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Effect of plasma insulin and branched-chain amino acids on skeletal muscle protein synthesis in fasted lambs

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The increase in fractional rate of protein synthesis (K_s) in the skeletal muscle of growing rats during the transition from fasted to fed state has been explained by the synergistic action of a rise in plasma insulin and branched-chain amino acids (BCAA). Since growing lambs also exhibit an increase in K_s with level of feed intake, the objective of the present study was to determine if this synergistic relationship between insulin and BCAA also occurs in ruminant animals. Six 30 kg fasted (72 h) lambs (8 months of age) received each of four treatments, which were based on continuous infusion into the jugular vein for 6 h of: (1) saline (155 mmol NaCl/l); (2) a mixture of BCAA (0·778 μmol leucine, 0·640 μmol isoleucine and 0·693 μmol valine/min·kg); (3) 18·7 μmol glucose/min·kg (to induce endogenous insulin secretion); (4) co-infusion of BCAA and glucose. Within each period all animals received the same isotope of phenylalanine (Phe) as follows: (1) L-[1-^{13}C]Phe; (2) L-phenyl-[ring $^{2}H_5$]-alanine; (3) L-[^{15}N]Phe; (4) L-[ring 2,6-^{3}H]Phe. Blood was sampled serially during infusions to measure plasma concentrations of insulin, glucose and amino acids, and plasma free Phe isotopic activity; biopsies were taken 6 h after the beginning of infusions to determine K_s in *m. longissimus dorsi* and *vastus* muscle. Compared with control (saline-infused) lambs, K_s was increased by an average of 40 % at the end of glucose infusion, but this effect was not statistically significant in either of the muscles sampled. BCAA infusion, alone or in combination with glucose, also had no significant effect on K_s compared with control sheep. K_s was approximately 60 % greater for *vastus* muscle than for *m. longissimus dorsi* ($P<0·01$), regardless of treatment. It is concluded that there are signals other than insulin and BCAA that are responsible for the feed-induced increase in K_s in muscle of growing ruminant animals.

Muscle protein synthesis: Insulin: Branched-chain amino acids

While insulin is the primary hormone responsible for maintenance of glucose homeostasis, it also has potent actions on protein metabolism. The increased protein anabolism in response to feeding previously fasted animals has been postulated to be mediated by the transient increases in insulin and amino acid concentrations that follow a meal (e.g. Millward *et al.* 1996; Svanberg *et al.* 1996*a*; Garlick *et al.* 1998). In ruminant animals, although muscle protein synthesis is increased during the transition from undernourished to fed states (Lobley *et al.* 1992; Crompton & Lomax, 1993), the differences between post-absorptive and postprandial states are less clearly defined, due to rumen fermentation. Therefore, the influence of insulin and amino acids on muscle protein metabolism may well be different in ruminant animals and be part of the explanation for the lower efficiency of N utilisation in ruminant animals compared with non-ruminant animals.

A series of carefully conducted studies in rats, reviewed by Garlick *et al.* (1998), have demonstrated that stimulation of muscle protein synthesis in response to feeding is caused by the synergistic actions of insulin and amino acids. In young fasted rats, intravenous insulin infusion increases muscle protein synthesis, but only at unphysiologically high levels of insulin (Garlick *et al.* 1983). This action of insulin was blocked by anti-insulin serum (Preedy & Garlick, 1986); co-infusion of branched-chain amino acids (BCAA) resulted in a nearly 10-fold increase in sensitivity to insulin infusion such that muscle protein synthesis was stimulated at physiological insulin concentrations (Garlick & Grant, 1988). Studies in young pigs have confirmed that muscle protein synthesis is increased in response to insulin infusion under conditions where glucose and amino acid concentrations are clamped (Wray-Cahen *et al.* 1998), but have suggested that these responses

Abbreviations: B + G, branched-chain amino acids + glucose; BCAA, branched-chain amino acid; BW, body weight; KS, fractional rate of protein synthesis; LD, *m. longissimus dorsi*; Phe, phenylalanine; VM, *vastus* muscle.

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are blunted as animals mature. In adult rats and human subjects muscle protein synthesis is insensitive to fasting or to insulin infusion (Fryberg *et al.* 1990; Baillie & Garlick, 1992; McNurlan & Garlick, 1994). Studies in adult human subjects have demonstrated that muscle protein degradation, rather than synthesis, is more sensitive to changes in nutrient supply and insulin than protein synthesis (Fryburg *et al.* 1990). In rats and human subjects, muscle protein metabolism can also be modulated directly by amino acids (Garlick *et al.* 1998).

