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Effects of insulin on amino acid uptake by the mammary gland of the pasture-fed lactating ruminant

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ABSTRACT

Twelve lactating ewes housed indoors were allocated to one of two treatment groups (hyperinsulinaemic euglycaemic clamp (HEC) or control) in a randomised block design experiment. Mammary amino acid uptake from plasma and utilisation for milk protein synthesis was measured during the fourth day of the HEC using the arterio-venous concentration difference (A-V) technique. There was no change in milk protein output during the HEC ($P>0.05$). The HEC induced a significant decrease in arterial concentrations of all essential amino acids (EAA) except for histidine and an increase in the extraction efficiency of isoleucine (HEC, 61 ± 3% vs. control, 52 ± 3%, $P<0.05$) and leucine (HEC, 64 ± 3% vs. control, 53 ± 3%, $P<0.05$). There was no significant change in extraction efficiency of other EAA or mammary blood flow. The ratio of amino acid uptake from plasma to output in milk protein suggested deficits in uptake for some EAA in both treatments, especially histidine. These results indicate that the mammary gland can adapt to a changing arterial supply of amino acids to maintain milk protein output by increasing extraction efficiency and/or a greater reliance on amino acids supplied by erythrocytes and/or plasma peptides.

Keywords: insulin; amino acids; mammary; lactating; ruminant; pasture.

INTRODUCTION

Insulin is a major anabolic hormone in the body, controlling uptake and subsequent metabolism of nutrients for different physiological processes. Early studies using bolus injections of insulin reduced milk and milk protein yield (Kronfeld *et al.*, 1963; Schmidt, 1966) but yields were restored to pre-treatment values with simultaneous glucose administration (Kronfeld *et al.*, 1963). Subsequent short term (2-10 h) studies with simultaneous insulin and glucose infusions (Hove, 1978; Laarveld *et al.*, 1981; Tesseraud *et al.*, 1992) demonstrated no effect on milk and milk protein yield. However, several recent experiments using the hyperinsulinaemic euglycaemic clamp technique (HEC) (during which insulin is infused to increase circulating concentrations of insulin with a simultaneous glucose infusion to maintain euglycaemia) over four days in concentrate fed cows resulted in an increase in milk protein yield (McGuire *et al.*, 1995). Yields were substantially increased when the cows were supplemented with additional protein (Griinari *et al.*, 1997; Mackle *et al.*, 2000). In contrast, when we used this approach in pasture-fed lactating ewes (Back *et al.*, 1998) we demonstrated that under HEC conditions there was no change in milk protein yield. However, there was a change in nutrient partitioning as milk protein yield was maintained despite dry matter intake (DMI) and hence crude protein intake decreasing up to 20%. This resulted in a change in the proportional utilisation of dietary protein for milk protein produced. But it is not clear how this apparent increase in protein utilisation was achieved. Did insulin influence the maintenance of amino acid (AA) supply to the mammary gland, and if so, by what mechanisms? Amino acid supply to the mammary gland could be maintained/increased by increasing AA extraction/uptake and/or blood flow. Either of these possibilities would ensure that milk protein yield could be maintained or possibly increased. Alternatively, it may be that the observed response was an adaptation by the mammary gland to the changing nutritional environment caused by the insulin infusion.

In the study reported here, we used the arterio-venous concentration difference (A-V) technique across the mammary gland to determine if mammary AA uptake/ extraction was altered during the insulin infusion.

