

Transsulfuration, protein synthesis rate and follicle mRNA in the skin of young Merino lambs in response to infusions of methionine and serine

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Methionine (Met) is usually the first limiting amino acid for sheep and supplements of Met may increase production of wool and meat. The wool response may be due to an increased supply of cysteine (Cys) from transsulfuration (TS) of Met. Met is catabolized through homocysteine to form Cys when the S from Met is transferred to serine (Ser). We hypothesized that providing additional Met would create a deficiency of Ser and that by simultaneously providing Met and Ser, TS and wool growth could be increased more than by providing Met alone. The effects of i.v. infusions of Met and Ser to young Merino lambs on TS, fractional synthesis rate (FSR) of protein in skin, follicle mRNA and wool growth were examined. Following 4 d of constant i.v. infusion of 3 g Met/d, or 10 g Ser/d or both, the isotope tracers: L-[3-¹³C]Cys, L-[ring-^d₃]phenylalanine (Phe) and L-[2,3,3-^d₃]Ser were infused over 8 h to allow for measurements of irreversible loss rate (ILR), and TS in whole body and skin. Skin biopsies were taken for measurement of FSR. Wool growth rate was measured using autoradiography. An infusion of Met significantly ($P < 0.05$) improved wool growth rate and increased skin FSR, Cys supply from TS and enhanced levels of follicle mRNA (from the K2.10 intermediate filament gene and three gene families encoding keratin associated proteins KAP1, KAP4 and KAP12). The extra Met lowered Ser ILR. The infusion of Ser doubled Ser ILR in the body and increased skin FSR calculated using the Cys tracer in plasma ($P < 0.05$). However, there were no significant ($P > 0.05$) changes in TS, skin FSR calculated using the Phe and Ser tracers, follicle mRNA or wool growth rate as a result of Ser infusion. While there were trends towards increased TS and FSR with Ser infusion, the overall lack of significant changes indicates a high capacity for the *de novo* synthesis of Ser.

Stable isotopes: Amino acids: Cysteine: Glycine: Wool: Sheep

Supplementation or infusion with methionine (Met) and/or cyst(e)ine (Cys) consistently increases concentrations of ultra-high S proteins and growth rate of wool in sheep, particularly when feed intake is around maintenance (Williams *et al.* 1988; Reis *et al.* 1990; Mata *et al.* 1995). Although wool proteins are rich in Cys (98–131 g/kg protein) and poor in Met (5–6 g/kg; Reis, 1979; MacRae *et al.* 1993), a supplement of Met generally has a superior effect to Cys on wool growth (Reis *et al.* 1990).

Met is catabolized mainly through the *S*-adenosylmethionine pathway, and then diverted either to synthesis of polyamines or to the synthesis of homocysteine. Homocysteine can be either remethylated to re-form Met or undergo further catabolism to form Cys after transsulfuration (TS). During TS, the S in Met is transferred to serine (Ser) to

produce Cys (Cooper, 1983). Improvement in wool growth following supplementation with Met could therefore be a result of increasing the amount of Cys from TS; a change in follicle function caused by the increased production of polyamines (Reis & Hynd, 1989; Hynd & Nancarrow, 1996) and/or a direct increase in Met supply for wool protein synthesis. To explain the higher response to Met than to Cys alone, a combination of the above would need to occur.

There is some evidence that the TS rate reaches a peak at a Met intake of about 4–6 g/d, and decreases at higher Met intakes (Benevenga & Egan, 1983; Pisulewski & Buttery, 1985). This may be due to insufficient enzyme activity to support higher levels of TS or to a lack of substrate (e.g. Ser) for TS. Ser concentrations in plasma are consistently

Abbreviations: Cys, cysteine; FSR, fractional synthesis rate; Gly, glycine; ILR, irreversible loss rate; Met, methionine; Phe, phenylalanine; Ser, serine; TS, transsulfuration.

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reduced by supplementation with Met. This has been reported in a number of species including sheep (Reis *et al.* 1990), cattle (Campbell *et al.* 1997) and human subjects (Frontiera *et al.* 1994). The results indicate that a supplement of Met results in the depletion of Ser, possibly through TS.

Ser may also be specifically required for wool growth and is found in high concentrations in wool protein (108 g/kg; Reis, 1979). Although Ser is a nonessential amino acid that is synthesized from glycine (Gly) and phosphoglycerate, it is not known if *de novo* synthesis is sufficient to satisfy the demands for both TS and wool growth.

