximately 30 min. In this set-up hindquarter and portal-drained viscera metabolism was measured simultaneously. The results of portal-drained viscera metabolism are discussed elsewhere.

Plasma flow was determined with the indicator dilution method using *para*-aminohippuric acid (PAH: iso-osmolaric, pH 7.40, Sigma A1422). A primed (0.15 ml/100 g body weight, 50 mM) continuous infusion (1.0 ml/100 g body weight/h, 5 mM) of PAH was simultaneously infused in the aorta and the superior mesenteric vein using a Gilson Minipuls 3 Peristaltic pump (Gilson Medical Electronics Inc., Villiers-le-Bel, France).

To study protein turnover, a primed (1 ml/100 g body weight, 1 μCi/ml) constant (1 ml·h^{-1}·100 g^{-1} body weight, 1 μCi/ml) infusion of L-[2,6-^{3}H]phenylalanine ([^{3}H]Phe, Sigma P 6053) was given in the right renal vein using the Gilson Minipuls pump. Measurements were taken at least 45 min after the priming dose, during steady-state conditions [19,23]

Sampling procedure

After a stabilization period of 10 min in which the rat was not handled, blood was slowly and simultaneously aspirated (1.0 ml per vessel at a rate of 1.0 ml/min) from the inferior caval vein, the portal vein and the common carotid artery. In some rats it was not possible to sample blood from one of the catheters, and therefore these animals could not be included in the calculations. The final number of rats in each group is given in the Tables. Samples were collected in heparinized cups (lithium–heparin micro-sample container CB100, Sarstedt, Essen, Germany) on ice. Immediately afterwards the right gastrocnemius muscle was rapidly excised, freeze-clamped with Wollenberger tongs [24] (total procedure in less than 30 s), put into liquid nitrogen and stored at −80 °C until further analysis. At the end of the experiment the anaesthetized rats were killed by opening of the diaphragm, to prevent spilling of radioactive blood.

Biochemical analysis and tissue processing

Blood and tissue samples were prepared, stored and processed as described previously [19,25]. In brief, haematocrit was obtained with a microfuge. Whole blood was deproteinized with trichloroacetic acid (10% w/v; Merck 807, Darmstadt, Germany) for spectrophotometric determination of PAH on a Cobas Mira S (Roche Diagnostica, Hoffman-La Roche, Basel, Switzerland). Plasma was obtained by centrifugation (8900 *g* at 4 °C for 5 min) and was deproteinized using trichloroacetic acid (50% w/v) before storage (−80 °C), for spectrophotometric determination by standard enzymic methods (Cobas Mira S) of urea and ammonia [22].

For determination of plasma amino acids, plasma was added to 5% sulphosalicylic acid (Brunschwig, Amsterdam, The Netherlands) for deproteinization and was determined after storage (−80 °C) by HPLC [26]. Plasma phenylalanine specific activity was determined by HPLC after its isolation on an ion-exchange column using a technique described previously [27]. For the determination of tissue ammonia and amino acid concentrations and tissue specific activities, muscle tissue was pulverized using a mortar and pestle precooled in liquid nitrogen. The tissue was further homogenized and deproteinized (sulphosalicylic acid) in a Mini-Beadbeater (Biospec Products, Bartlesville, OK, U.S.A). The homogenate was centrifuged (11000 *g* at 4 °C) and the supernatant frozen in liquid nitrogen and stored at −80 °C until further analysis. Tissue water content was determined after freeze-drying pulverized tissue [19].

Calculations

Hindquarter plasma flow and substrate flux

α-Amino nitrogen (α-AN) was calculated as the sum of the individual amino acids measured [26]. Plasma flow across the hindquarter was calculated using the PAH indicator dilution method [21]. Hindquarter substrate flux was calculated by multiplying the inferior caval vein−arterial concentration difference and the mean hindquarter plasma flow of the group. Flux is expressed in nmol·min^{-1}·100 g^{-1} body weight. A positive flux indicates net release; a negative flux reflects net uptake.