MATERIALS AND METHODS

Twelve lactating ewes were housed indoors in metabolism crates and individually fed fresh perennial ryegrass (*Lolium perenne*)-white clover (*Trifolium repens*) pasture *ad libitum* (as detailed by Back *et al.*, 1998) during the three day pre-experimental and the six day experimental period. All ewes underwent surgery around day 70 of gestation where, under general anaesthesia they were fitted with permanent aorta catheters and transit-time blood flow probes (Transonic Systems Inc., Ithaca, New York, USA) around the pudic artery. After recovery from surgery, the ewes were returned to pasture until three days post-lambing, when the lambs were removed and the ewes moved indoors. Four jugular catheters and one milk vein catheter were implanted (as detailed by Back *et al.*, 1998). One catheter in the left jugular vein was used to monitor blood glucose. In the right jugular vein, two catheters were implanted to enable simultaneous insulin and glucose infusions. The milk vein catheter was used for sampling venous blood leaving the mammary gland. The ewes were machine-milked twice a day (0730 and 1930 hours), with the aid of oxytocin administered via a jugular catheter to stimulate milk letdown (1 IU/ewe, Oxytocin V, Vetpharm (NZ)).

Ewes were allocated to a treatment group (± insulin) in a randomised block design and subjected to a four day HEC. The HEC was performed using bovine pancreas derived insulin (1μg/kg $BW^{0.75}$, Sigma Chemicals, St Louis, Mo.) in a sterile filtered 0.5% bovine serum albumin solution (Immuno Chemical Products (NZ) Ltd). Euglycaemia was maintained using a sterile 30% w/v glucose solution (food grade dextrose monohydrate; Pure Chem Co. Ltd, Thailand) via variable speed pumps. The blood glucose concentrations

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of the ewes were monitored using an Advantage Blood Glucose Meter (Boehringer Mannheim (NZ) Ltd), which allowed rapid (within 2 minutes) determination of blood concentrations for adjusting the glucose infusion rate to maintain euglycaemia.

Ewes were milked twice daily at 0730 and 1930 hours, and the yields weighed. Milk composition was predicted by near-infrared spectroscopy in transmission mode (Model 6500, NIRsystems Inc, Silver Spring, MD, USA). A sample was processed as skim milk, frozen at –85°C and analysed for individual milk proteins using a nephelometric assay (Collin *et al.*, 2001). Milk AA composition was determined in freeze-dried skim milk samples by ion-exchange HPLC (Shimadzu LC10A) after post-column derivatisation with ninhydrin after HCl hydrolysis (AOAC, 1990). Methionine concentrations were determined in the same way, in samples hydrolysed after oxidation with performic acid (AOAC, 1990).

Integrated A-V blood samples were taken by pump for 45 minutes. Five minutes before blood sampling the ewes were primed with a bolus injection of heparin. On the day before the HEC, two samples were taken commencing at 1200 and 1400 hours. On day 4 of the HEC, samples were taken during the final feeding period at 0730, 0900 and 1030 hours.

Plasma concentrations of insulin were measured using a double antibody radioimmunoassay (Flux *et al.*, 1984). Whole blood was centrifuged (at 3270g at 4°C for 15 minutes) and the resulting plasma was harvested and stored at -85°C until analysed. Amino acid concentrations (exclusive of cysteine) were determined on plasma samples that were mixed with 25µl of 3mM nor-leucine as an internal standard and then deproteinised by ultrafiltration (Centrisart, molecular weight cut-off 10,000; Sartorius AG, Gottingen, Germany). The ultrafiltrate was stored at –85°C until analysed by reverse phase HPLC separation of phenylisothiocyanate derivatives on a Waters PicoTag™ column used in conjunction with a Shimadzu LC10A HPLC system using a modified method of Bidlingmeyer *et al.*, (1984). Cysteine was determined by a modified automated method using acid ninhydrin (Gaitonde 1967) after plasma had been deproteinised by addition of 30% trichloroacetic acid (TCA), centrifuged at 40°C for 20 minutes and filtered through a 0.45µm cellulose syringe filter.

Blood flow was measured every day during the HEC in six ewes (three from each treatment) with transit-time ultrasonic blood flow probes.

The ewes were fed four times daily and the amount of feed offered recorded. Refusals from each feed were collected from the bins and floor, bulked separately, weighed and the bin refusals subsampled for analysis. Dry matter was calculated on both feed offered and refused and daily dry matter intake (DMI) was measured as offered minus refused. Daily pasture samples were analysed for crude protein (CP) by near infra-red reflectance spectrometry (Corson *et al.*, 1999).