In contrast to rodents and human subjects, neither systemic nor close-arterial infusions of insulin into fasted young lambs up to 5 months of age resulted in significant changes in muscle protein synthesis, but muscle protein degradation was decreased (Oddy *et al.* 1987; Douglas *et al.* 1991). Despite this lack of sensitivity to insulin administration, muscle protein synthesis is highly responsive to level of feed intake in 30 kg lambs (5–8 months old; Lobley *et al.* 1992; Crompton & Lomax, 1993; Hoskin *et al.* 2003).

Our previous studies in 6-month-old maintenance-fed wether lambs have demonstrated that a 6 h close-arterial infusion of insulin stimulates net protein gain across the hindlimb and that this effect can be mimicked by infusion of BCAA (Wester *et al.* 2000). However, we did not determine whether these effects were the result of changes in muscle protein synthesis, degradation or both. In view of the relatively few experiments carried out on ruminant animals and the lack of experiments exploring the relationships between insulin and amino acids in this species, the objective of the present study was to determine if the fractional rate of muscle protein synthesis responds to a 6 h increase in plasma insulin during infusions of glucose or BCAA. Muscle protein synthetic responses to the infusion treatments were measured by tracer kinetics using four different isotopes of phenylalanine (Phe), rather than using the same isotope on each occasion, as this creates the problem of increased 'background' labelling (e.g. Lobley *et al.* 1990).

Materials and methods

Animals and design

The present experiment was approved by the Ethical Review Committee of the Rowett Research Institute and conformed to UK legislation of the Animals (Scientific Procedures) Act 1986. Six Suffolk-cross wether lambs (29–34 kg body weight (BW), approximately 8 months of age) were fed 900 g grass pellets (10·5 MJ metabolisable energy/kg DM, 30 g N/kg DM, 900 g DM/kg)/d offered in two daily portions. This diet provided 1·5 × and 1·9 × maintenance requirements for metabolisable energy and protein respectively. Two d before the start of each infusion, a polyvinyl catheter (1·0 mm internal diameter, 1·6 mm outer diameter) was inserted into each external jugular vein: one for infusion of substances and the other for blood withdrawal. Lambs were then placed into metabolism cages and food, but not water, was withheld. Infusion treatments began 64 h after the last feed was offered to the lambs; this time is predicted to achieve a fasted state in ruminant animals (Lomax & Baird, 1983). After the last blood sample was taken, lambs were re-fed to their previous intake by using a continuous feeder. Lambs were returned to floor pens and fed 900 g grass pellets/d for 7 d before the next food withdrawal.

Treatments consisted of 6 h non-primed continuous infusions (0·5 g/min) of the following: (1) saline (155 mmol NaCl/l, control); (2) 0·778 μmol leucine + 0·640 μmol isoleucine + 0·693 μmol valine in saline (BCAA)/min·kg BW; (3) 18·7 μmol glucose in saline/min·kg BW; (4) BCAA and glucose (B + G) at the previous amounts. Glucose infusion rates were selected to double glucose entry rate (Kriel *et al.* 1992) and BCAA to achieve plasma concentrations observed in sheep fed 2–3 × maintenance N requirements (Lobley *et al.* 1998). In addition to treatments, isotopically labelled Phe was added to each infusate and was supplied at the rate of 67 nmol/min·kg BW. Treatments were allocated randomly across four periods and were infused on separate days with 10 d between subsequent infusions. A different isotope of Phe was used for each infusion period to eliminate the need for an initial background muscle biopsy to be taken before the start of each infusion. Isotopes of Phe were given in the following sequence: period 1, L-[1-^{13}C]Phe (^{13}C); period 2, L-phenyl-[ring $^{2}H_5$]-alanine ($^{2}H_5$); period 3, L-[^{15}N]Phe (^{15}N); period 4, L-[ring 2,6-^{3}H]Phe (^{3}H). Preparations of $^{2}H_5$ and ^{15}N were enriched ≥99 atom % while ^{13}C was 86 atom % (Isotec Inc., Miamisburg, OH, USA) and the specific radioactivity of L-[ring 2,6-^{3}H]Phe (Amersham Life Sciences, Amersham, Bucks., UK) was adjusted to approximately 32 MBq/mmol so that an equal mass of Phe was infused each period. Infusates were prepared and filter-sterilised on the day before use.

