

Metabolic hormones and tissue concentrations of mRNA for IGF-I in lines of sheep that differ in their protein synthesis response to feed intake

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Abstract

The rate of protein synthesis in the skin and muscle of sheep that have been genetically selected for high wool staple strength (SS) is less dependent on the level of dietary intake than that of low SS sheep. This study examined potential hormonal mediators of this difference in responsiveness. Sheep from SS+ and SS- genotypes were fed at 0.4, 1.1 or 1.8 times maintenance. Circulating concentrations of metabolic hormones and tissue concentrations of the mRNA for IGF-I were measured and compared with rates of protein synthesis measured previously. Plasma concentrations of GH, insulin, cortisol, thyroxine and IGF-I responded similarly to dietary intake in both genotypes, but SS+ sheep had higher plasma concentrations of

IGF-I at all levels of nutrition ($P < 0.05$). There were no interactions between diet and genotype. The concentration of mRNA for IGF-I was higher in the liver of SS+ sheep ($P < 0.05$), and tended to increase ($P = 0.06$) with nutrient intake, but there were no significant effects of genotype or nutrition in skin, muscle or gut. Concentrations of mRNA for IGF-I were not related to the rate of protein synthesis in any tissue examined. It was concluded that IGF-I did not drive the rate of protein synthesis directly, but it may mediate the responsiveness of protein synthesis rate, or protein degradation rate, to nutrient supply.

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Introduction

Groups of Merino sheep that differ genetically in their wool growth response to nutrient supply have been developed by Agriculture Western Australia (Greeff *et al.* 1997), as a result of flocks being bred for high or low staple strength (SS). The SS+ sheep have stronger wool staples, at least partly because the diameter of wool fibres changes less throughout the year in response to changes in feed supply from pasture (Adams *et al.* 1997). Paralleling their more constant wool growth, the rate of protein synthesis in both muscle and skin of the SS+ sheep also changes less in response to dietary intake, compared with the SS- sheep (Adams *et al.* 2000).

The present study examined potential hormonal mediators of this genetic difference. Concentrations of thyroxine, cortisol, insulin, growth hormone (GH) and insulin-like growth factor-I (IGF-I) were measured in plasma samples taken from SS- and SS+ sheep fed below, at or above maintenance. Paracrine effects of IGF-I in tissues may be more important than circulating concentrations (Hayden & Straus 1995, Moloney *et al.* 1998), so the concentration of IGF-I mRNA in skin, muscle, liver, rumen and jejunum was also measured. The rate of protein synthesis in these tissues was measured in the companion

study (Adams *et al.* 2000). The data enable simultaneous comparison of the rates of protein synthesis in individual tissues, the concentration of IGF-I mRNA in those tissues, and the relationship with circulating hormones.

Materials and Methods

The sheep and feeding regimes are described in detail by Adams *et al.* (2000). In brief, two experiments were carried out on 20–22-month-old castrate male sheep derived from the SS+ and SS- genetic selection lines from Agriculture Western Australia (Greeff *et al.* 1997). In the first experiment, sheep were fed at or below maintenance (M), while in the second experiment sheep were fed at or above M. The sheep grazed together on dry pasture until they were brought into an animal house and fed a ration of chopped oaten hay with 10% lupin seed and a mineral supplement.

Experiment 1

Two groups of 12 wethers aged 19 months representative of the SS+ and SS- genotypes were brought into the animal house on Day -21 and fed to M. On Day 0 each group was subdivided and feeding levels were adjusted so that they provided $0.44 \times M$ and $1.15 \times M$.

Animals were fitted with jugular catheters on Day 22 and blood samples were collected at 20-min intervals for 10 h on Day 23. Animals were given their daily feed ration immediately before sampling commenced. On Day 24 animals were injected rapidly with a 50% solution of glucose at the rate of 0.33 mg glucose/kg liveweight via an indwelling catheter. Blood samples were collected from the opposite jugular vein into fluoride oxalate for glucose analysis and into heparin for the insulin assay at -10, -5, 0, 5, 10, 15, 20, 30, 40, 60, 90, 120 and 180 min after injection.

