ISSN 0370-2731/2001 Proceedings of the New Zealand Society of Animal Production **61:** 244-247

γ-glutamyl transpeptidase and amino acid transport for milk protein production *in vivo*

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ABSTRACT

Activity of the enzyme γ-glutamyl transpeptidase (γ-GT) (EC 2.3.2.2) a putative facilitator of amino acid (AA) transport in the mammary gland, increases before lactation and decreases at weaning, suggesting a role in AA transport for milk protein synthesis. The effect of γ-GT inhibition by acivicin and supply of cyst(e)ine from N-acetylcysteine (NAC) on mammary AA uptake and milk production was studied in lactating goats. NAC increased ($P<0.05$) arterial supply of some AA but did not increase arterio-venous difference as a percentage of arterial supply (extraction %) and did not alter milk protein yield. Acivicin decreased ($P<0.05$) milk protein yield but this effect was not found when NAC was administered in combination with acivicin. This indicates that NAC, by indirectly enhancing intracellular cysteine, countered the effect of acivicin inhibition, suggesting a role for γ-GT in supply of cysteine for milk protein synthesis.

Keywords: goats; cysteine; amino acid transport; milk protein; acivicin; N-acetylcysteine.

INTRODUCTION

Understanding factors controlling milk protein synthesis is necessary before nutritional strategies to increase milk protein yield can be optimised in order to meet the Dairy Industry targets. Studies of substrate supply have identified some amino acids (AA) as restricting for increased protein production. In the cow, it has been shown that methionine, histidine, phenylalanine, leucine, and tyrosine are taken up from the blood in quantities insufficient to account for their output in milk, suggesting that these were potentially limiting for milk protein synthesis (Metcalf *et al.*, 1996). These authors also found that increases in supply of these AA does not necessarily increase AA uptake by the gland or result in increased milk protein output. This suggests that protein synthesis may be limited by other AA and/or other controlling factor(s).

The sulphur AA, methionine and cysteine, have been shown to be limiting for milk protein synthesis (Lee *et al.*, 1999) however, for analytical reasons, cysteine has often not been included in studies. The disulphide form, cystine, can be rapidly converted to cysteine inside the cell. Supply of cystine to cells has been shown to occur through γ-glutamyl transpeptidase (γ-GT) (EC 2.3.2.2), an enzyme of the γ-glutamyl cycle, which is involved in glutathione (GSH) metabolism (Thompson & Meister, 1975). The proposal of a role for γ-GT as an AA transporter has had both support (Cotgreave & Schuppe-Koistinen, 1994; Sweiry *et al.*, 1995) and criticism (Hanigan, 1998; Wolff *et al.*, 1998). Baumrucker *et al.* (1981) reported the presence of γ-GT in the plasma membrane of many secretory tissues including the mammary gland.

As part of a larger study, this experiment aims to define the role of γ-GT in cyst(e)ine supply for milk protein synthesis. We attempted to inhibit milk protein synthesis of the lactating goat using acivicin (L-(αS,5S)-α-amino-3-chloro-4,5-dyhydro-5-isoxazoleacetic acid), a well-documented inhibitor of γ-GT (Smith *et al.*, 1995), which has previously been used *in vivo* (Viña *et al.*, 1989). N-acetylcysteine (NAC) has been shown to increase intracellular thiols (Sen *et al.*, 1999) and was administered in an attempt to restore milk protein synthesis to normal. The present report describes the affect of acivicin and NAC on milk protein yield, and on net plasma concentrations of essential AA and tyrosine and cyst(e)ine, which can be considered essential in the mammary gland.

MATERIALS AND METHODS

Two Saanen and two Toggenburg goats (53-65 kg), non-pregnant and in mid-lactation, were surgically modified by implantation of a transit-time ultrasonic blood flow probe (Transonic Systems Inc., Ithaca, New York) around the right external pudic artery. A catheter was implanted into the same artery for infusion of NAC (BUFA B.V. Pharmaceutical Products, Holland) or physiological saline in controls. A catheter was also implanted into the mesenteric artery for arterial blood sampling. Temporary catheters were inserted into both caudal superficial epigastric (milk) veins for venous blood sampling and into one jugular vein for oxytocin (Vetpharm (NZ) Ltd., Glenfield, NZ) administration to aid milking. Animals were housed in metabolism crates in an air-conditioned room and fed chaffed lucerne hay *ad libitum* and 2 kg/day of grain-based concentrate (Moozlee, NRM, Auckland, NZ), by overhead continuous feeders with meadow hay and water freely available. Feed intake (dry matter: 1860 ± 44 g, n=4) was monitored daily, and was constant over the acclimation period (1-week) and experimental day.