Sampling and analyses

Blood (2 ml for 15 min samples, 6 ml for hourly samples) was withdrawn into heparinised syringes at 0, 15, 30, 45, 60, 90, 120, 180, 240, 300 and 360 min after the start of the infusion. Blood was immediately placed on ice and centrifuged within 10 min of collection at 1000 ***g*** for 15 min. Plasma was stored at −20°C until analysis. After the 360 min sample, but while the infusate was still being administered, lambs were placed into a portable crush and biopsies (about 0·5 g each) of *m. longissimus dorsi* (LD) and *vastus* muscle (VM) were taken under local anaesthesia. Sampled LD and VM were taken from alternate sides of the backbone and legs respectively between successive infusion periods. These muscles were chosen for sampling because they allow repeat biopsies of the same muscle at different sites and they also represent different major muscle groups on the carcass. At the end of the last infusion ([^{3}H]Phe), lambs were killed by intravenous overdose of pentobarbitone and muscle samples were excised, rinsed quickly with cold saline (155 mmol NaCl/l) and frozen in liquid N_2. Samples were stored at −70°C until analysis.

To measure free and protein-bound Phe in tissues, a 200 mg (400 mg for ^{3}H) sample of frozen muscle was homogenised in 4 ml sulfosalicylic acid (80 g/l) for 1 min on ice. Free Phe was separated from precipitated protein-bound

Phe by centrifugation at 1000 ***g*** for 10 min. The pellet was then rinsed three times with 5 ml sulfosalicylic acid to remove any contaminating traces of free Phe. The initial supernatant fraction was passed through a 1 ml column of Dowex AG 50W-X8 and the resin rinsed with 4 × 1 ml ultra-pure water before the Phe was eluted with 2 × 1 ml 2 M-NH_4OH. The eluant was lyophilised and stored at − 20°C until analysis. The washed pellet (containing protein-bound Phe) was dissolved for 1 h in 1 ml 0·6 M-NaOH and then transferred to a screw-topped test-tube containing 15 ml 5·6 M-HCl (4 M-HCl was used for samples containing $^2H_5$ to avoid loss of ^2H during hydrolysis). Tubes were sealed and placed in a heating block at 105°C for 16 h. Hydrolysates were dried under vacuum and resuspended in 2 ml 0·1 M-HCl. The remaining procedures depended on which isotope was to be analysed.

In protein hydrolysate samples containing ^{13}C and ^{15}N, where ion-exchange chromatography was necessary to isolate pure Phe, tyrosine, which would otherwise closely elute with Phe, was oxidised by overnight incubation in 3 ml performic acid at 0°C followed by addition of 0·3 ml HBr. Samples were evaporated under vacuum then resuspended in 1 ml 0·1 M-HCl and clarified through a 0·2 μm filter. Phe was separated by ion-exchange chromatography and isotope enrichment determined by dual-inlet isotope ratio MS following combustion (for ^{15}N) or decarboxylation with ninhydrin (for ^{13}C) as described previously (Lobley *et al.* 1990).

In protein hydrolysate samples containing $^2H_5$, Phe was converted enzymically to β-phenethylamine and measured as the heptaflurobutyryl derivative as described by Calder *et al.* (1992) using GC–MS. Tissue free pool enrichments of all stable isotope forms of Phe were measured by GC–MS as heptaflurobutyryl *n*-butyl esters (Calder & Smith, 1988). Plasma samples were deproteinised with sulfosalicylic acid (70 g/l final concentration) and the supernatant fractions were passed through a 0·5 ml Dowex AG 50W-X8 column and eluted with 2 × 1 ml 2 M-NH_4OH and lyophilised. Stable isotope enrichments of the plasma free pool were measured by GC–MS after conversion to the *t*-butyldimethylsilyl derivatives (Calder & Smith, 1988).

To measure ^3H, Phe in muscle supernatant fractions and protein hydrolysates was enzymically converted to β-phenethylamine and concentration was measured by fluorimetry (Waalkes & Udenfriend, 1957) and radioactivity by liquid scintillation counting. Phe in deproteinised plasma was separated by ion-exchange chromatography and concentration measured by reversed-phase HPLC (Waters Ltd, Watford, Herts., UK) after pre-column derivatisation with phenylisothiocyanate (Heinrikson & Meredith, 1984) and radioactivity by liquid scintillation counting.

Plasma free amino acid concentrations were determined following addition of an equal volume of 0·1 mM-L-norleucine and the supernatant fraction obtained after treatment with sulfosalicylic acid (final concentration 80 g/l) was analysed by automated ion-exchange chromatography and ninhydrin detection (Pharmacia Alpha Plus 2; Amersham Pharmacia Biotech UK Ltd, Little Chalfont, Bucks., UK). Immunoreactive plasma insulin concentration was determined by RIA (Midgley *et al.* 1969) using an antiserum to porcine insulin (IM38; Amersham International, Aylesbury, Bucks., UK) and porcine insulin as standards (Sigma, Poole, Dorset, UK). Plasma glucose concentration was measured using a colorimetric enzymatic (Trinder) method (Sigma).