The animals were killed on Day 36 by a lethal dose of phenobarbital and exsanguination. Samples of skin, *longissimus dorsi* muscle, liver, dorsal sac of the rumen, and jejunum were removed rapidly, frozen in liquid nitrogen and maintained at -80 °C until measurement of the mRNA for IGF-I.

Experiment 2

Two groups of 16 20-month-old wethers selected randomly from the SS- and SS+ flocks were introduced to the animal house on Day 15 and fed at M. On Day 0, each genotype was subdivided into two groups fed at 1.15 × M and 1.86 × M. Weekly blood samples were collected by single venepuncture to measure plasma thyroxine concentration. The sheep were fitted with jugular catheters on Day 29, and 20-min blood samples were collected for 10 h on Day 30, and the plasmas stored at -20 °C. The sheep were killed on Day 44 by overdose of phenobarbital and exsanguination and samples of skin, liver, muscle and jejunum were collected into liquid nitrogen as described above.

Assays

Concentrations of ovine GH and insulin in plasma samples were measured using the homologous RIAs described by Adams *et al.* (1996). The minimum detectable concentration of GH was 0.91 µg/l, and that of insulin was 0.065 µg/l. Concentrations of cortisol in plasma were measured by the RIA described by Atkinson & Adams (1988), with a minimum detectable concentration of 1.63 nmol/l. Thyroxine was measured in the RIA described by Adams *et al.* (1997), with minimum detectable concentration of 8 nmol/l.

Plasma concentrations of IGF-I were measured in single samples taken half-way through the 10 h blood sampling, using the RIA described by Adams *et al.* (1996), modified by extraction of IGF-I from circulating binding proteins by the HPLC gel filtration method described by Carr *et al.* (1995). Validation in our laboratory detected separation of binding proteins and immuno-assayable peptides in the fractions described by Carr *et al.* (1995). The minimum detectable concentration was 17.6 µg/l.

Plasma glucose was measured using a glucose hexokinase kit from Sigma Diagnostics (St Louis, MO, USA).

mRNA measurement

Total RNA was extracted from frozen tissue samples using a modification of the method of Chomczynski & Sacchi (1987). The integrity of the total RNA was assessed visually after ethidium bromide staining of the 28S and 18S ribosomal RNA separated by agarose gel electrophoresis, and the RNA was quantified by determining the absorbance at 260 nm.

Bluescript (Stratagene, La Jolla, CA, USA) plasmids containing a 271 bp ovine IGF-I cDNA (kindly provided by R S Gilmour, Auckland University Medical School), that included 187 bases of genomic intronic sequence and 84 bases of exon 3 coding sequence (Dickson *et al.* 1991) were linearized with HindIII, and antisense probes synthesized and labelled (MaxiScript kit, Ambion Inc., Austin, TX, USA) using T7 polymerase and [α -³²P]uridine triphosphate. The probe (~450 b) was checked on a polyacrylamide gel to ensure that the *in vitro* transcription reaction was successful and that there were no premature termination products present (Fig. 3). The yield and specific activity of the probe were also determined. Ribonuclease protection assays were performed using a RPA II kit (Ambion Inc.) with some modifications. In brief, known quantities of total RNA (15–50 µg) were hybridized (42 °C; overnight) with approximately 4 fmol (2×10^6 c.p.m.) of probe. After hybridization 0.5 U RNase A and 20 U RNase T1 (cloned) were added and the tubes incubated at 37 °C for 30 min to digest non-hybridized RNA. Sodium dodecylsulphate and proteinase K (Ambion Inc.) were added at a final concentration of 0.4% and 0.2 mg/ml respectively, and the tubes incubated at 50 °C for 1 h. Protected RNA hybrids were isolated by ethanol precipitation and separated according to size on a 6% polyacrylamide/8 M urea denaturing gel. Dried gels were exposed to X-ray film (BioMax Film, Kodak, Cambridge, UK) at -80 °C for 24 h for liver, skin and muscle, and 48 h for jejunum. In Experiment 1, protected RNA hybrids were quantified using densitometry. In Experiment 2, bands corresponding to protected hybrids were excised from the dried gel, and measured in scintillant (Filter-Count, Packard, Canberra, Australia), on a liquid scintillation analyser. Expression of IGF-I mRNA was represented as the IGF-I equivalents/g total RNA by dividing the sample count by the counts for a known amount of probe, and multiplying this by the amount of RNA hybridized.