On non-experimental days, goats were machine milked twice daily. Goats were milked four times at 1.5-hour intervals using oxytocin (1 IU) on the day prior to experimentation in addition to morning and afternoon milking. On experimental days, goats were milked using oxytocin every 0.5 hours 4 times prior to infusion, then hourly until acivicin (Sigma-Aldrich) or control treatment, then every 1.5 hours to the end of infusion.

Each animal received four treatments (control (saline) acivicin, NAC, NAC and acivicin) in a cross-over design. Two goats underwent treatment per experimental day with a 1-week rest between experiments. NAC in saline (0.4 mg/ml) or saline was infused (1 ml/min) for 7.5 hours via the pudic artery catheter. Acivicin was administered 10 minutes after the gland was thoroughly stripped of milk by

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hand. Acivicin (5 ml 14 mg/ml saline) or saline was injected into the right hand side (RHS) lumen of the mammary gland via the teat using 150 mm of polyethylene tubing, (ID 0.58 mm OD 0.96 mm; Dural Plastics & Engineering) connected to a syringe by a 23G blunt needle. Saline (5 ml) was injected via the teat into the left hand side (LHS) of the gland. The gland was gently massaged for 5 min. Three 15 ml blood samples were collected immediately after acivicin or saline administration, from arterial and venous catheters by perstaltic pump continuously over 30, 60, then 120 minutes.

Milk fat was estimated by microcentrifugation (Fleet & Linzell, 1964) and total protein in skim milk was analysed by near infrared transmittance spectrophotometry. Blood for GSH measurement was treated with SDS/EDTA to a final concentration 0.19% (w/v)/2.25 mM, and trichloroacetic acid (TCA) to 7.5 % (w/w) before centrifugation (3270 g, 15 min, 4°C). Supernatants were stored at -85°C until analysis. Benzofurazan (7-fluoro-2,1,3-benzoxadiazole-4-sulphonate; Dojindo Laboratories, Kumamoto, Japan) derivatised blood sample supernatants were separated by HPLC and GSH detected by fluorescence (Lee *et al.*, 1993). Plasma was prepared by centrifugation of blood (3270 g, 15 min, 4°C). Cyst(e)ine was determined in untreated plasma after acid ninhydrin treatment using a method modified from Gaitonde (1967). Plasma was treated with SDS/EDTA (0.17% (w/v)/2.1 mM) and DTT (3.7 mM) with norleucine (70 mM) as internal standard. TCA (7.5% (w/w)) was added before centrifugation (3270 g, 15 min, 4°C). Supernatants were filtered through 0.45 µm membranes and stored at -85°C. AA in this prepared plasma were separated by HPLC after derivatisation using phenylisothiocyanate (modified from Bidlingmeyer *et al.*, 1984).

The extraction of AA by the gland were calculated as the difference in concentration between arterial (A) and venous (V) plasma expressed as a percentage of the concentration of A (extraction %). Analysis of variance and 5 % least significant differences (Genstat 5) were used to determine the significance of treatment and gland effects, and the occurance of interactions between these factors.

RESULTS

Acivicin administration did not affect total milk yield, but milk protein concentration decreased on both sides of the gland and protein yield decreased on the LHS (Table 1).

Protein concentration was significantly decreased by NAC, in milk taken from both sides of the gland while total milk yield increased on the RHS, but protein yield was unchanged. NAC combined with acivicin did not significantly affect milk protein concentration or milk yield. NAC treatment caused milk fat concentration to significantly decrease on the LHS but the decrease observed on the RHS was not significant. Fat yield and concentration were unaffected by acivicin or acivicin in combination with NAC.

In comparison with control treatments, acivicin administration increased histidine V concentrations and leucine A concentrations (Table 2). Acivicin did not alter histidine extraction %. Extraction % of phenylalanine by both sides of the gland, and leucine and tryptophan by the LHS, were decreased with acivicin treatment (Table 2).