Calculations and statistical analyses

Rates of skeletal muscle protein synthesis were calculated using one of two estimates for the precursor amino acid pool. The precursor pool was estimated as: (1) the $area_p$ under the isotopic activity × time curve of free Phe in plasma; or (2) the $area_m$ under the isotopic activity × time curve of free Phe in muscle:

$$area_p = S_{max}(kt + e^{-kt} - 1)/k, \quad (1)$$

where t is time in days, S_{max} is the isotopic activity at time t, and k is the rate constant of the isotopic activity × time curve of free Phe in plasma taken as the average of k across animals, isotopes and treatments. The isotopic activity × time curve of free Phe in plasma was described using a curve-fitting program that calculated S_{max}, the isotopic activity of free Phe at equilibrium (or 'plateau'), and k, the rate constant. For all isotopes and lambs, plasma free pool isotopic activity reached equilibrium between 60 and 100 min of infusion.

The $area_m$ under the isotopic activity × time curve for the muscle free Phe precursor pool was obtained from the ratio of enrichments for the endpoint muscle sample and the plasma values × area under the plasma curve. Because a non-primed continuous tracer infusion was used, the area under the isotopic activity × time curve needs to be calculated to estimate accurately the amount of label available for incorporation into protein:

$$area_m = \text{muscle free Phe isotopic activity} \times (kt + e^{-kt} - 1)/k, \quad (2)$$

where t is time in days and k is the rate constant of the of the isotopic activity × time curve of free Phe in plasma taken as the average of k across animals, isotopes and treatments. The use of k from the isotopic activity × time curve of free Phe in plasma assumes that the rate constants for labelling in plasma and muscle free-pools are similar (Nicholas *et al.* 1977). The rate of protein synthesis (K_s; %/d) was calculated as described by equation (3):

$$K_s = 100 \times (\text{muscle protein-bound Phe isotopic activity})/\text{area}, \quad (3)$$

where area is that of selected precursor pool (K_{sp} or K_{sm}, based on plasma (1) or muscle (2) free Phe pools respectively).

Whole-body irreversible loss rate (mmol/h) of the plasma free Phe was calculated as:

$$\text{irreversible loss rate} = \text{isotope infusion rate}/S_{max}. \quad (4)$$

Because the treatments were not replicated equally across periods, data were analysed using the Residual Maximal Likelihood (REML) procedure in GenStat 5 (release 3.2; Lawes Agricultural Trust, Rothamsted, Herts., UK) with animal and animal × period as random effects and

muscle site × (treatment and period) as fixed effects. A Wald statistic was generated to test fixed effects and predicted means were compared using a two-tailed *t* test.

Results

The present experiment was designed to test if insulin stimulates the fractional rate of muscle protein synthesis in fasted lambs and whether this is enhanced by BCAA. To examine these objectives, glucose was infused systemically, to raise endogenous insulin secretion, with and without concurrent infusion of BCAA. Plasma glucose concentration doubled within the first 60 min when glucose was infused and reached an asymptote 180 min into infusion at approximately 3-fold greater than the basal value (Fig. 1(a)). Throughout the glucose infusion, plasma insulin concentration increased steadily, doubling within the first hour, and reaching a maximum of approximately 9-fold after 6 h of infusion (Fig. 1(b)). The pre-infusion saline and BCAA-only insulin values were below the sensitivity of the assay used (≤ 16·4 pM).

Comparison of the values with the control at the start of infusion (0 h) or the end (6 h) demonstrates that the amino acid mixture alone increased the concentration of the total plasma BCAA 2-fold, whereas glucose-only infusion resulted in a decrease to approximately 63 % of control values ($P < 0{\cdot}05$; Table 1). However, for B + G plasma BCAA concentrations were unchanged from control values, i.e. the reduction in BCAA as the result of endogenous insulin was negated by the introduction of exogenous BCAA. The plasma concentration of Phe did not change with treatment except for B + G, where there was a 26 % decrease by the end of the infusion ($P < 0{\cdot}05$). For many other amino acids, there were reductions in plasma concentrations associated with the infusion of glucose regardless of whether or not BCAA were co-infused. In contrast, alanine and serine concentrations were increased by glucose infusion.