Whole-body and hindquarter protein turnover

Whole-body protein turnover was calculated by dividing the venous infusion rate of [^{3}H]Phe by the arterial specific activity of [^{3}H]Phe at steady state [28]. Hindquarter protein turnover was calculated by the arteriovenous dilution of [^{3}H]Phe across the hindquarter using a three-pool compartment model [18]. In this three-pool compartment model, measurement of the arterial, venous and intracellular specific activity enables the intracellular disposal (*D*, rate of protein synthesis) and production (*P*, rate of protein breakdown) of phenylalanine to be estimated. This reflects *in vivo* intracellular protein turnover, because metabolism of phenylalanine in muscle is restricted to synthesis and breakdown of protein [28,29].

In the present study we did not measure the enrichment of the amino acyl-tRNA of phenylalanine, the true precursor pool for protein synthesis, but used the enrichments of the arterial and the intracellular pool as surrogate values [30]. Since the specific activity in the arterial pool exceeds that of the tRNA pool, calculations using arterial specific activity as precursor pool labelling will underestimate protein synthesis and can be seen as lower limits [30]. The intracellular specific activity is generally lower than the tRNA pool and calculations with this pool will therefore overestimate the true rate of protein synthesis and can be viewed as upper limits. A recent study by Ljungvist et al. [31] suggests the intracellular specific activity to be a more valid and constant surrogate value than the plasma specific activity.

Group comparisons and statistical analysis

Differences between groups were tested using ANOVA. Bonferroni *post hoc* analysis between appropriate control groups was used for variables that showed significant differences ($P < 0.05$ was considered significant). Thus, *post hoc* comparisons were made between NORM-saline

and MSO-saline to test the effect of MSO. The MSO-saline group was compared with the MSO-GLN group to test for the effect of glutamine administration during MSO treatment. To control for the metabolic effects of administration of a single amino acid the MSO-GLN group was compared with the MSO-ALA group. Statistical analysis was performed using the SPSS Statistical Software Package, version 7 (SPSS Inc., Chicago, IL, U.S.A.). Data are presented as means ± S.E.M.

RESULTS

Arterial concentrations (Table 1)

Administration of MSO resulted in a 40% reduction in plasma glutamine concentration, which was successfully attenuated by glutamine infusions. MSO treatment did not alter the plasma levels of other amino acids, ammonia and urea. Glutamine infusion increased plasma ammonia and urea, and decreased tyrosine and α-AN concentrations compared with saline controls. However, there were no differences in plasma urea, phenylalanine, tyrosine and α-AN levels compared with alanine controls. Plasma glutamate and alanine were lower after glutamine supplementation compared with the control groups, but no other effects were demonstrated.

Muscle tissue concentrations (Table 2)

Muscle tissue water content did not differ between groups. MSO treatment successfully reduced intracellular muscle tissue glutamine concentration to 60% of NORM-saline controls. MSO treatment had no effect on intracellular muscle ammonia concentration or on concentrations of other amino acids. Supplementation of glutamine attenuated the fall in intracellular muscle glutamine, resulting in higher tissue levels compared with both control groups. Glutamine-treated rats also had decreased intracellular muscle tissue tyrosine and α-AN concentrations compared with saline controls, but not compared with alanine-treated controls (Table 2).

Table 1 Arterial concentrations

Values are means ± S.E.M. BCAA: sum of the branched-chain amino acids valine, leucine and isoleucine; α-AN: sum of all amino acids. Statistics: ANOVA and Bonferroni for comparison between appropriate control groups ($P < 0.05$): NORM-saline versus MSO-saline, [a]$P < 0.05$; MSO-saline or MSO-ALA versus MSO-GLN, [b]$P < 0.05$. NS, not significant.