There are, therefore, a number of ways in which Met and Ser may influence wool growth either independently or interactively. In this study the effects of infusions of Met and Ser to Merino lambs on whole body TS, fractional synthesis rate (FSR) of protein in skin, follicle mRNA, wool growth, and possible TS in the skin were examined.

Materials and methods

Animals and management

Sixteen 5-month-old female Merino lambs, weighing 27 kg (SD 1.0), were used in this study. The animals were maintained in individual pens and fed on 650 g DM/d of a diet that contained 750 g chopped oaten hay/kg, 230 g lupin seed/kg and 20 g Siromin/kg (Compass Farm Feeds Pty Ltd, Mt Compass, SA, Australia), for 14 d. The metabolizable energy intake was estimated to be $1.1 \times$ maintenance (Agricultural Research Council, 1984). At the end of the 14 d adjustment period, the sheep were put into individual metabolism cages and fed on the same total amount of diet in equal portions at intervals of 1.5 h for 12 d. The lambs were divided into four equal-sized groups and allocated to one of four treatments (see later) for the last 5 d (treatment period) in the metabolism cages. Two jugular catheters were inserted into each sheep 2 d before the treatment period for an infusion of amino acids and sampling blood respectively. At the end of the treatment period, the lambs were moved back into individual pens and fed on the same amount of the same diet for a further 14 d.

Treatments and infusion procedures

The experimental design was a 2×2 factorial with groups as follows: control, saline only; +Met, 3 g Met/d; +Ser, 10 g Ser/d; +Met+Ser, 3 g Met+10 g Ser/d. For the +Met group, 6.25 g L-Met was dissolved in 200 ml sterilized MilliQ water (Millipore Corporation, Yonezawa, Japan) and made up to 1 litre with sterile saline. For the +Ser and +Met+Ser groups, 20.8 g Ser, or 6.25 g Met and 20.8 g Ser were dissolved in 1 litre of sterilized MilliQ water. These high concentrations of the amino acids would probably have an osmolarity similar to saline. The control group was infused with sterile saline only. In all groups, 1.25 g L-phenylalanine (Phe) and 0.31 g L-Cys were added per 1 litre solution, which were equal to the amounts of the corresponding tracers infused on the last day to each group. The solutions were infused into the jugular vein via a catheter at a constant rate of 20 ml/h for 4 d. All the

amino acids were obtained from the Sigma Chemical Company (Sydney, Australia).

Measurement of amino acid kinetics

On day 5, the infusates (220 ml) were prepared with the partial replacement (on an equal weight basis) of Cys, Phe and Ser with 0.05 g L-[3- 13 C]Cys, 0.2 g L-[ring- d_5]Phe, and 0.14 g or 0.28 g L-[2,3,3- d_3]Ser (for nil Ser or +Ser groups respectively) (Cambridge Isotope Laboratories, Inc., Andover, MA, USA; 99%, 99% and 98% atom percent excess for Cys, Phe and Ser respectively). A portion of each solution (25 ml) was injected i.v. via a jugular catheter as a priming dose at the start of the infusion, and this was then followed by a constant infusion of the tracer solutions over a period of 8 h. The flow rates (about 20 g/h) of the isotope solutions over the last 4 h were recorded for calculation of infusion rates of each isotope. Infusion rates of the tracers were 38 μ mol/h for Cys, 98 μ mol/h for Phe and 109 or 224 μ mol/h for Ser (with or without an addition of Ser).

One blood sample and one skin biopsy from the midside flank were taken on day 4 of the treatment period (one day before the tracer infusion) and used for determination of the background of isotopes. On the day of tracer infusion, blood samples of 10 ml each were drawn via the contralateral catheter into EDTA tubes at 30, 60, 90, 120, 150, 180, 210, 240, 300, 360, 420 and 480 min after the start of the infusion. Plasma was harvested and stored at -80° . Skin biopsies were taken under local anaesthetic from the midside of the left flank using a trephine (10 mm diameter) at 480 min, rinsed in saline, frozen in liquid N_2 and stored at -80° .

Wool growth

The fibre length growth rate and diameter were measured by means of an intradermal injection of L-[35 S]Cys followed by autoradiography (Downes *et al.* 1967). The intradermal injections of L-[35 S]Cys were carried out on the day the sheep were first placed into metabolism cages, again 7 d later (immediately before the initiation of treatments) and for a third time 5 d later (at the end of the treatment period). A total of fifty fibres were measured from each animal using an image analysis system (Video Pro, Leading Edge, SA, Australia).

