

Effects of peroral alanine administration in lactating ewes with decreased availability of glucose

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(Received 17 February 1997 – Revised 18 April 1997 – Accepted 29 April 1997)

The metabolic effects of a phlorizin-induced drainage of glucose were studied in six lactating ewes with or without peroral alanine drenches in a study of crossover design. Phlorizin gave rise to a small, but significant, elevation of plasma β -hydroxybutyrate. The plasma level of alanine decreased by about 30 % due to the phlorizin injections and alanine was negatively correlated to β -hydroxybutyrate. The plasma level of free fatty acids increased due to phlorizin. Plasma insulin and glucose concentrations were not significantly affected by phlorizin while glucagon level showed a small but significant increase. Peroral alanine drenches to phlorizin-treated ewes gave rise to a transitory elevation of alanine in plasma. The plasma level of free fatty acids was about 40 % lower in phlorizin-treated ewes receiving alanine and β -hydroxybutyrate tended to be lower ($P < 0.08$). We suggest that β -hydroxybutyrate, apart from its function as an oxidative fuel, might play an important role by limiting glucose oxidation and protein degradation in skeletal muscles during periods of negative energy balance in ruminants. Furthermore, it is suggested that alanine supplementation decreases lipolysis and ketogenesis in lactating ewes.

Alanine: Pancrease: β -Hydroxybutyrate

Ruminants are largely dependent on hepatic gluconeogenesis for their glucose supply. Lactating ruminants have a major demand placed on them to provide enough glucose to meet glucose requirements for both milk synthesis and other organs and tissues of the body. Studies with lactating sheep have shown that the glucose requirements of the mammary gland account for about 60 % of the glucose used by the animal (Bergman & Hogue, 1967). The principal substrate for glucose synthesis in the fed ruminant is propionate coming from the rumen microbial degradation of feed carbohydrates. During periods of glucose deficit skeletal muscle protein utilization, in order to provide amino acids for hepatic gluconeogenesis, becomes an important event (Heitmann & Bergman, 1980). However, during long periods of glucose deficit sustained degradation of muscle protein would deplete the body reserves, which is hazardous for the animal. Thus, body protein as well as glucose must be efficiently spared. Mechanisms for adaptation to prolonged catabolic states have evolved in several species (for a review, see Thompson & Wu, 1991). During such periods oxidation of free fatty acids (FFA), mobilized from the body reserves, increases and ketone bodies, β -hydroxybutyrate (BHBA) and acetoacetate, are formed. These compounds can readily be used as oxidative fuels in many tissues (for a review, see Robinson & Williamson, 1980). Ketone bodies not only contribute as oxidative fuels, they also act as metabolic signals. This is partly achieved by their effects on amino acid metabolism. Alanine and glutamine are quantitatively the most important amino acids released from the skeletal muscle (Felig, 1975; Reynolds & Huntington, 1988). Intravenous infusion of BHBA reduces the circulating concentration of alanine in man, sheep and dogs (Sherwin *et al.* 1975; Radcliffe *et*

al. 1983; Umpleby *et al.* 1988). The reduced alanine concentration has been attributed to an inhibitory effect of ketone bodies on alanine production in skeletal muscle and therefore its subsequent release to the circulation (Umpleby *et al.* 1988; Wu & Thompson, 1988). On the other hand an anti-ketogenic action of alanine has been observed in man, rats and sheep (Weik & Zander, 1975; Ozand *et al.* 1977; Koeslag *et al.* 1985). Alanine has also been shown to stimulate the utilization of ketone bodies in rats (Reed *et al.* 1984). Alanine might also affect the energy metabolism indirectly in sheep due to its stimulatory effects on the release of pancreatic hormones (Kuhara *et al.* 1991).

Phlorizin has been used to study the metabolic effects of decreased glucose availability in lactating dairy cows (Amaral-Phillips *et al.* 1993). Phlorizin causes glucosuria by preventing the reabsorption of glucose in the renal tubules (Horsburgh *et al.* 1978). The purposes of the trial reported here were to investigate how lactating ewes adjust to a glucose drain caused by phlorizin, by measuring key plasma metabolites, insulin and glucagon, and to study if peroral loads of alanine could escape rumen metabolism and affect the intermediary metabolism of ewes with decreased availability of glucose.

MATERIALS AND METHODS

Animals

Six ewes of the Swedish domestic breed (body weight ranging from 65 to 75 kg) in weeks 3–6 of lactation nursing two or three lambs each were used. They were kept indoors together with their lambs. The ewes were given 0.6 kg DM hay and 1–1.5 kg DM concentrate, depending on the number of lambs they nursed, twice daily at 08.30 hours and 15.00 hours. The hay contained 9.1 MJ metabolizable energy (ME) and 64 g digestible crude protein (DCP)/kg DM. The concentrate contained 13.1 MJ ME and 96 g DCP/kg DM. The diet was formulated to supply 100% and 95% of the DCP and ME requirements respectively according to the Swedish recommendations for lactating sheep (Spörndly, 1995). The ewes always consumed the feed so there were no significant feed refusals in the morning before the feeding. Water and salt were available *ad libitum*.