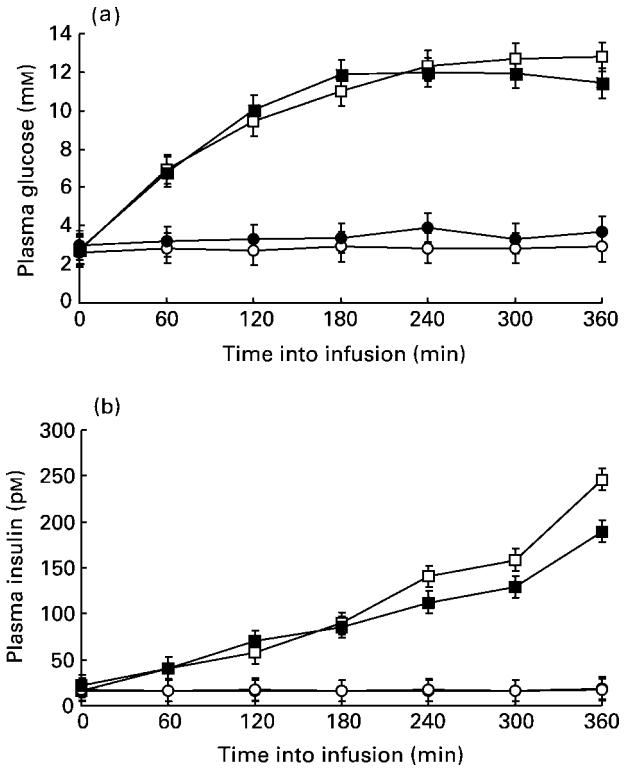

Fig. 1. Plasma glucose (a) and insulin (b) concentrations in lambs infused for 6 h with saline (○; 155 mmol NaCl/l), branched-chain amino acids (●), glucose (□) or co-infusion of branched-chain amino acids and glucose (■). Values are means with their standard errors shown by vertical bars (six lambs per group). For details of procedures, see p. 403.

The fractional rate of muscle protein synthesis was approximately 60 % greater for VM than for LD ($P < 0{\cdot}01$) regardless of treatment. This difference in fractional protein synthetic rate was independent of the precursor pool selected. Treatments with glucose or BCAA, alone or in combination, had no significant effects on skeletal muscle protein synthetic rate, although there was a trend towards a 47 and 30 % increase in VM and LD respectively (Table 2). The whole-body irreversible loss rate of Phe was lowest for BCAA and greatest for glucose, with B + G and the control intermediate ($P < 0{\cdot}05$; Table 2).

Discussion

The aim of the present study was to examine if insulin would stimulate protein synthesis in skeletal muscle of fasted, growing sheep and determine whether co-infusion of BCAA would enhance this action of insulin. This objective arose from the studies of Preedy & Garlick, (1986), who showed that supraphysiological levels of insulin administration were necessary to stimulate skeletal muscle protein synthesis in the fasted young rat, but the amount of insulin required to achieve maximal stimulation was reduced to physiological levels when AA were co-infused. Later it was found that the maximal stimulatory action of a complete mixture of amino acids could be mimicked when only leucine, isoleucine and valine were infused (Garlick & Grant, 1988). That these three amino acids may serve to signal post-hepatic tissues to an impending increase in nutrients is logical, considering that BCAA largely escape hepatic uptake in relation to other amino acids (Heitmann & Bergman, 1980; Wray-Cahen *et al.* 1997).

Previous studies in ruminant animals on the effects of insulin and amino acids on muscle protein synthesis have focused on neonatal or adult animals, and have not examined synergism between insulin and BCAA. The present study and our previous reports (e.g. Crompton & Lomax, 1993; Wester *et al.* 2000; Hoskin *et al.* 2001) have investigated the control of muscle mass in the 30 kg growing lamb (at approximately 40 % of their mature weight and prepubertal), since improvements in lean tissue gain at this weight would have profound effects on carcass composition when they grow to a commercial slaughter weight of about 44 kg. Our previous study (Wester *et al.* 2000) demonstrated that hindlimb protein gain was increased by close arterial infusions of insulin or BCAA at physiological doses, but did not provide evidence of a synergism between insulin and BCAA when co-infused. Since this change in protein gain could have been the result of alterations in protein synthesis or degradation, or both, we examined the hypothesis that muscle protein synthesis was sensitive

Table 1. Plasma free amino acids (μmol/l) before and after a 6 h infusion of saline (155 mmol NaCl/l control), branched-chain amino acids (BCAA), glucose or co-infusion of branched-chain amino acids and glucose (B + G)*

