Volume 237, number 1,2, 133-136 FEB 06291 September 1988

Inhibition of protein breakdown by glutamine in perfused rat skeletal muscle

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Received 22 July 1988

We have assessed the effects of glutamine (Gln) availability on protein breakdown in perfused rat hindlimb by measuring net phenylalanine (Phe) production (an index of protein balance), the dilution of [^{15}N]Phe labelling (an index of mixed protein breakdown) and rate of production of 3-methylhistidine (3-MeH) (an index of myofibrillar breakdown). 15 mM Gln significantly inhibited net protein loss and protein breakdown compared to rates obtained in its absence (net protein loss, 200 ± 230 vs 2080 ± 200 nmol Phe/hindlimb per h; protein breakdown, 4566 ± 480 vs 1614 ± 180 nmol Phe/hindlimb per h; both $p < 0.01$). Insulin (100 μU/ml) inhibited protein breakdown but less than Gln. The effects on protein breakdown of Gln and insulin together were not additive, suggesting a common mode of action. Production of 3-MeH (mean 20.3 ± 2.8 nmol/hindlimb per h) was unaffected by Gln or insulin. Gln appears to inhibit protein breakdown of soluble rather than myofibrillar protein in muscle.

Glutamine; Anabolism; Myofibrillar protein; Soluble protein; (Muscle)

1. INTRODUCTION

It is now well recognised that wasting of the musculature often accompanies bodily injury (whether due to elective surgery or accidental injury), endocrine disease, sepsis, primary myopathy and cancer [1]. In such conditions a common finding is a lower-than-normal concentration of free Gln in the intramuscular water [1,2]. We have presented evidence elsewhere [3,4] that the characteristics of transport of Gln in skeletal muscle are such (sodium dependency and hormonal sensitivity) that known changes in the hormonal and ionic milieu occurring with disease and injury will tend to promote Gln efflux from muscle. In attempting to decide whether the correlation between low intramuscular Gln concentration and net protein wasting of muscle was fortuitous or causal, we discovered a positive relationship between the fractional rate of muscle protein synthesis and the intramuscular Gln concentration, manipulated by varying the availability of Gln in the perfused rat hindlimb preparation [5]. The effect was substantial, comprising a 2-fold change in muscle protein synthesis over the range of intramuscular Gln of 7–35 μmol/g protein (about 2–12 mM in the intracellular water). Although the effect was independent of insulin (i.e. occurred in its absence), the sensitivity of protein synthesis to Gln was greater in the presence of high physiological concentrations of insulin (100 μU/ml). Since the maintenance of the protein mass of a tissue depends on the balance between rates of protein synthesis and breakdown, we were naturally interested to determine whether the anabolic effect of Gln on protein synthesis might extend to an inhibition of protein breakdown in the perfused rat muscle preparation. An inhibitory effect of Gln on protein breakdown has been reported for cultured muscle cells [6].

The experiments described below were designed to answer the question by measuring (i) the rates of

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

appearance into the perfusate of unlabelled Phe and 3-MeH as indices of net protein loss and myofibrillar protein breakdown, respectively (Phe is a constituent of all protein, and may be recycled into synthesis; 3-MeH is a constituent of actin and myosin only and is not reutilised) and (ii) the rate of dilution of the labelling of [^{15}N]Phe during the course of the perfusion (an indication of mixed protein breakdown).

2. MATERIALS AND METHODS

Post-absorptive female Wistar rats (250 ± 15 g body wt) were studied between 08:30 and 16:00 h, having had free access to food and water on the previous night. The rats were anaesthetised using pentobarbital (Sagatal, May & Baker, Dagenham, Essex; 6 mg/100 g body weight i.p.) before surgical preparation for perfusion of the hindquarters in the manner described by Ruderman et al. [7]. The perfusion apparatus was as described by MacLennan et al. [5]. The perfusate consisted of Krebs-Henseleit bicarbonate buffer, pH 7.4, equilibrated with 95% O_2/5% CO_2 to which were added sufficient ^{15}N-labelled Phe (99% ^{15}N, Tracer Technology, Somerville, MA) to bring the initial perfusate Phe enrichment to 7–8 gatom% excess (APE). All other amino acids (except the branched-chain amino acids (BCAA) which were at one third of normal plasma concentrations, Gln which was either absent or present at 15 mM and 3-MeH which was absent) were present at normal rat plasma concentrations [5]. Such manipulations result in intracellular Gln concentration in gastrocnemius muscle of about 2 and 10 μmol/g muscle respectively, compared to 4.8 μmol/g muscle in gastrocnemius muscle sampled in vivo from anaesthetised rats.