	NORM-saline ($n = 8$)	MSO-saline ($n = 8$)	MSO-ALA ($n = 9$)	MSO-GLN ($n = 9$)	ANOVA P value
Ammonia (μmol/l)	174 ± 12	200 ± 25[b]	193 ± 21[b]	292 ± 34	0.005
Urea (mmol/l)	3.5 ± 0.3	3.2 ± 0.1[b]	8.5 ± 0.9	11.6 ± 1.8	0.000
Glutamate (μmol/l)	101 ± 6	88 ± 11	93 ± 5[b]	70 ± 6	0.017
Glutamine (μmol/l)	452 ± 42[a]	279 ± 29[b]	294 ± 17[b]	464 ± 90	0.021
Alanine (μmol/l)	455 ± 58	536 ± 67	602 ± 47[b]	392 ± 41	0.034
Tyrosine (μmol/l)	87 ± 7	83 ± 6[b]	54 ± 3	44 ± 2	0.000
Phenylalanine (μmol/l)	136 ± 9	140 ± 8	132 ± 4	124 ± 4	NS
BCAA (μmol/l)	279 ± 13	274 ± 27	277 ± 15	264 ± 26	NS
α-AN (μmol/l)	2948 ± 129	3064 ± 226[b]	2628 ± 124	2285 ± 225	0.014

Table 2 Muscle tissue amino acid concentrations and percentage water

Values are means ± S.E.M. BCAA: sum of the branched-chain amino acids valine, leucine and isoleucine; α-AN: sum of all amino acids; ww, wet weight. Statistics: see Table 1. NORM-saline versus MSO-saline, [a]$P < 0.05$; MSO-saline or MSO-ALA versus MSO-GLN, [b]$P < 0.05$. NS, not significant.

	NORM-saline ($n = 8$)	MSO-saline ($n = 8$)	MSO-ALA ($n = 9$)	MSO-GLN ($n = 9$)	ANOVA P value
%H_2O (ww)	76.6 ± 0.2	76.6 ± 0.2	76.6 ± 0.2	76.5 ± 0.3	NS
Ammonia (μmol/kg ww)	633 ± 104	608 ± 62	583 ± 57	807 ± 130	NS
Glutamate (μmol/kg ww)	1134 ± 94	1257 ± 219	1587 ± 138	1539 ± 161	NS
Glutamine (μmol/kg ww)	1883 ± 246[a]	1247 ± 105[b]	1074 ± 67[b]	2137 ± 215	0.000
Alanine (μmol/kg ww)	2418 ± 134	2608 ± 222	2560 ± 162	2021 ± 216	NS
Tyrosine (μmol/kg ww)	145 ± 10	149 ± 7[b]	100 ± 5	103 ± 11	0.000
Phenylalanine (μmol/kg ww)	149 ± 8	152 ± 7	145 ± 7	150 ± 4	NS
BCAA (μmol/kg ww)	227 ± 16	269 ± 40	250 ± 18	275 ± 52	NS
α-AN (μmol/kg ww)	28551 ± 642	29356 ± 564[b]	26628 ± 727	26595 ± 1044	0.022

Table 3 Hindquarter plasma flow, ammonia and amino acid fluxes

Values are means ± S.E.M. BCAA: sum of the branched-chain amino acids valine, leucine and isoleucine; α-AN: sum of all amino acids; bw, body weight. Statistics: see Table 1. MSO-saline or MSO-ALA versus MSO-GLN, [b] $P < 0.05$. NS, not significant.

	NORM-saline ($n = 8$)	MSO-saline ($n = 8$)	MSO-ALA ($n = 9$)	MSO-GLN ($n = 9$)	ANOVA *P* value
Plasma flow (ml · min^{-1} · 100 g^{-1} bw)	4.2 ± 1.5	5.5 ± 0.4[b]	5.3 ± 1.4	2.9 ± 0.6	0.022
Ammonia (nmol · min^{-1} · 100 g^{-1} bw)	115 ± 100	78 ± 53	105 ± 61	39 ± 62	NS
Glutamate (nmol · min^{-1} · 100 g^{-1} bw)	−42 ± 32	30 ± 56	−20 ± 45	92 ± 32	NS
Glutamine (nmol · min^{-1} · 100 g^{-1} bw)	136 ± 66	224 ± 153	220 ± 87	228 ± 96	NS
Alanine (nmol · min^{-1} · 100 g^{-1} bw)	23 ± 36	98 ± 27	43 ± 27	43 ± 13	NS
Tyrosine (nmol · min^{-1} · 100 g^{-1} bw)	12 ± 12	54 ± 18	33 ± 21	9 ± 7	NS
Phenylalanine (nmol · min^{-1} · 100 g^{-1} bw)	13 ± 25	45 ± 29	6 ± 12	11 ± 13	NS
BCAA (nmol · min^{-1} · 100 g^{-1} bw)	58 ± 33	176 ± 74	28 ± 51	54 ± 54	NS
α-AN (nmol · min^{-1} · 100 g^{-1} bw)	824 ± 341	2457 ± 776	1975 ± 674	1023 ± 396	NS