Analyses

Plasma samples. Ser, Cys and Phe in both infusate and plasma were prepared for analysis of enrichments of the isotopes using a GC-mass spectrometer (HP 5970 MSD interfaced with HP 5890 GC, Hewlett Packard Co., Wilmington, DE, USA). The sample preparation used anion exchange (AG-1 resin, OH^{-1} form) and has been detailed by Liu & Figliomeni (1998). The amino acids were processed as *t*-butyldimethylsilyl derivatives (Calder & Smith, 1988), and analysed under electron-impact conditions using the selective-ion mode. Briefly, Ser was monitored from the fragment ions at m/z 390 ($m+0$) and 393 ($m+3$), Cys at m/z 406 ($m+0$), 407 ($m+1$ from ^{13}C -Cys) and 409 ($m+3$ from d_3 -Ser), and Phe at m/z 234 ($m+0$) and 239 ($m+5$). Gly

was also monitored at m/z 218 and 219 to examine the conversion of Ser to Gly. Met was monitored at m/z 320 for the measurement of concentration in plasma.

Norleucine was added to each plasma sample as an external standard for the calculation of amino acid concentrations. It was monitored at m/z 200. The calibration curves were produced using serial increments of each amino acid against the ratio fragment ion of the amino acid : norleucine ion (m/z 200). The concentrations for unknown samples were calculated from the measured ratios.

Skin biopsies. The isotope enrichments for Ser, Cys, and Phe, in both free amino acid and protein pools, were analysed in skin biopsies as described by Liu & Figliomeni (1998). Briefly, the biopsies were homogenized and deproteinized with acetone. The supernatant was processed following the same procedure as used for plasma samples, and the amino acids were eluted with 2 M-HCl. The eluate was dried (under vacuum) to remove the acid and reconstituted with water. A portion was taken and processed for formation of *t*-butyldimethylsilyl derivatives (Calder & Smith, 1988). Norleucine and dithiothreitol were added at the beginning of homogenization as an external standard and antioxidant respectively.

The protein pellet was thoroughly washed with sulfosalicylic acid (60 g/l) to remove the remaining amino acids, and hydrolysed with 6 M-HCl at 110° for 24 h (Lobley *et al.* 1992). Phenol and dithiothreitol were added as antioxidants. Adequate amounts of Cys from the hydrolysate for GC-mass spectrometry analysis were obtained with dithiothreitol protection, although the extent of recovery of Cys from hydrolysis was not measured. The acid was then removed under vacuum, and the dried hydrolysate was reconstituted in water. A portion was processed following the same anion change procedure as described above, and the eluate was further processed for formation of *t*-butyldimethylsilyl derivatives. GC-mass spectrometry analysis was applied to the derivative for enrichment of Ser($m+3$), Cys($m+1$) and Cys($m+3$). Another portion was incubated with tyrosine decarboxylase to convert phenylalanine to phenylethylamine (Calder *et al.* 1992), which was further analysed as *t*-butyldimethylsilyl derivative for enrichment of [ring- d_3] using GC-mass spectrometry (Liu *et al.* 1998).

Follicle mRNA responses were measured using a gene-specific probe for the intermediate filament keratin protein K2.10, and gene family probes for the keratin associated proteins KAP1, KAP4 and KAP12. The K2.10, KAP 1 and KAP4 probes were as described by Fratini *et al.* (1994). The KAP12 probe was a 640 bp coding region probe detecting a novel Cys-rich keratin associated protein family (B. Powell, unpublished results). mRNA was prepared by the guanidium method of Chomczynski & Sacchi (1987). Northern gel analysis and dot blot analyses were done according to Sambrook *et al.* (1989). DNA probes were labelled with 32 P-dCTP using the Amersham Megaprime DNA labelling kit (Amersham International, Amersham, Bucks., UK). For the analysis of treatment effects on mRNA levels the amount of RNA in each sample was first normalized using either a 28S rRNA probe (for total RNA loading) or a trichohyalin probe (for follicle RNA loading). Similar results were obtained with both normalization methods. The levels of the specific

mRNA were adjusted to reflect equivalent RNA loadings and the relative changes in the mRNA were derived from the ratio values obtained before : after treatment.

Calculations. The irreversible loss rate (ILR, mmol/h) of Ser, Cys or Phe in the whole body was calculated using the formula (Lobley *et al.* 1996):

$$ILR = \left(\frac{MPE_i}{MPE_s} - 1 \right) \times I,$$

where MPE_i and MPE_s are the enrichments (%) of Ser($m+3$), Cys($m+1$) or Phe($m+5$) in the infusate and in the plasma samples that were taken from 240 until 480 min of the infusion, and I is the rate of isotope infusion (mmol/h) over the same period of time. With a priming dose of isotopes, enrichments in plasma reached a plateau 180, 240 and 300 min after dosing for Ser, Phe and Cys respectively.