In addition [1-^{13}C]Leu (99% [^{13}C]Leu, Tracer Technology) was added to the perfusate to approx. 10 APE. After initiation of the perfusion at a flow rate of 6 ml/hindquarter per min, the preparation was allowed to stabilize over a period of 20 min at the end of which gastrocnemius muscle from the left leg was partially dissected free (blood supply intact) and clamp-frozen using Wollenberger tongs cooled in liquid N_2. The left iliac artery and vein were ligatured and the flow rate of the right half of the hindquarter reduced to 3 ml/min. During the equilibration period, the perfusate was discarded, but over the subsequent 40 min of perfusion, perfusate was collected. Each aliquot was thoroughly mixed and samples taken for measurement of amino acid concentration (by use of a standard amino acid analyser (Biotronik) using lithium buffers and fluorimetric detection), and for gas chromatographic-mass spectrometric analysis of enrichment of the Phe with ^{15}N and Leu with ^{13}C [8]. D_3-3-MeH (Tracer Technology) was added to the perfusate as internal standard (4.5 μmol 99% D_3). The concentration of 3-MeH in 20-ml aliquots of perfusate was measured after precipitating out perfusate albumin using 10% perchloric acid, neutralising with 2 M KOH, 0.5 M KCl desalting was performed by means of ion-exchange chromatography using Dowex-50W X8 (H^+ form), by washing with 1 M HCl then distilled water. The amino acid fraction was eluted with 4 M NH_4OH, the ammonia evaporated under nitrogen and then freeze-dried. The residue was dissolved in 0.1 M pyridine then passed through Dowex-50W X8 (H^+) according to Haverberg et al. [9]. The column was prewetted with 0.2 M pyridine. Neutral and acidic amino acids were eluted with 0.2 M pyridine (16 ml) and elution of 3-MeH was achieved using 1 M pyridine (8 ml). The eluate was concentrated by rotary evaporation prior to quantitation of 3-MeH using selected ion monitoring GC/MS of the *tert*-butyldimethylsilyl derivative [8]. Chromatographic separation was performed on an RSL-300 fused silica capillary column (25 m × 0.25 mm Alltech Associates, Deerfield, IL) coupled directly to the ion source of a Finnigan 1020B GCMS. Helium was used as a carrier gas; sample injection was in the spitless mode. The column was held at 140°C for 1 min then ramped to 280°C at 20°C per min.

The difference in concentrations of amino acids and the enrichment of Phe were used to calculate various indices of protein turnover by comparison of these values in an aliquot collected between 40 and 60 min perfusion with those of the first aliquot collected between 0 and 20 min perfusion. Net protein loss was calculated from the increase in Phe production, myofibrillar protein breakdown from the production of 3-MeH and the rate of mixed protein breakdown from the dilution of [^{15}N]Phe by unlabelled Phe entering the perfusate. The changes in [^{13}C]Leu enrichment were used for comparison although it was recognised that since Leu may be metabolised to its keto acid, the dilution of [^{13}C]Leu would not provide a specific index of protein breakdown. At the end of 1 h perfusion, the gastrocnemius muscle of the right leg was clamped frozen as the left leg had been earlier. Muscle tissues were analyzed for ATP and lactate by standard enzymatic methods [10]; free amino acids were extracted from the tissue using 10% perchloric acid. Concentrations of muscle free amino acids and enrichments of Phe with ^{15}N and Leu with ^{13}C were estimated as described above. Concentrations of intramuscular 3-MeH were not measured.

3. RESULTS

The perfused preparation was stable throughout the 1 h perfusion following the equilibration period. Oxygen consumption and production of CO_2 and H^+ were constant and of the expected magnitude (305 ± 35, 218 ± 28, 2.6 ± 1.1 nmol/hindlimb per min, respectively, means ± SD). Muscle ATP and lactate concentrations were identical in muscle sampled after the initial equilibration periods and after 1 h perfusion and were similar to values in vivo (36 ± 9 and 9 ± 4 μmol/g protein, respectively). Intramuscular concentrations of Gln were, in the absence of added perfusate Gln, 16.5 ± 3.5 μmol/g protein, and in the presence of 15 mM Gln, 62.4 ± 8.8 μmol/g protein. Muscle Glu concentrations (14.4 ± 2.8 μmol/g protein) were not significantly different, in the absence or presence of Gln. There were no significant differences in the intramuscular concentrations of Val, Iso and Leu (0.62 ± 0.19, 0.35 ± 0.10, 0.53 ± 0.13 μmol/g protein, respectively), the

Table 1

Effect of Gln on indices of muscle protein breakdown and net loss of protein

Perfusate conditions	Intramuscular Phe (nmol/g protein)	Net Phe release (nmol Phe/hindlimb per h)	Mixed protein breakdown (nmol Phe/hindlimb per h)	3-Methylhistidine release (nmol 3-MeH/hindlimb per h)
0 Gln, 0 Ins	640 ± 170	2080 ± 200	4566 ± 480	19.1 ± 4.8
15 mM Gln, 0 Ins	570 ± 70	200 ± 230[b]	1614 ± 180[b]	24.6 ± 8.4
0 Gln, 100 μU/ml Ins	590 ± 90	1840 ± 175	3232 ± 340[a]	18.6 ± 2.4
15 mM Gln, 100 μU/ml Ins	450 ± 140[a]	720 ± 140[b]	1661 ± 150[b]	18.8 ± 2.8

Hindlimbs from 250 ± 15 g rats were perfused as in [5] with all perfusate AA except glutamine (0 or 15 mM), BCAA (1/3 normal) and 3-methylhistidine (absent) at plasma concentrations. [^{15}N]Phe was added to give starting enrichment of 7–8 gatom% excess. Perfusate was sampled after 20 min equilibration and again after 60 min; hindlimb muscle was sampled at 60 min. Mixed protein breakdown was calculated from the dilution of [^{15}N]Phe. [a] $p<0.05$; [b] $p<0.01$ comparison of values with those for 0 Gln, 0 insulin. Results are means ± SD for 6 preparations

BCAA, in preperations studied in the absence or presence of 15 mM Gln.