Table 4 Whole-body rate of appearance (Ra) of phenylalanine and hindquarter protein synthesis (D_{lw} and D_{up}) and breakdown (P_{lw} and P_{up})

Values are mean ± S.E.M. Protein kinetics shown as net balance, lower (D_{lw}) and upper bounds (D_{up}) of protein synthesis and lower (P_{lw}) and upper bounds (P_{up}) of protein breakdown. Statistics: see Table 1. NS, not significant.

	NORM-saline ($n = 8$)	MSO-saline ($n = 8$)	MSO-ALA ($n = 9$)	MSO-GLN ($n = 9$)	ANOVA *P* value
Whole-body Ra (nmol Phe · min^{-1} · 100 g^{-1} bw)	681 ± 26	749 ± 34	782 ± 40	668 ± 47	NS
Protein kinetics (nmol Phe · min^{-1} · 100 g^{-1} bw)					
D_{lw}	51 ± 28	45 ± 20	104 ± 20	46 ± 11	NS
D_{up}	92 ± 55	64 ± 28	183 ± 37	107 ± 38	NS
P_{lw}	64 ± 9	90 ± 31	110 ± 22	58 ± 18	NS
P_{up}	105 ± 33	109 ± 40	189 ± 37	118 ± 44	NS
Net balance	13 ± 25	45 ± 29	6 ± 12	11 ± 13	NS

Hindquarter plasma flow and fluxes (Table 3)

MSO treatment did not alter hindquarter plasma flow. The hindquarter flow was significantly decreased in MSO-GLN rats compared with MSO-saline controls, but was not different from alanine controls. There were no significant differences in efflux of amino acids across the hindquarter between the appropriate control groups (Table 3).

Protein kinetics (Table 4)

No significant differences in whole-body rate of appearance of phenylalanine could be demonstrated. Calculation of muscle protein synthesis by the three-pool compartment model (D_{lw} and D_{up}) could not demonstrate impaired protein synthesis after MSO treatment. Although glutamine supplementation successfully attenuated intracellular muscle glutamine concentrations, it did not alter muscle protein synthesis. Administration of alanine, however, increased D_{lw} and showed a tendency to increase D_{up} ($P = 0.1$) compared with the glutamine-supplemented rats.

Muscle protein breakdown (P_{lw} and P_{up}) was not influenced by MSO treatment or by glutamine infusions. Administration of alanine showed a tendency to increase protein breakdown (P_{lw}: $P = 0.054$ and P_{up}: $P = 0.083$). As a consequence of the unchanged rates of protein synthesis and breakdown after MSO treatment with and without glutamine infusions, the net balance of phenylalanine across the hindquarter was not different between the control groups.

DISCUSSION

In the present study, MSO was successfully used to decrease plasma and intracellular muscle tissue glutamine concentrations. Subcutaneous infusion of glutamine in MSO-treated rats attenuated the fall in both plasma and intracellular muscle glutamine concentrations. Although

the intracellular muscle glutamine level differed largely between the experimental groups we did not observe changes in the rates of muscle protein synthesis and breakdown measured with the three-pool compartment model. Recently we showed that in rats the three-pool compartment model can detect differences in rates of muscle protein synthesis and degradation of at least 20% [19]. The present study showed that if there were any detectable differences in rates of protein synthesis or degradation, this was probably only in the alanine group. Therefore, this study cannot support a significant role of the intracellular concentration of glutamine in the regulation of muscle protein synthesis.