The amount of mass transferred from Ser to Cys (TS, mmol/h) was calculated using the formula:

$$TS = \frac{ILR_{cys} \times MPE_{cys(m+3)}}{MPE_{ser}},$$

where ILR_{cys} is the ILR of Cys based on the enrichment of Cys($m+1$) in the plasma samples, MPE_{ser} is the plasma enrichment of Ser($m+3$) and $MPE_{cys(m+3)}$ is the plasma enrichment of Cys($m+3$) which was derived from [2,3,3- d_3]Ser. All the values above were converted from mmol/h to mmol/d.

The fractional synthesis rates (FSR) of protein (%/d) were calculated using the isotope enrichment in protein of skin biopsies, and the enrichment in free amino acids in plasma samples. The calculation of FSR was performed using the following formula (Slater *et al.* 1995):

$$FSR (\%/d) = \frac{MPE_t - MPE_0}{MPE_p} \times 3 \times 100,$$

where MPE_0 and MPE_t refer to the isotope enrichment in protein-bound amino acid in the skin biopsies which were taken at 0 min (background sample) and 480 min. MPE_p is the mean of isotope enrichment of free amino acid in plasma samples which were drawn from 0 min until 480 min (total thirteen samples), calculated from the area under the enrichment curve (calculated by integrating the isotope enrichment curve fitted to the data points) divided by a total time of 480 min. The factor of three is used to convert the data obtained over 480 min to 24 h to allow for a common unit for FSR (%/d). It should be noted that for an 8 h infusion period, the loss of protein-bound isotope by degradation of newly synthesized protein may be significant, and this is not accounted for in the above formula. That is, calculated MPE_t is likely to be lower than it actually is. Also, plasma was used as the precursor pool for protein synthesis, and the isotope enrichment in plasma at a constant infusion is usually higher than that in the intracellular space (Lobley *et al.* 1992). For these reasons, the isotope enrichment ratio skin homogenate (only sampled at 480 min) : plasma was also used to adjust FSR values, and they are presented separately in the present report.

Statistical analysis

Factorial ANOVA was used to examine the treatment effects with Met and Ser as factors. Analyses of all the data, except for follicle mRNA responses, were performed using Systat for Windows (version 5.0, Systat Inc., Evanston, FL, USA). The pretreatment wool measurements were used as covariates for the statistical analysis of treatment effects on wool length and diameter. For the data on follicle mRNA responses, some data points were missing because of the failure to recover mRNA from the samples, and the analysis was performed using Genstat 5.3 (The Numerical Algorithms Group Ltd, Oxford, UK).

Results

Irreversible loss rates of serine, phenylalanine and cysteine and transsulfuration

ILR of Ser was significantly ($P < 0.001$) increased by an i.v. infusion of Ser. During the i.v. infusion of 10 g Ser/d, the

ILR of Ser was increased by 108.8 mmol/d (or 11.4 g/d) without an infusion of Met, and by 55.6 mmol/d (or 7 g/d) with Met. An i.v. infusion of 3 g Met/d (or 20 mmol/d) resulted in a significant ($P = 0.02$) decline in Ser ILR of 32 mmol/d (main effect for Ser, Table 1). ILR of Phe ranged from 33 to 43 mmol/d. There were no significant ($P > 0.05$) changes in Phe ILR during the infusions of Met and Ser. ILR of Cys tended to increase (4.9 mmol/d) with an infusion of Met ($P = 0.08$). The infusion of Ser did not influence Cys ILR. There were no significant treatment effects on the amounts of Cys from TS or the percentage of TS contributing to Cys ILR.