NAC treatment increased A and V concentrations of AA, except methionine, phenylalanine and valine, which decreased only in V samples and histidine which was not affected (Table 2). Extraction % of AA decreased on both sides of the gland, with the exception of histidine, which

TABLE 1: Mean milk yield and composition from each side of the mammary gland of four lactating goats in the absence and presence of acivicin and/or N-acetylcysteine (NAC) compared with control.

	Control		Acivicin		NAC		NAC + Acivicin		LSD
	Left	Right	Left	Right	Left	Right	Left	Right	
Total yield (g/h)	50.4^{ab}	46.7^{a}	48.5^{a}	48.6^{a}	56.2^{b}	55.9^{b}	49.9^{ab}	48.1^{a}	6.54
Protein (g/100g)	2.98^{b}	3.05^{b}	2.56^{a}	2.68^{a}	2.70^{a}	2.72^{a}	2.90^{b}	2.94^{b}	0.168
Protein yield (g/h)	1.58^{c}	1.50^{bc}	1.28^{a}	1.33^{ab}	1.53^{c}	1.51^{bc}	1.48^{bc}	1.43^{abc}	0.194
Fat (g/100g)	4.77^{c}	4.47^{bc}	4.43^{bc}	4.45^{bc}	3.78^{a}	3.89^{ab}	4.93^{c}	4.86^{c}	0.641
Fat yield (g/h)	2.51^{a}	2.22^{a}	2.23^{a}	2.27^{a}	2.23^{a}	2.31^{a}	2.52^{a}	2.39^{a}	0.449

$^{a, b, c}$ Means in the same row without a common superscript differ (P<0.05)

Means are from 4 samples at different times per goat.

TABLE 2: Arterial and venous concentrations (mean µM ± SEM) of amino acids in plasma on each side of the mammary gland of four lactating goats in the absence and presence of acivicin or N-acetylcysteine (NAC) compared with control.

Amino Acid	Control			Acivicin			NAC		
	Arterial	Venous		Arterial	Venous		Arterial	Venous	
		Left	Right		Left	Right		Left	Right
Histidine	32 ± 2.7	26 ± 1.9	26 ± 2.0	36 ± 2.3	30 ± 1.9	30 ± 2.0	31 ± 3.9	27 ± 4.1	27 ± 4.0
Lysine	73 ± 6.0	42 ± 5.1	42 ± 5.1	67 ± 4.7	38 ± 4.6	36 ± 4.0	90 ± 10	62 ± 11	60 ± 11
Methionine	20 ± 2.2	9 ± 1.3	9 ± 1.4	21 ± 2.7	11 ± 1.9	11 ± 2.0	24 ± 2.1	15 ± 2.0	14 ± 2.0
Phenylalanine	46 ± 2.8	31 ± 1.9	30 ± 1.9	44 ± 2.1	32 ± 1.5	30 ± 1.6	51 ± 2.4	37 ± 2.5	37 ± 2.4
Threonine	53 ± 5.3	29 ± 3.7	30 ± 3.8	53 ± 4.5	32 ± 2.7	31 ± 3.0	68 ± 6.0	47 ± 6.0	45 ± 6.1
Tryptophan	27 ± 1.1	25 ± 0.8	25 ± 0.9	27 ± 1.9	25 ± 1.8	25 ± 1.9	32 ± 1.9	29 ± 1.2	29 ± 1.0
Isoleucine	79 ± 8.8	45 ± 5.0	45 ± 5.1	80 ± 6.5	49 ± 5.5	46 ± 4.9	96 ± 7.8	64 ± 6.1	63 ± 5.8
Leucine	103 ± 9.0	56 ± 5.3	55 ± 4.9	141 ± 19	83 ± 11	79 ± 12	127 ± 6.9	82 ± 5.2	79 ± 3.4
Valine	181 ± 21	126 ± 13	121 ± 9.8	182 ± 11	138 ± 8.1	141 ± 11	215 ± 16	164 ± 13	164 ± 12
Tyrosine	56 ± 3.7	43 ± 3.1	43 ± 3.1	61 ± 4.5	50 ± 5.0	49 ± 4.8	78 ± 8.6	67 ± 8.9	67 ± 8.6
Cyst(e)ine	142 ± 6.4	134 ± 7.6	136 ± 9.9	124 ± 2.9	119 ± 3.3	120 ± 2.4	108 ± 6.4	104 ± 5.0	100 ± 5.5

Means are from 3 samples at different times per goat.