In periods of catabolic stress, like during trauma or sepsis, intracellular muscle glutamine levels decline by more than 50% and plasma glutamine levels may fall up to 30% [1,2]. The fall in muscle tissue glutamine levels was hypothesized to result from the characteristic kinetic properties of the muscle membrane transporter for glutamine [32]. Jepson et al. [5] proposed a link between the size of the muscle glutamine pool and the rate of muscle protein synthesis. This potential link suggested possibilities of therapeutic use of glutamine, aiming at reversing the loss of muscle glutamine and thereby limiting the loss of protein in injury, sepsis and chronic disease [32].

A number of clinical studies of parenteral glutamine supplementation after surgical stress proved that it raised the depleted intracellular muscle glutamine concentrations [8,9,33,34], but no solid evidence was provided that this rise in glutamine concentration enhanced muscle protein synthesis. These studies showed improved nitrogen balance [8,9] and sparing of intramuscular polyribosomes [9,33,35], a gross *in vitro* parameter of muscle protein synthesis. However, the nitrogen-sparing effect in these studies is of such a degree that it can be largely explained by the increase of the free glutamine (and alanine [8]) concentration in muscle, leaving very little room for net protein sparing. Conflicting with these studies is a recent report by the group of Wolfe [36] who administered dichloroacetate to increase muscle glutamine concentrations in patients with burn wounds. Although dichloroacetate administration successfully increased intracellular glutamine levels by 32%, this did not affect muscle protein synthesis determined by the three-pool compartment model. One of the reasons why these studies in humans could not detect differences in protein kinetics could be that, although the intracellular glutamine concentrations were successfully raised compared with the control groups, the absolute magnitude of these concentrations was still far below healthy control concentrations [9,33,36].

Experimental studies in animals to verify the hypothesis that intramuscular glutamine levels have a regulating role in muscle protein kinetics yielded conflicting conclusions. *In vitro* experiments using perfused hind limbs and incubated muscles suggested a direct relation between acute changes in muscle glutamine concentration and muscle protein kinetics [6,7,37]. In these reports, addition of glutamine (15 mM) to the perfusate, a mixture of amino acids at plasma concentrations, stimulated protein synthesis [6,37] and reduced protein breakdown [7] in muscles of healthy animals. Recently, Fang et al. [11] showed that glutamine requires the presence of other amino acids in the perfusate to regulate protein breakdown. They pointed out that adding glutamine (15 mM) to an amino-acid-free medium had no influence on rates of protein synthesis and breakdown in incubated muscles from both septic and non-septic rats, despite the fact that it markedly increased intracellular glutamine levels. When glutamine was added to a medium containing a mixture of amino acids at physiological levels, total muscular proteolytic rates were inhibited, but rates of muscle protein synthesis were not affected. Interestingly, Wu and Thompson [37] showed that the stimulating effect of 15 mM glutamine on the protein synthesis of isolated chick muscles was decreased from 58% to 19% in the absence of tyrosine. The data of these *in vitro* studies suggest that the relationship between intramuscular glutamine levels and protein turnover rates may be circumstantial rather than causal.

Experimental studies measuring *in vivo* muscle protein synthesis were also unable to support the hypothesis that intracellular muscle glutamine concentration *per se* has a regulatory role in muscle protein metabolism. In these studies, parenteral supplementation of glutamine to endotoxin-treated rats [12], and to rats with aseptic abscesses [13,14], increased muscle glutamine levels but could not improve rates of muscle protein synthesis *in vivo*. The use of stress models to verify the relation between intracellular muscle glutamine levels and muscle protein kinetics can be criticized, since an observed relationship could be coincidental because of the confounding influence of a common additional factor on these parameters, like changes in hormonal and inflammatory mediators [5]. In our study we tried to minimize the influence of confounding factors by using a model of decreased plasma and muscle glutamine levels by administration of MSO, an effective glutamine synthetase inhibitor [16,17]. Our data confirmed the observations made in the *in vivo* stress models, that raising depleted intracellular muscle glutamine levels by infusions with glutamine has no effect on rates of muscle protein synthesis. In order to use this model to understand human pathophysiology, at least two issues should be kept in mind. Firstly, in the present model intracellular glutamine levels are depleted to 40% of control values, compared with depletion of intracellular glutamine levels of up to 70% during critical illness in humans [36]. Secondly, in the present study muscle tissue glutamine concentrations were successfully raised to normal levels, whereas attempts in humans to replenish tissue glutamine