Fractional synthesis rate of protein in the skin and wool growth

FSR of protein in the skin and wool production are shown in Tables 2 and 3. FSR values varied depending on which amino acid tracer was used, and which precursor pool was selected. Using plasma as the precursor pool, the FSR values

Table 1. The irreversible loss rate (ILR) of serine (Ser), phenylalanine (Phe) and cysteine (Cys) in Merino lambs infused with Met, Ser or Met + Ser. ILR calculation was based on isotopic enrichment of amino acids in plasma* (Values are means for four lambs in each group with their pooled standard error)

	Control	+ Met	+ Ser	+ Met + Ser	ANOVA	
					SEM	P†
Ser ILR (mmol/d)	87.7	76.7	196.5	143.3	11.9	Met: 0.02 Ser: < 0.001
Phe ILR (mmol/d)	43.4	36.6	42.9	33.4	5.8	Met: 0.18 Ser: 0.76
Cys ILR (mmol/d)	20.8	25.9	22.0	26.6	2.6	Met: 0.08 Ser: 0.70
Cys from TS (mmol/d)	3.25	4.72	4.92	4.50	0.91	Met: 0.57 Ser: 0.44
Total TS : Cys ILR (%)	15.7	18.2	22.6	16.9	3.4	Met: 0.86 Ser: 0.46

TS, transsulfuration.

* For details of procedures see p. 403.

† $P > 0.1$ for all interactions.

Table 2. Fractional protein synthesis rate (FSR, %/d) in the skin of Merino lambs infused with methionine (Met), serine (Ser) or Met + Ser. The values were calculated using either plasma as the precursor pool (FSRp) or the skin homogenate (FSRh)*

(Values are means for four lambs in each group with their pooled standard error)

	Control	+ Met	+ Ser	+ Met + Ser	ANOVA	
					SEM	P†
From plasma						
FSRp(Ser)	11.4	17.3	21.1	18.9	2.73	Met: 0.47 Ser: 0.04
FSRp(Phe)	19.5	26.7	24.5	23.0	2.2	Met: 0.23 Ser: 0.77
FSRp(Cys)	20.0	30.9	29.6	33.4	2.6	Met: <0.01 Ser: 0.02
From skin homogenate						
FSRh(Phe)	52.2	71.4	55.3	65.6	5.6	Met: 0.02 Ser: 0.81
FSRh(Cys)	39.3	67.4	57.3	62.8	5.9	Met: 0.01 Ser: 0.28

Phe, phenylalanine; Cys, cysteine.

* For details of procedures see p. 403.

† $P > 0.1$ for interactions not shown.

Table 3. Fibre length growth ($\mu\text{m}/\text{d}$), fibre diameter (μm) and fibre volume ($\mu\text{m}^3/\text{d}$) in Merino lambs infused with methionine (Met), serine (Ser), or Met + Ser*
(Values are least square means for four lambs in each group and the pooled standard error is the largest value of all groups)

	Control	+ Met	+ Ser	+ Met + Ser	ANOVA	
					SEM	P†
Fibre length	478.2	509.5	498.3	507.4	6.2	Met: 0.005 Ser: 0.15 Met × Ser: 0.08
Fibre diameter	17.6	21.1	18.0	20.0	0.8	Met: 0.007 Ser: 0.65
Fibre volume	129.2	175.6	134.4	160.2	13.9	Met: 0.023 Ser: 0.68

* For details of procedures see p. 402.

† $P > 0.1$ for interactions not shown.

calculated from Cys were higher than those from Phe ($P < 0.05$) which, in turn, were higher ($P < 0.05$) than from Ser (group means 28.5, 23.4 and 17.2 %/d respectively). Using the skin homogenate as the precursor pool, similar FSR values were obtained from both the Cys and Phe tracers.

The infusions of Met consistently increased skin FSR compared with the control although the increments in FSR values using plasma Ser and Phe lacked statistical significance. The infusion of Ser resulted in a tendency to increase FSR values only when the FSR values were calculated from plasma Cys ($P = 0.02$) (Table 2).

Wool length growth rate ($P < 0.005$), fibre diameter ($P = 0.007$) and the volume of wool grown ($P < 0.05$) were significantly increased by Met infusion. Ser infusion resulted in a trend towards an small increase in length growth rate only when Met was not supplied (Met × Ser, $P = 0.078$) but no effect on any other wool measurements were observed (Table 3).

Amino acid concentrations in plasma and in skin

Infusions of Met and Ser resulted in marked increases in the concentrations of the infused amino acid in both plasma and skin, but no significant changes in the concentrations of Cys or Phe (Table 4).

The concentrations of Met and Phe in the skin homogenate were 1.3- to 2.3-fold higher than those in plasma, while the concentrations of Cys and Ser, two nonessential amino acids, in the skin were 4–5-fold or 6–9-fold higher than in plasma respectively (Table 4).