Amino acid	Baseline (0 h)	Control (6 h)	BCAA (6 h)	Glucose (6 h)	B + G (6 h)	SED
Leu	167·1^{a}	159·4^{a}	342·6^{b}	53·8^{c}	131·8^{a}	12·7
Ile	98·4^{a}	99·7^{a}	234·5^{b}	30·9^{c}	84·2^{a}	8·3
Val	240·8^{a}	239·5^{a}	511·0^{b}	99·6^{c}	254·8^{a}	19·4
Phe	42·3^{a}	44·8^{a}	41·2^{a}	40·3^{a}	33·1^{b}	2·0
Ala	113·6^{a}	124·5^{a}	136·5^{a}	164·3^{b}	166·2^{b}	9·5
Arg	115·6^{a}	107·4^{a}	110·8^{a}	72·3^{b}	81·6^{b}	5·6
Asn	46·5^{a}	44·2^{ab}	40·7^{ab}	34·7^{b}	36·2^{b}	3·3
Asp	4·4	3·3	3·5	4·1	4·0	0·9
Citrulline	182·5^{a}	146·4^{ab}	158·6^{ab}	92·9^{c}	129·8^{b}	11·4
Cystine	2·9	2·3	2·9	2·3	2·4	0·6
Glu	54·6^{a}	49·3^{ab}	48·6^{ab}	39·5^{b}	39·0^{b}	4·6
Gln	218·0^{a}	215·4^{a}	214·5^{a}	163·0^{b}	181·2^{b}	11·8
Gly	576·0^{a}	581·4^{a}	521·9^{ab}	465·5^{bc}	413·1^{c}	32·3
His	35·8	36·0	34·8	35·7	35·2	1·8
Lys	138·3^{a}	119·4^{ab}	108·7^{bc}	91·8^{c}	86·7^{c}	7·9
Met	20·7^{a}	18·5^{ab}	16·5^{bc}	15·2^{c}	11·9^{d}	1·1
Ornithine	79·4^{a}	76·0^{a}	77·7^{a}	44·5^{b}	48·6^{b}	7·3
Pro	78·6^{a}	69·3^{ab}	67·2^{ab}	62·4^{ab}	49·7^{b}	7·4
Ser	57·9^{ab}	55·5^{a}	53·4^{a}	71·9^{b}	71·1^{b}	4·7
Thr	108·3^{a}	104·2^{a}	88·6^{ab}	70·1^{bc}	53·8^{c}	7·6
Tyr	40·1^{a}	40·7^{a}	33·3^{b}	31·7^{b}	24·8^{c}	1·9

^{a,b,c,d}Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$).
* For details of procedures, see p. 403.

Table 2. Muscle protein synthetic rates in *vastus* muscle and *m. longissimus dorsi*, and whole-body phenylalanine irreversible loss rate (ILR) as affected by a 6 h infusion of saline (155 mmol NaCl/l, control), branched-chain amino acids (BCAA), glucose or co-infusion of branched-chain amino acids and glucose (B + G)†

	Control	BCAA	Glucose	B + G	SED	
					Muscle	Treatment
K_{sm} (%/d)*‡						
Vastus muscle	1·5	1·8	2·2	1·7	0·3	0·2
M. longissimus dorsi	1·0	0·9	1·3	1·3		
K_{sp} (%/d)*§						
Vastus muscle	1·2	1·4	1·8	1·4	0·2	0·2
M. longissimus dorsi	0·8	0·7	1·0	1·0		
Whole-body Phe ILR (mmol/h)	2·33^{ab}	1·93^{a}	2·54^{b}	2·00^{ab}		0·12

^{a,b}Mean values within the same row with unlike superscript letters were significantly different ($P < 0·05$).
* $P < 0·01$ (effect of muscle group).
† For details of procedures, see p. 403.
‡ K_{sm} is the rate of muscle protein synthesis calculated using the area under the isotopic activity × time curve for muscle free phenylalanine as the precursor amino acid pool.
§ K_{sp} is the rate of muscle protein synthesis calculated using the area under the isotopic ativity × time curve for plasma free Phe as the precursor amino acid pool.

to insulin and BCAA. We chose to elevate insulin concentrations by infusing glucose, since this avoided complications with hypoglycaemia and the increased plasma glucose concentration would not limit any response in protein synthetic rate that might be expected to occur.

Over the 6 h glucose infusion, plasma insulin gradually increased from very low fasted values to levels similar to those reported for sheep fed at maintenance intake (< 150 pM) after 4 h of infusion (Cole *et al.* 1988; Kriel *et al.* 1992; Wester *et al.* 2000). Despite this, skeletal protein synthesis was not significantly altered in either of the two sampled skeletal muscles when measured at the end of the 6 h infusion, although on average there was 40 % increase in protein synthesis (approaching significance) during glucose infusion. Oddy *et al.* (1987) and Douglas *et al.* (1991) did not observe any stimulation in skeletal muscle protein synthesis in fed or fasted young lambs (12–16 kg) after attaining physiological (or greater) increases in plasma insulin levels in response to a 5 h systemic insulin infusion. Insulin infusion into adult goats under euglycaemic clamp and hyperaminoacidaemic conditions has similarly failed to induce significant alterations in muscle protein synthesis (Tesseraud *et al.* 1993; Tauveron *et al.* 1994; Bequette *et al.* 2002). The lack of a significant effect of insulin in the present study may have been due either to the short 2 h period over which plasma insulin levels attained levels equivalent to the fed state or there may have been insufficient stimulation by the physiological increase in insulin achieved by glucose infusion. In adult human subjects, administration of supraphysiological doses of insulin across the forearm stimulates protein synthesis (Biolo *et al.* 1995; Hillier *et al.* 1998), but in contrast, supraphysiological doses of insulin failed to increase muscle protein synthesis in fasted lambs of

about 1 month of age (Oddy *et al.* 1987). Therefore, the evidence suggests that insulin insensitivity may be a feature of ruminant animal muscle protein synthesis, although the non-significant changes reported in the present study suggest that this is worthy of further investigation.