TABLE 3: Mean extraction % (A-V difference as a % of A) of amino acids from plasma by each side of the mammary gland of four lactating goats for control, acivicin and/or N-acetylcysteine (NAC).

Amino Acid	Control		Acivicin		NAC		NAC + Acivicin		LSD
	Left	Right	Left	Right	Left	Right	Left	Right	
Histidine	19.2^{bcd}	18.8^{abc}	16.4^{ab}	17.7^{abc}	13.5^{a}	14.8^{ab}	22.2^{cd}	24.6^{d}	5.70
Lysine	42.8^{b}	43.6^{bc}	43.9^{bc}	48.0^{cd}	34.8^{a}	37.0^{a}	48.1^{cd}	50.1^{d}	5.08
Methionine	54.6^{bc}	56.3^{c}	47.2^{ab}	48.8^{abc}	42.5^{a}	43.1^{a}	46.5^{ab}	52.2^{bc}	8.11
Phenylalanine	33.8^{b}	34.1^{b}	27.6^{a}	30.9^{a}	27.7^{a}	28.1^{a}	30.3^{ab}	32.7^{a}	5.55
Threonine	45.5^{c}	45.5^{c}	39.0^{abc}	43.2^{bc}	34.0^{a}	36.3^{ab}	42.6^{bc}	45.1^{c}	7.67
Tryptophan	9.4^{b}	9.2^{ab}	5.1^{a}	6.0^{ab}	8.8^{ab}	9.2^{ab}	7.1^{ab}	7.9^{ab}	4.18
Isoleucine	41.8^{b}	42.9^{b}	40.0^{b}	43.4^{b}	33.2^{a}	33.3^{a}	40.8^{b}	43.6^{b}	4.53
Leucine	44.8^{c}	46.0^{c}	40.3^{b}	44.7^{c}	35.8^{a}	37.3^{ab}	44.9^{c}	48.2^{c}	3.93
Valine	27.5^{a}	29.9^{a}	24.2^{a}	22.5^{a}	23.6^{a}	23.3^{a}	22.8^{a}	23.30^{a}	8.98
Tyrosine	22.9^{b}	22.8^{b}	18.4^{ab}	20.8^{ab}	17.3^{a}	16.6^{a}	19.0^{ab}	20.5^{ab}	5.10
Cyst(e)ine	6.1^{a}	4.3^{a}	4.4^{a}	2.9^{a}	3.2^{a}	7.4^{a}	3.5^{a}	7.3^{a}	8.64

[a, b, c] Means in the same row without a common superscript differ ($P<0.05$)

Means are from 3 samples at different times per goat.

decreased on the LHS only, and valine and tryptophan, which were unaffected (Table 3). NAC in combination with acivicin, increased histidine extraction by the RHS of the gland and lysine on both sides of the gland (Table 3).

Cyst(e)ine concentrations of both A and V plasma were lower with NAC and acivicin compared with control treatment values (Table 2). Cyst(e)ine extraction % by the RHS of the gland appeared to decrease with acivicin and increase with NAC but these observations were not statistically significant. NAC in combination with acivicin did not affect cyst(e)ine extraction % (Table 3).

GSH was not affected by treatment but numerical increases were observed in A blood GSH concentration and extraction % (mean μm ± SEM; control LHS, -1.6 ± 3.9, RHS -4.6 ± 4.5; NAC LHS 3.0 ± 2.5, RHS 0.8 ± 1.0) with NAC treatment. Since the control treatment values were negative this suggests a trend towards decreased GSH release.

DISCUSSION

Previous reports of AA concentrations in A plasma (Henderson & Peaker, 1983; Bequette *et al.*, 1997) and A-V differences (Ranawana & Kellaway, 1977; Backwell *et al.*, 1996) are similiar to those for goats in this experiment. There were no reported values for tryptophan concentration in plasma from goats but the degree of extraction shown here (LHS 9.4 %, RHS 9.2 %) was not dissimilar to that for cows (4.3 %; Pacheco-Rios *et al.*, 1998).