Statistical analysis

Results of the two experiments were analysed statistically by analysis of variance or by repeated measures analysis of variance using Systat (Wilkinson 1992), with intake and genotype as factors and experiment as a covariate. Plasma concentrations of GH were not normally distributed, and were converted to logarithms before analysis. Back transformed means are presented. The pulsatile pattern of GH release was analysed by the Pulsar algorithm as described

Table 1 Mean (and s.e.m.) plasma concentrations of metabolic hormones in the low and high staple strength (SS) sheep fed at different levels of maintenance (M)

	Feeding level ^a			SS Group ^b		S.E.M.	Probability	
	0.4 × M	1.1 × M	1.8 × M	Low	High		Feed ^a	Group ^b
Mean GH (µg/l)	11.0	4.2	2.1	4.5	4.6	0.4	0.001	0.8
Pulses of GH/10 h	5.8	4.8	3.3	4.6	4.7	0.3	0.001	0.9
Insulin (µg/l)	0.52	1.05	1.19	0.89	0.95	0.09	0.001	0.5
Insulin-like growth factor-I (µg/l)	175	209	268	202	232	10	0.001	0.03
Cortisol (nmol/l)	5.31	7.16	9.71	6.98	7.81	0.47	0.002	0.2
Thyroxine (nmol/l)	71.5	67.5	82.9	76.2	71.7	3.0	0.003	0.2

Data were analysed by repeated measures two-way ANOVA; ^acombined values of SS+ and SS- groups, ^bcombined values of the three feeding levels.

previously (Adams *et al.* 1996). Tissue concentrations of IGF-I mRNA were normalized within individual tissues across the two methods of quantification before statistical analysis.

Results

Hormones and metabolites

The plasma concentrations of GH, cortisol, insulin and thyroxine were all significantly affected by the level of feeding ($P < 0.01$), but not by genotype (Table 1). Plasma concentrations of IGF-I were also affected by feeding level ($P < 0.001$), and were higher in the SS+ group (Table 1; $P < 0.05$). The higher plasma IGF-I in the SS+ group occurred at all feeding levels, so there was no statistically significant interaction between SS genotype and feeding level for any of these hormones.

There were significant interactions between feeding level and time of sampling for GH ($P < 0.001$). The values presented in Fig. 1 indicate that plasma concentrations of GH were suppressed at the beginning of the sampling

period, immediately after feeding, and then rose more in the sheep fed at $0.44 \times M$ than in those fed at $1.8 \times M$. There was also a significant interaction between time of sampling, genotype and feed ($P < 0.01$). This interaction was explored by calculating the s.e.m. of the plasma GH concentration for individual sheep to estimate the variability of individual secretion patterns. These tended to be more variable ($P = 0.06$) in the SS+ than in the SS- group.

Plasma concentrations of insulin decreased with time after feeding ($P < 0.001$), with the decrease being greatest in the sheep fed at $0.4 \times M$ (interaction $P < 0.001$). Plasma concentrations of cortisol did not change significantly over the 10-h sampling period. Plasma concentrations of thyroxine changed over the 9-week sampling period ($P < 0.001$), being highest in the samples taken in the first week, and then declining over the next two weeks before increasing again. Values increased more in the second half of the experiment in the group fed at $1.8 \times M$ than in the other two groups, so there was a significant interaction between feed and time ($P < 0.001$). All these effects were similar in the two genotypes.