Ratio of cysteine(m+3): cysteine(m+1) in various pools

The ratio of Cys(m+3) (derived from TS):Cys(m+1) (as a tracer for all Cys except Cys from TS) was calculated from Cys MPE(m+3):Cys MPE(m+1). The definition of Cys MPE(m+3) is $\text{MPE}(m+3) \div (\text{MPE}(m0) + \text{MPE}(m+3))$ and

Table 4. The concentrations of amino acids in plasma and in the free amino acid pool of the homogenate of the midside skin in Merino lambs*

(Values are means for four lambs in each group with their pooled standard error)

	Control	+ Met	+ Ser	+ Met + Ser	ANOVA	
					SEM	P†
Plasma ($\mu\text{mol}/\text{l}$)						
Ser	104	105	164	125	15.4	Met: 0.21 Ser: 0.017
Phe	37.2	47.3	45.5	43.3	4.8	Met: 0.34 Ser: 0.60
Met	16.9	36.9	24.8	37.8	2.6	Met: 0.001 Ser: 0.09
Cys	38.2	45.9	43.1	36.9	6.0	Met: 0.60 Ser: 0.73
Skin homogenate ($\mu\text{mol}/\text{kg}$ wet weight)						
Ser	835	847	991	996	58	Met: 0.88 Ser: 0.02
Phe	85.8	75.1	62.5	74.3	6.9	Met: 0.94 Ser: 0.10
Met	37.7	79.3	31.4	60.0	5.6	Met: 0.001 Ser: 0.04
Cys	166	206	191	202	15	Met: 0.11 Ser: 0.49

Met, methionine; Ser, serine; Phe, phenylalanine; Cys, cysteine.

* For details of procedures see p. 403.

† $P > 0.1$ for interactions not shown.

Table 5. Proportion of [2,3,3-d₃]cysteine from transsulfuration to [3-¹³C]cysteine in various metabolic pools of Merino lambs infused with methionine (Met), serine (Ser) or Met+Ser*
(Values are means for four lambs in each group with their pooled standard error)

	Control	+Met	+Ser	+Met+Ser	ANOVA	
					SEM	P†
Plasma	0.106	0.166	0.145	0.219	0.033	Met: 0.06 Ser: 0.19
Skin free-amino-acid pool	0.127	0.217	0.161	0.255	0.035	Met: 0.02 Ser: 0.32
Skin protein pool	0.701	0.938	0.954	1.136	0.155	Met: 0.20 Ser: 0.17

* For details of procedures see p. 405.

† $P > 0.1$ for interactions not shown.

Table 6. Response of follicle mRNA (from four genes or gene families, K2.10, KAP1, KAP12 and KAP4) in the skin of Merino lambs infused with methionine (Met), serine (Ser) or Met+Ser. The responses were expressed as the ratio of mRNA during the treatment to the background (before the treatment)*

(Values are means for four lambs in each group with their pooled standard error)

	Control	+Met	+Ser	+Met+Ser	ANOVA	
					SEM	P†
K2.10	0.90	1.90	0.95	1.40	0.18	Met: 0.001 Ser: 0.13 Met×Ser: 0.07
KAP1	0.93	1.70	1.05	1.38	0.23	Met: 0.015 Ser: 0.54
KAP12	1.03	2.90	1.00	1.92	0.32	Met: <0.001 Ser: 0.07 Met×Ser: 0.09
KAP4	0.95	2.50	1.00	1.73	0.22	Met: <0.001 Ser: 0.06 Met×Ser: 0.04

* For details of procedures see p. 403.

† $P > 0.1$ for interactions not shown.

Cys MPE(m+1) is $MPE(m+1) \div (MPE(m0) + MPE(m+1))$. Since MPE(m+3) and MPE(m+1) are very small compared with MPE(m0) and the difference between them is small, therefore $(MPE(m0) + MPE(m+3)) \approx (MPE(m0) + MPE(m+1))$. Thus:

$$\frac{MPE(m+3) \div (MPE(m0) + MPE(m+3))}{MPE(m+1) \div (MPE(m0) + MPE(m+1))} \approx \frac{MPE(m+3)}{MPE(m+1)}$$

This ratio was used as an index of the extent of TS in various metabolic pools. The values are shown in Table 5. The ratio in plasma varied from 0.106 to 0.219, and in the free amino acid pool of the skin homogenate varied from 0.127 to 0.255. The infusion of Met had a significant effect on the ratio. In the skin protein pool, the ratio increased substantially, about 4–6-fold that in the free amino acid pool. No statistically significant differences due to treatments were observed in the skin protein pool although higher ratios tended to occur in the Met- or Ser-infused sheep.