The data generated in this experiment were analysed as a randomised block design. As the ewes were blocked according to lambing date, analyses included HEC as a fixed treatment effect with block as a random effect and time as a repeated factor. Analyses were performed using the procedure GLM from the statistical package SAS (1996). Results are expressed as least squares means ± standard errors. All treatment and block effects were tested, and means and probability values were generated to compare between treatments. Normality of the data, tested by plotting the standardised residuals against the standardised predicted values of the response variables, verified the normality assumption of the ANOVA model.

RESULTS

By day 4 of the HEC, plasma insulin concentrations had significantly increased in the insulin treated ewes compared to the control ewes (397 ± 19 vs. 182 ± 19 pg/ml, $P<0.001$). Milk yield measured on the day before the HEC (the pre-infusion day) was significantly higher in the control ewes (1719 ± 78g) than in HEC ewes (1429 ± 78g). By day 4 of the HEC, there was no significant change in milk yield in either the control (1631 ± 78g) or HEC ewes (1367 ± 78g). Because of the higher milk yield, control ewes also had a higher CP yield on the pre-infusion day (98.5 ± 7.8g) than the HEC ewes (73.2 ± 7.8g). However, concentrations of CP were not significantly different on the pre-infusion day (control 5.6 ± 0.1% vs. HEC, 5.5 ± 0.1%) or by day 4 of the HEC (control 5.4 ± 0.4% vs. HEC, 5.2 ± 0.1%). There was no effect of the HEC on the concentration of β-casein, β-lactoglobulin, κ-casein or immunoglobulins by day 4.

Feed intake (DMI) was not significantly different between the HEC and control ewes on the day before the HEC (control 1.44 ± 0.008 kg vs. HEC, 1.47 ± 0.008 kg). By day 4 of the HEC, there tended ($P=0.08$) to be a difference in DMI (control, 1.51 ± 0.08 kg vs. HEC, 1.31 ± 0.08kg). There was no difference in CP intake, either on the pre-infusion day (control 314 ± 15g vs. HEC, 302 ± 15g), or day 4 of the HEC (control 315 ±15g vs. HEC, 279 ± 15g).

Arterial plasma concentrations of EAA and NEAA are presented in Table 1. Arterial concentrations of all EAA measured (except for histidine) were significantly lower in the insulin treated ewes during day 4 of the HEC. The concentration of branch chain AA (BCAA; leucine, isoleucine, valine) decreased by 16 %, and the remaining EAA decreased on average by 17%. Overall concentrations of the non-essential AA (NEAA) were decreased by 17%. This was mainly due to concentrations of asparate, glutamate, glycine and proline being significantly lower in the insulin treated ewes. Despite a numerical decrease, concentrations of serine, asparagine, glutamine, alanine and tyrosine were not significantly different between the HEC and control ewes. Glycine was the only AA that was significantly higher in the HEC ewes.

Mammary extraction efficiency data are presented in Table 1. Among the EAA the HEC treatment significantly increased the mammary extraction efficiencies of isoleucine and leucine by 20%. In contrast, the extraction efficiency of tyrosine was significantly lower in the insulin treated group. There was a numerical increase in extraction efficiencies of lysine (17%) and valine (12%) but these were not statistically significant. There were low extraction efficiencies for cysteine and histidine in both the HEC and control ewes. For the NEAA, the extraction efficiency of serine was significantly lower in the insulin clamp group. The observed differences in the HEC ewes for glutamine,

TABLE 1: Comparison of amino acid arterial plasma concentrations, extraction efficiencies and uptake:output ratio (LSM ± SEM) between treated and control ewes on day 4 of the hyperinsulinaemic euglycaemic clamp.