γ-GT has been shown to have affinity for most AA but not tyrosine (Thompson & Meister, 1977; Morita *et al.*, 1994). In dispersed cell cultures, the γ-GT inhibitor acivicin decreased milk protein synthesis (S.L Johnston, unpublished data). Administration of acivicin to the RHS of the gland was expected to decrease extraction of AA except tyrosine by this side, but in fact caused a significant decrease in extraction % of leucine, phenylalanine, and tryptophan by the LHS. That the side of the gland other than the treated side was affected indicates a systemic effect of acivicin absorbed through the treated udder-half. The injection of saline up the teat canal without effect indicates that the administration technique itself was not inducing an effect. It is difficult to explain why the treated side was not affected to the same extent, however, acivicin significantly decreased protein yield in milk from the LHS of the gland with a trend to decreased protein yield on the RHS. Increased number of goats in this study may have produced a significant acivicin affect.

In this experiment cyst(e)ine concentrations in A plasma were almost 2-fold higher than those previously reported by Lee *et al.* (1999). γ-GT has a high affinity for both cystine and cysteine so a decrease in plasma extraction was expected with acivicin administration. Although reduced cyst(e)ine extraction was observed on both sides of the gland with acivicin administration, this was not significant, thus, it has not been possible to confirm the decrease in cystine uptake caused by acivicin that has been shown previously in cultured cells (Cotgreave & Schuppe-Koistinen, 1994; Sweiry *et al.*, 1995).

Increased blood flow may increase substrate supply. The blood flow probes failed in two animals but data collected from the other two throughout the experiment indicate that acivicin and NAC treatments did not affect mammary blood flow.

Inhibition of γ-GT has been shown to decrease GSH removal from the cell because of inhibition of a carrier or inhibition of intracellular γ-GT (Griffith & Meister, 1979). Treatment with acivicin in this experiment did not affect GSH release, which may be due to the inability of acivicin to penetrate the cell membrane, suggesting that decreased intracellular GSH is from γ-GT inside the cell.

While NAC was infused only to the RHS of the gland, AA on both sides of the gland were affected, indicating a whole-body effect. The increase in milk yield with NAC treatment was probably because of increased water in milk and may result from increased lactose production, which was not measured in this study. There was no decrease in protein yield associated with decreased AA extraction with NAC, which may indicate that the decrease in AA uptake was insufficient to affect protein synthesis. Despite reduced uptake of AA, in the presence of NAC, probably through the increased supply of cyst(e)ine, protein synthesis continues as normal. Intracellular pools (Clark *et al.*, 1980) including recycled AA from milk protein degradation inside the gland (Oddy *et al.*, 1988) are a likely source of other AA.

The decrease in extraction of AA that resulted with NAC treatment is difficult to explain. It is possible that when the cell is replete with cysteine, derived from NAC in this experiment, a signal is produced that acts to down-regulate uptake of other AA by conventional transport systems. NAC alone decreased extraction of histidine and lysine, and acivicin had no effect, whereas NAC infusion in

combination with acivicin increased uptake of these AA, presumably via conventional systems. This indicates that the NAC-derived signal for down-regulation of AA uptake was through the γ-glutamyl cycle. This is in contrast to studies that have shown the γ-glutamyl cycle up-regulates AA uptake through the intermediate, 5-oxoproline (Viña *et al.*, 1989).

NAC increased total milk yield but acivicin administration reversed this effect. Similarly, the decrease in protein yield with acivicin was prevented with NAC and suggests acivicin decreased cyst(e)ine uptake while NAC increased intracellular concentration allowing protein synthesis to continue. This indicates that γ-GT has an important regulatory role in the supply of cyst(e)ine for milk protein synthesis. Further studies may allow modification of γ-GT activity to increase milk protein yield.

ACKNOWLEDGEMENTS

The authors thank Dr Gordon Reynolds for surgical preparations, Dr Warren McNabb for assistance, Dr David Pacheco Rios for statistical advice; and Bruce Sinclair, Bryan Treloar and Dean Corson for analytical assistance. This research was supported by the Foundation for Science, Research and Technology.

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