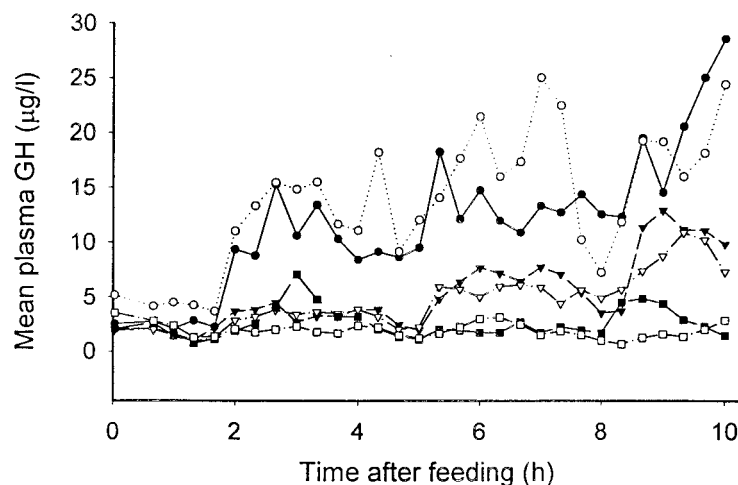


Figure 1 Mean concentration of GH in SS+ (solid symbols) and SS- (open symbols) sheep fed at $0.4 \times M$ (●, ○), $1.1 \times M$ (▼, ▽) and $1.8 \times M$ (■, □).

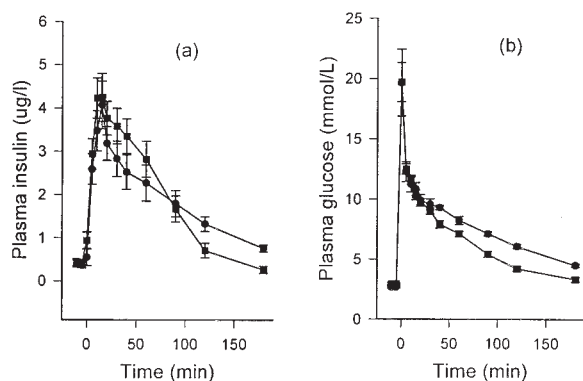


Figure 2 Plasma concentration of insulin (a) and glucose (b) in sheep fed at $0.4 \times M$ (●) or $1.1 \times M$ (■) after bolus injection of 0.3 mg/kg liveweight glucose.

Following bolus injection, the peak concentration of glucose in plasma declined more slowly in the group fed at $0.4 \times M$ in Experiment 1 ($P < 0.01$; Fig. 2). Similarly, feed intake affected the rate of decline in the plasma concentration of insulin from the peak value ($P < 0.05$), with initially higher values in the sheep fed at $1.1 \times M$ which then declined below those of sheep fed at $0.4 \times M$ (Fig. 2). There was no effect of genotype, or interaction between genotype and feeding level, for any characteristic of glucose or insulin concentrations.

mRNA for IGF-I

Tissue concentrations of IGF-I mRNA equivalents were higher in the liver of SS+ sheep ($P = 0.03$), and tended to increase with increasing feed levels ($P = 0.06$, Table 2). Neither feed nor genotype had a statistically significant effect on IGF-I mRNA concentration in other tissues.

The total amount of IGF-I message differed among the tissues. In Experiment 2, the expression of IGF-I mRNA (mean \pm s.e.m.) was 0.20 ± 0.03 , 1.10 ± 0.21 , 0.66 ± 0.07 and 0.21 ± 0.02 IGF-I equivalents/g total RNA, for liver, skin, muscle and jejunum respectively. Liver and jejunum had significantly lower and skin had significantly higher IGF-I expression/g total RNA, com-