	Arterial concentration		Extraction Efficiency[1]		AA uptake:output ratio[2]	
	HEC	Control	HEC	Control	HEC	Control
	(μM)		(%)			
Essential Amino Acids						
Arginine	76±4.8[a]	98±4.8[b]	51±4.5	50 ± 3.9	2.66±0.324	2.26±0.275
Cysteine	70±3.5[a]	83±3.5[b]	6±6.2	20± 6.2	nd	nd
Histidine	58±3.6	58±3.6	15±5.3	21±4.7	0.83±0.290	0.86±0.246
Isoleucine	75±2.8[a]	87±2.8[b]	61±2.9[a]	52±2.6[b]	1.38±0.084	1.33±0.080
Leucine	126±4.3[a]	156±4.3[b]	64±2.8[a]	53±2.5[b]	1.18±0.066	1.13±0.078
Lysine	73±3.8[a]	96±3.8[b]	58±4.6	50±3.9	0.88±0.051	0.79±0.043
Methionine	28±1.0[a]	33±1.0[b]	61±3.6	56±3.3	1.35±0.115	1.31±0.097
Phenylalanine	51±1.4[a]	61±1.4[b]	41±2.2	43±2.1	0.96±0.086	0.94±0.073
Threonine	105±4.5[a]	134±4.5[b]	29±1.9	31±1.7	1.00±0.044	0.99±0.052
Tyrosine	71±2.1	71±2.1	32±3.0[a]	42±2.7[b]	1.06±0.084	1.08±0.071
Valine	158±5.4[a]	190±5.1[b]	46±2.4	41±2.1	1.66±0.010	1.54±0.085
Non Essential Amino Acids						
Alanine	141±4.4	137±4.7	26±2.6	23±2.4	1.14±0.053[a]	0.78±0.045[b]
Asparagine	62±2.6	68±2.6	40±2.7	40±2.3	nd	nd
Asparatic acid	6.2±0.40[a]	9.2±0.40[b]	35±5.0	46±4.5	0.06±0.015	0.06±0.013
Glutamine	360±21.9	378±20.8	4±3.5	6±3.1	nd	nd
Glutamate	87±2.7[a]	138±2.7[b]	56±3.1	51±2.8	0.39±0.021	0.42±0.018
Glycine	494±17.2[a]	439±17.2[b]	-2 ±3.2	-2 ±2.9	0.24±1.020	0.65±0.865
Proline	103±3.1	116±3.1	27±2.5	26±2.5	0.45±0.044	0.33±0.037
Serine	67±3.2	73± 3.2	30±7.3[a]	59±6.5[b]	0.71±0.072	0.69±0.052

[1] Extraction efficiency (%) was calculated as A-V/A x 100.
[2] Uptake:output ratio was calculated as A-V of AA/proportion of AA in milk protein (Davis *et al.*, 1978).
[3] nd is not determined.

glutamate, alanine and proline were not significantly different from control animals.

There was no significant difference in blood flow between the HEC and control ewes over the four days of the HEC. Blood flow measured on day four during the A-V blood sampling was 799 ± 45 ml/min in the control ewes vs. 788 ± 42 ml/min in the HEC ewes.

There was no significant difference in the ratio of AA uptake:output in milk protein in either EAA or NEAA (except for alanine) between the HEC and control ewes (see Table 1). The uptake:output ratio was less than 1:1 for histidine, lysine and phenylalanine in the HEC and control ewes. For the NEAA, the ratio was less than 1:1 for all NEAA, except for alanine in the HEC ewes.

DISCUSSION

The HEC technique was used in conjunction with the A-V plasma difference technique across the mammary gland of lactating ewes to determine uptake and subsequent utilisation of AAs for milk protein synthesis. In this study, insulin concentrations were doubled, which is in contrast to results obtained in our previous work (Back *et al.*, 1998) and the studies by McGuire *et al.* (1995), Griinari *et al.* (1997) and Mackle *et al.* (2000), where concentrations were raised four-fold. Although circulating insulin concentrations were still significantly increased, there was no increase in milk yield, milk protein yield or concentration or proportions of individual milk proteins. However, milk protein output was maintained despite a decrease in DMI. While this decrease in intake was smaller than in our previous study, this may also be related to the lower increase in circulating insulin concentrations.