Responses of follicle mRNA in the skin

The infusion of Met significantly increased the levels of mRNA from four follicle gene families, the KAP1, KAP4 and KAP12 families of cysteine-rich proteins and the K2.10

intermediate filament gene in the skin ($P < 0.01$). There was also a significant interaction between Met and Ser on KAP4 indicating a reduction in response to Met when Ser was also provided ($P < 0.05$). Ser infusion alone did not result in any significant responses in follicle mRNA (Table 6).

Discussion

Infusion of methionine

The effects of an infusion of Met on wool growth, ILR of Cys, total TS, the ratio Cys(m+3):Cys(m+1), the concentrations of Cys in plasma and in the skin and protein synthesis rate in the skin were measured. The infusion of 3 g Met/d for 5 d improved wool growth rate. The results agree with other reports (Williams *et al.* 1988; Mata *et al.* 1995). The stimulating effect of Met on wool production was through the enhanced protein synthesis rate in the skin, particularly when the synthesis rate was calculated using the Cys tracer or when the skin homogenate was used as the precursor pool. The higher FSR values from Cys-tracer compared with the other tracers may indicate that Met differentially stimulated synthesis of Cys-rich protein components in the wool (Reis, 1979). This is supported by the higher increase in the mRNA for the cortical cysteine-rich

proteins (KAP12 and KAP4) compared with the mRNA for the intermediate filament protein (K2.10) in response to Met infusion. Further, the response in skin protein synthesis may be a part of an increase in whole-body anabolism as reported in goats when Met was supplemented (Muramatsu *et al.* 1994), and/or the superior amino acid sparing-effects resulting when Met, rather than other essential amino acids, was given to pigs fed on an amino acid deficient diet (Fuller & Garlick, 1994).

The infusion of Met increased TS as reflected by the higher ratios Cys(m+3):Cys(m+1). There was also a trend towards increases in Cys ILR, the calculated total amount of Cys from TS and Cys concentrations in plasma and skin; however, the variability in these measurements was high and the results were not statistically significant. This lack of statistical significance was due to high individual variation between the lambs, particularly when a variable was calculated from several measurements (e.g. the amount of TS).

The importance of the Met TS pathway for wool production is well recognized but the amount of Cys from TS relative to Cys requirement for wool growth is unknown. Wool growth rate in our lambs can be estimated to be about 5.2 g/d from the results of Masters *et al.* (1998). Thus, a total of about 0.68 g Cys would be retained in this wool if it contained 131 g Cys/kg (Reis, 1979). The increase in wool growth rate of 36% from the infusion of Met (Table 3) requires an extra 0.25 g (2 mmol) Cys retention in wool/d. The infusion of Met resulted in an increase of Cys from TS of only 1.47 mmol/d in the circulating system. Using the fraction of 6% skin blood flow out of the cardiac output (Harris *et al.* 1989), the provision of extra Cys from TS to the skin will be small and insufficient to account for the additional wool growth. Our measurement of Cys from TS as the percentage of Cys ILR ranged between 16 and 22%, and agreed with other reports. For example, 5–22% in Merino ewes (Williams *et al.* 1988) and 4–18% in British cross-bred wethers (Pisulewski & Buttery, 1985). The difference between the Cys required for increased wool growth and the increased Cys from TS would suggest: (1) Met improves the utilization efficiency of Cys by skin for wool growth by stimulating protein synthesis rate; (2) Cys from TS in the skin *per se* may be an important source as discussed in the following text.

Transsulfuration in the skin

The recent finding of enzymes for TS (cystathionine β -synthase and cystathionase) in the skin of sheep (J. Lee, personal communication) supports the existence of TS in this tissue. Using follicle culture technique, TS has also been confirmed in the skin of cashmere goats (M Souri, cited by Galbraith, 1998). In our study, the increases of 11–31% in the ratio Cys(m+3):Cys(m+1) in the free amino acid pool of skin compared with plasma may indicate a proportionally higher rate of TS is occurring in the skin than in the whole body. The 4–6-fold increase in the ratio in skin protein compared with the free amino acid pool in the skin could indicate compartmentalization of TS in the skin and that the Cys from TS in the skin is readily incorporated into wool protein. Otherwise the Cys(m+3):Cys(m+1) ratio would be the same in the protein and homogenate pools.

Similar results were observed in pregnant Merino ewes when [35 S]Met and [3,3- 3 H $_2$]Cys were infused and [35 S]Cys was measured as an estimate of TS in the body (Williams *et al.* 1988). In that experiment the ratio [35 S]Cys:[3 H]Cys in the wool was 9–15-fold higher than that in plasma. The TS in the skin *per se* is probably an important source of Cys for wool growth and its quantitative importance needs to be further investigated.