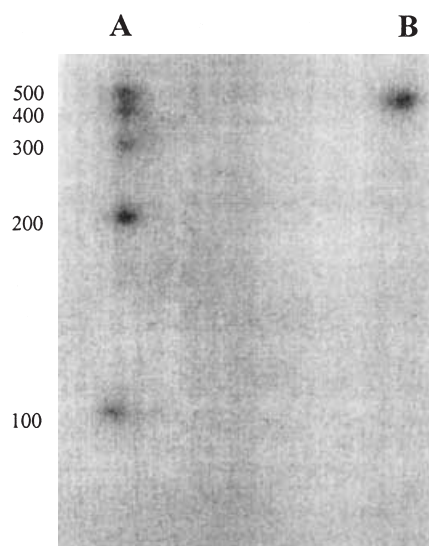


Figure 3 ^{32}P -UTP labelled (A) RNA markers of Sigma RNA marker templates (R4142) and (B) ovine antisense IGF-I probe containing 83bp Exon 3 of ovine IGF-I gene in Bluescript plasmid, produced by *in vitro* transcription with T7 polymerase.

pared with muscle. The yield of total RNA from frozen samples differed markedly between tissues. Average yields of RNA (mean \pm s.e.m.) were 2.43 ± 0.15 , 0.12 ± 0.01 , 0.22 ± 0.01 and 2.43 ± 0.26 mg total RNA/g tissue, for liver, skin, muscle and jejunum respectively. Thus, the relative amounts of IGF-I mRNA/g of liver, skin, muscle and jejunum were 1.00, 0.28, 0.30 and 1.05 respectively. Similar results, with slightly different values, were obtained in Experiment 1. Standardization across the two experiments prevented accurate comparisons of the amount of mRNA between tissues in Table 2.

Using simple correlations, plasma concentrations of IGF-I were significantly ($P < 0.05$) related to the amount of IGF-I mRNA in liver, muscle and skin (Table 3). Although the concentration of IGF-I in plasma was related to the level of nutrition, feed intake was not strongly related to the concentration of IGF-I mRNA in any tissue except liver (Table 3). Concentrations of mRNA for

Table 2 Mean (and s.e.m.) expression of IGF-I mRNA (arbitrary units) in the low and high staple strength (SS) sheep fed at different levels of maintenance (M)

	Feeding level ^a			SS Group ^b			Probability	
	$0.4 \times M$	$1.1 \times M$	$1.8 \times M$	Low	High	s.e.m.	Feed ^a	Group ^b
Liver	1.98	2.48	3.40	2.35	2.93	0.25	0.06	0.03
Skin	1.73	2.22	2.10	1.84	2.30	0.25	0.44	0.15
Muscle	1.12	0.98	1.42	1.05	1.24	0.07	0.40	0.94
Jejunum	0.33	0.44	0.42	0.37	0.46	0.03	0.51	0.24

Data were analysed by two-way ANOVA; ^acombined values of SS+ and SS- groups, ^bcombined values of the three feeding levels.

Table 3 Simple correlation matrix for plasma concentrations of IGF-I, its mRNA in tissues, and the level of nutrition

	<u>Muscle mRNA</u>	<u>Skin mRNA</u>	<u>Jejunum mRNA</u>	<u>Liver mRNA</u>
Skin mRNA	0.35**			
Jejunum mRNA	0.43**	0.27		
Liver mRNA	0.10	-0.07	0.28*	
Plasma IGF	0.39**	0.29*	0.25	0.38**
Feed intake	0.19	0.14	0.20	0.36**
Protein FSR ^a	0.07	-0.02	0.07	-0.23

^aProtein fractional synthesis rate determined in the same tissues by Adams *et al.* (2000).

* $P < 0.05$, ** $P < 0.01$.

IGF-I were correlated among muscle, skin and jejunum, but were not related to the rate of protein synthesis in any organ (Table 3).

Discussion

The SS+ sheep had higher plasma concentrations of IGF-I than the SS- line, and this was accompanied by higher concentrations of mRNA for IGF-I in the liver. This difference was not due to the mean levels of GH, which were similar in the two genotypes. Plasma concentrations of GH have a major effect on both plasma IGF-I and the concentration of IGF-I mRNA in liver (Hua *et al.* 1993, Brameld *et al.* 1996). The secretion of GH tended to be more variable in the SS+ sheep, but this is not likely to have caused the difference in IGF-I because a disrupted pulsatile pattern of GH secretion is less effective in maintaining plasma IGF-I (Veldhuis *et al.* 1995).