The A-V difference technique was utilised to determine if AA utilisation by the mammary gland was influenced by higher insulin concentrations during the HEC. The decrease in arterial concentrations of EAA (except for histidine and threonine) during the HEC is consistent with changes in arterial concentrations in the study by Mackle *et al.* (2000) in concentrate-fed cows. However, in that study, concentrations of histidine were also lowered. There is a large difference in the severity of the reduction in arterial concentrations between these two studies. In our study concentrations of BCAA decreased by 16% compared to 55% in the concentrate-fed cows of Mackle *et al.* (2000). The remaining EAA decreased by 17%, which is similar to the 23% reduction reported by Mackle *et al.* (2000). It is not clear what causes this decrease in AA concentrations but it may be as a result of decreased release of AA from splanchnic tissues (Biolo & Wolfe, 1993). The magnitude of effect may be dependent on the absolute change in insulin concentrations i.e. in our study the decrease in arterial AA concentrations may have been less because of the smaller increase in insulin concentration.

An increase in mammary gland extraction of AA and/or blood flow are two ways that AA supply for milk protein synthesis can be maintained and/or increased. In this study, the HEC ewes showed an increased extraction efficiency of two BCAA, isoleucine and leucine by 20% but there was no increase in mammary blood flow. This is in contrast to Mackle *et al.* (2000), where there was a greater increase in extraction of BCAA (40%), arginine and lysine (20%) and also an increase in mammary blood flow. This greater response may again have resulted from the higher insulin concentrations induced in the concentrate fed cows.

As there was no significant change in mammary blood flow from the HEC, a ratio of AA uptake relative to output in milk was calculated by dividing A-V differences for individual AA by their content in milk protein (g/100g) (see Davis *et al.*, 1978). Individual ratios are expressed relative to the average of phenylalanine+tyrosine. The ratio of uptake:output of these two AA is close to one as they are not catabolised by the mammary gland (Verbeke *et al*., 1972). The ratios are in agreement with previous studies (reviewed by Bequette *et al*., 1998) that showed EAA such as arginine and the BCAA are taken up in excess of their output by the mammary gland. However, the analysis of plasma samples shows insufficient uptake of histidine and lysine to account for their output in milk protein as shown by Bequette *et al.* (1999), Pacheco-Rios *et al.* (1999), and Mabjeesh *et al*. (2000). Pacheco-Rios *et al*. (1999) demonstrated AA uptake from plasma was insufficient for milk protein output of histidine, lysine, phenylalanine and tyrosine. But, when whole blood was analysed in that study, the uptake of lysine, phenylalanine and tyrosine was sufficient to account for the output of these AA in milk protein and the contribution of erythrocytes was seen to be between 5-15%. For histidine, there was a deficit when measured in either plasma or whole blood and it appears that for this AA the plasma free pool may not be the main precursor pool. Concurrently, there may be a greater dependency on another alternative source such as blood borne peptides (Bequette *et al.*, 1999). In lactating goats, Bequette *et al.* (1999) showed that between 5-25% of the supply of lysine, methionine, phenylalanine and tyrosine for casein synthesis came from vascular peptides.

The ratio for methionine was high, given that as a Group 1 amino acid the uptake:output ratio would be expected to be around 1:1. This indicates that methionine was taken up in excess by the mammary gland. However, further work is required to elucidate whether this is real or a result of analytical procedure.

This study showed the mammary gland was able to maintain milk protein output despite a decrease in arterial AA supply, supporting the theory that the mammary gland has the ability to directly respond to modified precursor supply to the gland. How the gland was able to identify the deficit in arterial supply allowing it to increase extraction is not clear. If the decrease in arterial supply of AA is indicative of reduced AA release from splanchnic tissue (Biolo and Wolfe, 1993), does this signal the mammary gland to increase extraction? There is evidence of communication between the gut and gland (Davis *et al.*, 1996). Alternatively, do the mammary cells respond to the decreased AA concentration at mammary level as suggested by Bequette *et al.* (2000)? In reality, it may be that there is a combination of both mechanisms and further work is required to determine this.

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