have only been partly successful [9,33,36]. Whether this phenomenon is due to interspecies differences in glutamine transmembrane transport or splanchnic glutamine extraction, or to differences in the amount of glutamine administered, is unknown and needs further research.

In the present study, the net balance of phenylalanine across the hindquarter remained unchanged after MSO treatment. This contrasted with a previous study by our group, in which an increased efflux of phenylalanine after a four-fold higher dose of MSO was observed [15]. It is possible that in the present experiment an increased net balance has remained undetected by the variation of the data (type II error). Part of the variation in the model is accounted for by the variation in blood flow. However, the variation in blood flow measurements within the current study is in the same range as reported previously [15,19,21,22,25].

In the present study we did not measure the rate of incorporation of tracer in muscle protein to calculate the fractional synthetic rate. In humans and rabbits [36,38–40] protein synthesis measured with the three-pool compartment model gives comparable results and similar variability to protein synthesis measured with the incorporation model. However, only one study compared the variability of values of muscle protein synthesis determined with the three-pool compartment model with values obtained with the incorporation model in rats [19]. Overnight fasted and moderately fasted rats had similar rates of protein synthesis measured by either the three-pool compartment model or the incorporation model. In the present experiment fasting periods can be compared with the 16-h period of starvation. However, the rate of variability of the three-pool compartment model was twice as high and this model should only be applied to evaluate acute metabolic changes that are expected to induce changes in rates of protein synthesis of at least 20%.

In the present study MSO treatment did not result in significant alterations in protein kinetics. However, even if MSO treatment induced an increased muscle protein breakdown compared with the control (saline) rats it would not prevent a rejection of the central hypothesis. Phenylalanine net balance did not differ between the glutamine- and alanine-treated groups, although the latter had markedly lower intracellular muscle glutamine levels. One of the reasons why others found a stimulating effect of glutamine administration could be that they did not control for the effect of additional carbon and nitrogen sources, beyond that of a saline-infused group. Therefore, the rats infused with the iso-osmolar alanine solution may be a more appropriate control group for the glutamine-infused rats.

In the present study we observed an unusual magnitude of intracellular glutamine concentrations in the NORM-saline group. In this group the average intracellular alanine concentration was higher than the intracellular glutamine concentration. This finding is in contrast to most of the other reports in the literature and also to previous studies performed by our group [15,19,21,23]. A closer analysis of the data of the NORM-saline group showed that in 5 of the 8 rats the glutamine concentration was lower than the alanine concentration. Therefore, a consistent error during the data processing seems unlikely. The observed low intracellular glutamine concentrations could represent the outer limits of normal variation in (Wistar) rats. Evidence for this was found in a series of experiments in Wistar rats by Ardawi et al. [4,10,41,42]. As in our study, they have a single report [4] in which the intracellular muscle glutamine concentration was lower than the alanine concentration in the (pair-fed) control group. Moreover, the 'normal' controls in the present study received subcutaneous saline supplementation and it is therefore difficult to compare their intracellular amino acid concentrations with data from the literature.

In conclusion, the present study showed that reduced plasma and intracellular muscle tissue glutamine concentrations after MSO treatment were successfully raised after subcutaneous glutamine infusions. Although intracellular muscle glutamine levels differed largely between the experimental groups in this *in vivo* model, no changes in rates of muscle protein synthesis and breakdown and whole-body protein turnover were observed. Therefore, we have to conclude that *in vivo* the intracellular muscle concentration of glutamine is unlikely to be a major regulating factor in muscle protein kinetics.

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