The lack of a significant response of muscle protein synthesis to increased insulin levels during glucose infusion may have been the result of the 64 % decrease in BCAA concentrations, which has been proposed to increase muscle tissue sensitivity to insulin. A decline in BCAA concentration in response to insulin (or glucose) infusion was observed in young fasted rats (Garlick & Grant, 1988), but B + G increased BCAA concentrations 1·6–1·8-fold compared with control animals and stimulated muscle protein synthesis. To test this hypothesis, we infused BCAA separately or with glucose, but there was no evidence of any effect of BCAA or B + G on muscle protein synthesis. Infusion of BCAA alone raised circulating levels approximately to those found in lambs fed 2- to 3-times N equilibrium (Lobley *et al.* 1998). When BCAA were administered with glucose, the BCAA infusion simply prevented the fall in BCAA concentration observed when glucose alone was infused. In essence, the B + G treatment acted as a hyperinsulinaemic–euglycaemic–BCAA clamp. In the present study BCAA concentrations were similar in the control and the B + G infusions. Papet *et al.* (1992) did not observe any changes in muscle protein synthesis when BCAA were infused into suckling lambs, but in contrast, Schaefer *et al.* (1986) reported a significant increase in adult sheep infused with leucine at twice the rates used in the present study. However, neither of these studies were combined with insulin or glucose infusion.

The studies by Garlick in young rats were able to explain the feeding-induced increase in muscle protein synthesis in terms of a rise in plasma insulin and BCAA. Studies in rats and human subjects have demonstrated that the sensitivity of muscle protein synthesis to feeding and to insulin is a feature of young animals and is not observed in adults (Garlick *et al.* 1998). However, it is very unlikely that the 30 kg lambs studied here had simply become refractory to insulin during development, since muscle protein synthesis in much younger lambs did not respond to insulin (Oddy *et al.* 1987). Furthermore, our studies in 30 kg growing sheep have demonstrated repeatably that muscle protein synthesis is highly sensitive to the level of feed intake (Lobley *et al.* 1992; Crompton & Lomax, 1993: Hoskin *et al.* 2003). Thus, muscle protein synthesis increased 3·5-fold when feed intake was from 0·2 to 3·0 × maintenance energy intakes (Crompton & Lomax, 1993). Interestingly, the mouse also differs from rats and human subjects in that muscle protein synthesis is sensitive to food deprivation or insulin administration even in adult animals (Sandstrom *et al.* 1995; Svanberg *et al.* 1996*b*). The present results clearly demonstrate that in ruminant animals the sensitivity of muscle protein synthesis to feed intake is not due to alterations in insulin and BCAA *per se*. It is possible that other hormones, such as IGF-1 or cortisol, are responsible for these feeding-induced changes (Crompton & Lomax, 1987; Millward *et al.* 1996). Douglas *et al.* (1991) demonstrated that IGF-1 infusions stimulate muscle protein synthesis in post-weaning lambs.

We chose to concentrate on the interaction between BCAA and insulin, since BCAA, and in particular leucine, have been demonstrated to influence the action of insulin in rats (Garlick *et al.* 1998). However, it is possible that amino acids other than the BCAA may play a role, since muscle protein synthesis has been reported to be stimulated by amino acid infusion alone in human subjects and pigs (Bennet *et al.* 1990; Watt *et al.* 1992*a*; Svanberg *et al.* 1996*a*). During glucose infusion, circulating levels of Phe, arginine, asparagine, glutamate, glutamine, glycine, lysine, methionine, ornithine, proline, threonine and tyrosine were significantly decreased, and in some cases co-infusion of BCAA further decreased this effect of glucose (Phe, methionine, tyrosine). Additional glutamine supply has been shown to increase muscle protein synthesis in human subjects (Barua *et al.* 1992) and rodents (Watt *et al.* 1992*b*) and an increase in N retention in response to abomasal infusion of glutamine has been reported in cattle (Reecy *et al.* 1996). Therefore, a limitation of amino acids to muscle may have limited the response to insulin during glucose infusion.