Infusion of serine

The amount of Ser in the circulating blood was high (ILR 9.2 g/d) in sheep receiving the saline infusion only and this was doubled by the infusion of Ser. This large increase did not result in the expected changes in TS. This could indicate a high capacity of the body to synthesize Ser *de novo*. In the skin homogenates, the concentration of Ser was 9-fold that in plasma. In comparison, Cys was 4–5-fold and Met and Phe were about 2-fold higher in plasma than skin homogenates. Furthermore the homogenate:plasma isotope ratios for Ser, Cys and Phe were 0.11, 0.38 and 0.39 respectively in this study. The lower isotope ratio for Ser indicated an existence of a high proportion of unlabelled Ser in the skin, probably originating from *de novo* synthesis.

There is an interconversion between Ser and Gly through both the action of Ser hydroxymethyltransferase and the Gly cleavage system in mammals. To examine the conversion of Ser to Gly, Gly(m+1) in plasma samples was also monitored in this study. The enrichment of Gly(m+1) over the last 4 h of infusion varied from 0.24 to 0.34%. After dividing the enrichment of Ser(m+3) in plasma, the corrected Gly(m+1) in plasma was 9–10%, i.e. 9–10% Gly was derived from Ser. No differences were found between the treatments. We cannot calculate the total amount of Gly generated from Ser because Gly ILR was not measured. However, the concentrations of Gly in plasma (400–640 μ M) and in the skin (1418–1558 mmol/kg wet skin) were higher than Ser, indicating that Gly ILR would be high and that there was considerable amount of Ser converted to Gly in our lambs. For the conversion of Gly to Ser, a figure of 25% Ser originating from Gly has been found in human subjects (Arends *et al.* 1995) and 4.9% in fetal sheep (Cetin *et al.* 1991), but very little in cross-bred lambs (G. E. Loble, personal communication). In homogenates of the skin sample taken at 480 min there was no detectable increase in Gly(m+1), possibly due to limitations in sensitivity of the GC-mass spectrometry used.

The information from this experiment indicates that the sheep has a high capacity to synthesize Ser *de novo* and that this synthesis is sufficient to support both TS and wool growth.

Follicle mRNA responses

The K2.10 gene encodes an intermediate filament keratin protein and the KAP1, KAP4 and KAP12 genes encode keratin intermediate filament associated proteins (Powell & Rogers, 1997). Intermediate filaments are the major structural elements of wool keratinocytes, comprising about half the protein mass of the wool fibre, and they are the products of eight genes. The other half of the wool fibre mass is

primarily composed of the intermediate filament associated proteins, the products of over fifty genes derived from at least ten gene families. Whereas the K2.10 probe was specific for one of the eight wool intermediate filament keratin genes, the three KAP probes were able to cross-react with other members of their gene families and therefore measure the net responses of those families (Fratini *et al.* 1994; B. Powell, unpublished data). Infusions of Met significantly increased the expression of all these mRNA corresponding with the increases observed in wool growth. These results can be compared with the findings by Fratini *et al.* (1994) where mRNA for the KAP4 gene but not K2.10 responded to an i.v. infusion of Cys. The differences may help to explain why the increase in wool growth from a supplement of Met is, in most cases, greater than from Cys alone. Met works on most of the proteins while Cys works mostly on high and ultra-high S proteins. The significant responses of mRNA for the KAP genes to Met supports the earlier discussion that substantial amounts of Met are converted to Cys in this experiment.

The relationship between protein synthesis, wool growth and the mRNA activities in response to the Met infusion indicates that Met influenced transcription and possibly translation rates. A further study on this aspect will help describe the mechanism of Met on protein synthesis and wool production.

Limitation of using a single amino acid constant infusion technique to measure skin fractional synthesis rate

The various components of skin protein (which include wool growth) may not be synthesized in equal amounts. The method we used measured the synthesis rate of both skin and wool protein combined. Wool protein has a different amino acid composition than skin protein and is higher in Cys and Ser (MacRae *et al.* 1993). Although wool protein accounts for about 20–30% of the total protein synthesis in the skin (Nash *et al.* 1994; Liu *et al.* 1998), it strongly influences amino acid requirements of the skin. This probably limits the use of a single amino acid to trace protein synthesis in both skin and wool, as shown in this study where FSR values calculated using the isotope enrichment in plasma varied according to the tracer used.

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