In the absence of perfusate Gln or insulin, the intramuscular concentration of Phe became elevated (as was tyrosine, not shown) and this was reflected in a rise of perfusate Phe concentration (table 1). The rise in Phe production rate was used as an index of the net balance of protein, i.e. protein breakdown minus protein synthesis. The release rate observed in the absence of Gln was almost completely attenuated by its presence; insulin had a smaller effect and their presence together caused no significant additional diminution in the net rate of appearance of unlabelled Phe.

Insulin and Gln, when presented separately, substantially diminished the dilution of [^{15}N]Phe by release of unlabelled phenylalanine (table 1).

Dilution of [^{13}C]Leu was also diminished; the ratio of final to initial [^{13}C]Leu labelling was 0.68 ± 0.09 for preparations without Gln or insulin and 0.57 ± 0.07 in the presence of Gln and insulin ($p<0.05$). As for the effect of the net appearance of Phe, the effect of Gln and insulin together on the dilution of ^{15}N was no greater than the effect of either alone. The rate of appearance of 3-MeH was unaffected by the absence or presence of Gln and insulin.

4. DISCUSSION

The present results were obtained in a preparation of mixed skeletal muscle which was able to maintain physiological rates of oxygen consumption and CO_2 production as well as normal concentrations of ATP. Muscle lactate concentration and production rate were also within the range which we observe in the muscle of anaesthetised rats in vivo [11].

The lack of any changes in plasma or perfusate Glu or BCAA on addition of Gln at 15 mM suggests that, as expected from the low activities of glutaminase in rat muscle, there is no possibility of increased production of BCAA as a result of addition of Gln. Thus any effects of Gln on protein turnover are independent of changes in BCAA. The present results show quite clearly that Gln does affect the protein balance of perfused rat muscle as indicated by the lower release of Phe in the presence of elevated concentrations of Gln, the effect being to inhibit the net loss of amino acids. We have previously shown that Gln stimulates protein synthesis in muscle [5] and a substantial part of the inhibition of net loss is presumably via this mechanism; nevertheless the only means by which Gln could conceivably inhibit the dilution of tracer [^{15}N]Phe, as was observed, is by an inhibition of muscle protein breakdown. This effect was substantial, but was not as great as the effect of Gln on net Phe balance suggesting that the stimulation of protein synthesis, as observed previously [5], is more sensitive to Gln than the inhibition of protein breakdown. The effects of insulin and Gln appeared not to be additive suggesting that they act through a common mechanism. The effects of Gln and insulin on [^{13}C]Leu dilution supported these interpretations.

Assuming that Phe comprises 4% of muscle protein and that each hindlimb, perfused from the iliac bifurcation, contains about 8 g muscle comprising about 18% protein, then the rate of mixed muscle

protein breakdown observed in the absence of Gln or insulin is about 20% per day. In the presence of Gln and insulin, the rate appears to fall to about 7% per day. We have previously observed rates of muscle protein synthesis in the presence of 15 mM Gln in an identical preparation to be about 15% per day suggesting that there would be accretion of hindlimb muscle of about 5–8% per day, values which may be observed in rats of this size under some circumstances in vivo [12].

The production rate of 3-MeH was identical under all circumstances. This suggested that although the effect of high concentrations of Gln was to inhibit muscle protein breakdown, the protein subject to proteolysis did not include that from which 3-MeH is derived, i.e. the actin and myosin of the myofibrillar apparatus. These observations are thus further examples of a particular kind of differential response between the soluble sarcoplasmic protein which appears to be susceptible to a variety of positive and negative influences on its rate of proteolysis and myofibrillar protein which is much less susceptible [13–15].

The present results are consonant with those obtained in a different system by Smith who showed in a brief report that Gln could inhibit release of tracer previously incorporated into protein in cultured L6 myoblasts [6]. Further, so far as we know, they constitute the first observation of an effect of Gln in inhibiting muscle protein breakdown in mature mixed-fibred mammalian skeletal muscle. The demonstration of an inhibitory effect on muscle protein breakdown adds further weight to the idea derived from the correlation between muscle protein wasting and low intramuscular Gln concentration as well as the demonstration of a positive relationship between muscle Gln and protein synthetic rate, that intramuscular Gln concentration plays an important part in the control of the size of the muscle mass.

Acknowledgements: This work was supported by grants from Lipha Ltd, British Diabetic Association, Action Research, Muscular Dystrophy Group of Great Britain, Biomedical Research Committee of SHHD, University of Dundee and The Wellcome Trust.

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