Two muscles were chosen for biopsy to determine if any alterations in protein synthesis in response to insulin and BCAA were common to different major muscle groups. In rats, muscle protein synthesis was positively correlated with the content of slow oxidative fibres (Garlick *et al.* 1989; Baillie & Garlick, 1991). Lobley *et al.* (1990) in sheep, and Dawson *et al.* (1991) in cattle, found no difference in muscle protein synthetic rate between MV and LD, but Lobley *et al.* (2000) reported significant differences between these two muscles in cattle. Maltin *et al.* (2001) reported that VM in cattle contained a higher percentage frequency and area of slow oxidative fibres than LD, which is consistent with the higher rates of protein synthesis in VM reported in the present study. Davis *et al.* (2001) reported that the stimulation of protein synthesis by insulin in neonatal pigs, with amino acids and glucose clamped at fasting levels, was greater in LD that in the more oxidative maseter muscle; this is in contrast to the present study, in which a greater (47 *v.* 39 %), but non-significant, increase in protein synthetic rate in VM compared with LD was observed.

In sheep infused with BCAA alone, whole-body Phe irreversible loss rate (flux) was non-significantly lower (17 %) than in control sheep, and significantly lower (24 %, $P<0·05$) than in glucose-infused animals, indicating a decrease in whole-body protein degradation. Studies that examined the effects of oral administration of BCAA on overnight-fasted human subjects also found that whole-body Phe flux was decreased by 20 %, despite no change in net Phe balance across the leg (Ferrando *et al.* 1995). In contrast, intravenous infusion of a wider mixture of amino acids to post-absorptive human subjects increased protein synthesis and decreased protein degradation across muscles of both the forearm and leg, despite no change in plasma insulin concentration (Svanberg *et al.* 1996*a*). Svanberg *et al.* (1996*a*) contended that specific amino acids triggered the changes in protein balance. In older animals any anabolic effects of insulin may be due to decreases in muscle proteolysis, as has been observed

for adult human subjects (Fryburg *et al.* 1995). The limited information available for ruminant animals favours reduced proteolysis, but most results are from adult animals (e.g. Tesseraud *et al.* 1993; Tauveron *et al.* 1994; Larbaud *et al.* 1996).

Our previous studies have demonstrated that close arterial insulin or BCAA infusions at physiological levels stimulate net protein gain across the hindlimb of maintenance-fed 30 kg sheep. Close arterial administration has the advantage of not altering systemic levels of glucose or non-infused amino acids. The results of the present study therefore suggest that our previous observations are more likely to have been the result of a decrease in muscle protein degradation rather than an increase in muscle protein synthesis.

Effects of exogenous insulin on muscle protein metabolism seem to be greatest in the fasted state, as observed in rats (Garlick & Grant, 1988), sheep (Oddy *et al.* 1987) and human subjects (Fryburg *et al.* 1995). This may be because metabolism is very sensitive to insulin, with maximal responses observed at relatively low concentrations (Jepson *et al.* 1988). Thus, the initial concentration of insulin in circulation must be very low if metabolic responses to insulin infusion are to be observed experimentally, e.g. as observed during fasting. Following meal ingestion, absorption of glucose signals release of insulin. In non-ruminant animals, this insulin secretion occurs at the same time that peripheral tissues, including skeletal muscle, receive other nutrients derived from meal absorption, i.e. energy and protein absorption is synchronous. Since BCAA escape liver metabolism (Wray-Cahen *et al.* 1997), they are likely candidates for nutrient modulators of muscle sensitivity to insulin to promote maximal anabolism when energy and protein supply are optimal. In ruminant animals, however, energy and protein supply are likely to be asynchronous, particularly in meal-fed animals, since rumen energy fermentation causes meal-related changes in circulating insulin concentrations while amino acid absorption into the portal vein is relatively constant. Furthermore, in normal grazing ruminant animals, nutrients appear in the portal vein at a relatively constant rate, resulting in far less fluctuations in insulin and amino acids. Therefore, ruminant animals may have evolved alternative mechanisms from the insulin and BCAA signals, to allow muscle protein synthesis to be regulated by the composition and level of dietary intake.

The results presented in the present report demonstrate that elevation of insulin, with or without increased concentrations of BCAA, failed to stimulate muscle protein synthesis in fasted lambs. Therefore, the sensitivity of sheep muscle protein synthesis to level of feed intake cannot be explained in terms of insulin secretion or alterations in insulin sensitivity by BCAA, as has been suggested in the rat. This species difference in the regulation of muscle protein synthesis may be the result of the more constant pattern of nutrient absorption and insulin secretion due to rumen fermentation and leads the questions of whether the differences observed between ruminant animals and non-ruminant animals are the result simply of diet and regimen, or if there is a more basic physiological explanation.

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