It is possible that the higher circulating IGF-I and liver IGF-I mRNA in the SS+ sheep were secondary to a difference in one or more of the six IGF binding proteins (IGFBPs). The role of IGF-I in co-ordinating tissue function within individual organs is highly dependent on local variations among the IGFBPs and associated proteases (Perks & Wathes 1996, Batch *et al.* 1996). In addition, circulating concentrations of IGF-I in sheep are affected by plasma concentrations of IGFBPs, particularly BP3 (Brier 1999). Relationships among IGF and its BPs are complex (Lemmey *et al.* 1997), and concentrations of mRNA for some of the IGFBPs appear to be directly influenced by nutrition (Higashi *et al.* 1998). Whatever the mechanism, the fundamental genetic difference between the lines involved the IGF system.

The two lines of sheep in this study differed in mean IGF-I concentration, but not in mean rate of protein synthesis, indicating that IGF-I did not affect the rate of protein synthesis directly. This conclusion is supported by the failure to observe a significant correlation in any tissue between the relative concentration of mRNA for IGF-I

and the rate of protein synthesis (Table 3). A similar conclusion was reached after direct measurement of the concentration of IGF-I peptide in tissues (Hua *et al.* 1993). Furthermore, Svanberg *et al.* (1998), using dwarf mice, also concluded that nutrition did not drive protein synthesis through stimulation of IGF-I mRNA. However, differences in the IGFBPs, or in the efficiency of translation of IGF-I mRNA (Thissen *et al.* 1991, Hua *et al.* 1993), may also contribute to the lack of relationship with protein synthesis rate.

The main functional difference between the two genetic lines is a reduced sensitivity of protein synthesis rate to dietary intake in the SS+ sheep. Adams *et al.* (2000) found that protein synthesis rate was relatively higher in SS+ sheep fed below M, but not if fed above M, when compared with SS- sheep. Since the only hormonal difference between them was the increased mean concentration of IGF-I in SS+ sheep, it is likely that IGF-I was involved in mediating the animal's response to the level of nutrition.

The concentration of IGF-I in plasma increased with feed intake ($P < 0.01$), but the increase in its mRNA approached statistical significance only in the liver (Table 2). This is consistent with other reports that the level of nutrition did not affect the concentration of mRNA for IGF-I in muscle of sheep (Oldham *et al.* 1996) or swine (Brameld *et al.* 1996). An intra-abomasal infusion with casein caused a transient increase in IGF-I mRNA in muscle of cattle that disappeared by 14 days (Moloney *et al.* 1998). Despite there being little relationship to feeding level, concentrations of IGF-I mRNA were significantly correlated among muscle, skin and jejunum (Table 3). These relationships may reflect a similar response to some other stimulus, such as a recent peak of GH concentration (Lemmey *et al.* 1997).

There is considerable evidence, summarized by Bell *et al.* (1998), that IGF-I has a primary effect on protein degradation rate. Protein degradation rate is sensitive to a number of hormones, including IGF-I (Oddy & Owens 1996), cortisol (Rooyackers & Nair 1997) and insulin (Oddy 1993). Certainly, any increase in protein synthesis rate caused by infusion with IGF-I is usually only temporary (Lobley *et al.* 1997). In the SS+ sheep used in the current study, changes in whole body protein degradation rate were even less responsive to nutrition than synthesis rate (Adams *et al.* 2000), which would also explain the greater loss of liveweight by SS+ sheep than SS- sheep when fed below M (Adams *et al.* 1997). Furthermore, greater differences in protein degradation rate than in synthesis rate have been observed after genetic selection of both sheep (Oddy *et al.* 1995) and cattle (Oddy *et al.* 1998) for differences in protein deposition rate. We conclude that the most likely basic difference between the SS+ and SS- genotypes is the sensitivity of protein degradation rate to dietary intake, resulting from a genetic difference in IGF-I status.

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