Experimental procedures

The six ewes were randomly divided into two groups and received two different treatments in a two-period crossover study. Each period included an initial 12 h period to establish baseline measurements in which blood was collected from the jugular vein at 08.00, 14.00 and 20.00 hours. Both groups were then subjected to phlorizin injections subcutaneously in the scapular region (5 mg/kg body weight; Sigma Chemical Co., St Louis, MO, USA). The phlorizin was dissolved in 1 ml propylene glycol. This treatment was repeated every 12 h, (at 08.00 and 20.00 hours) for 60 h. In each period one group of ewes also received peroral drenches with 400 mmol L-alanine (Rexim/Degussa, Courbevoie, France) dissolved in 300 ml water immediately after each phlorizin injection. Thus, in each period one group only received phlorizin injections (phlo) and the other group received phlorizin injections followed by alanine drenches (phlo + ala). There was an interval of 1 week between the two periods. Blood samples were collected every second hour (08.00–20.00 hours) immediately following the last phlorizin injection in both phlo and phlo + ala groups. All blood samples were kept on ice until plasma was obtained by centrifugation within 30 min after sampling. The plasma was then divided into portions and frozen at -20° until analysed. Urine was collected from the animals when they urinated spontaneously during baseline measurements and when they were treated with phlo and phlo + ala.

Analyses

All analyses except the glucagon analysis were made on heparinized plasma. Plasma for glucagon contained K₃-EDTA with 500 kalikrein units of aprotinin (Trasylol, Bayer, Leverkusen, Germany) per ml.

The alanine concentration was determined in deproteinized plasma according to the method of Williamson (1985). BHBA was determined using an enzymic technique (310-A, Sigma Chemical Co.). Glucose was analysed enzymically using a Kodak DT 60 II analyser (Kodak, Rochester, NY, USA). Insulin and glucagon in plasma were determined by radioimmunoassay using commercial assay kits (Pharmacia RIA 100, Pharmacia Diagnostics AB, Uppsala, Sweden and GL-32K, Linco Research, Inc., St Louis, MO, USA respectively). Intra-assay and inter-assay CV were 9.1% and 8.1% for insulin respectively. Intra-assay CV for glucagon was 5.7% while the inter-assay coefficient was not determined.

Glucose in urine was determined qualitatively (Labstix, Bayer Diagnostica, Gothenburg, Sweden). The lowest detection limit was 5.5 mmol/l. FFA in plasma were analysed using an enzymic technique (Boehringer Mannheim GmbH, Mannheim, Germany).

Statistical analysis

Data were analysed by ANOVA using the general linear model procedure of Minitab Statistical Software (Release 9, Statistical Software, Birmingham, West Midlands). The differences between means of the three measurements during the day before treatment began were tested *v.* means of three measurements, at equal times, during the third day of treatment. The statistical model included ewe, day, period and treatment. The significance of the differences between phlo and phlo + ala groups on the third day of treatment were also assessed based on means of the seven measurements during that day. The model included ewe, period and treatment. Simple regression analyses were performed using Minitab Statistical Software.

RESULTS

In Table 1 baseline levels and levels on the third day of the two treatments respectively of FFA, BHBA, alanine, insulin, glucagon and glucose are shown.

Treatment with phlorizin was associated with increases in the plasma levels of FFA and BHBA in both phlo- and phlo + ala-treated sheep (Table 1). On the other hand, the mean plasma level of alanine showed a striking decrease in both phlo- and phlo + ala-treated animals (Table 1). The mean plasma levels of glucose and insulin were not significantly affected on the third day while plasma glucagon level increased slightly (Table 1).

In Fig. 1 the baseline concentrations of alanine, BHBA, FFA, glucose, insulin and glucagon and the concentrations on the third day of treatment are shown. The peroral alanine supplementation gave rise to a peak in plasma alanine concentration 2 h post-supplementation followed by a decline to the pre-supplementation level after 6 h. When phlorizin was given alone there was no alanine peak. Thus, mainly due to the initial peak, the alanine level on the third day of treatment was higher in phlo + ala sheep than in phlo sheep (Fig. 1 and Table 2). The plasma glucose level was slightly higher in those phlorizin-treated sheep that received alanine than in those that only received phlorizin (Fig. 1 and Table 2). At 2 h after both phlo and phlo + ala treatments the BHBA plasma concentration reached a peak (Fig. 1). The average BHBA level tended ($P < 0.08$) to be higher in the

Table 1. Plasma levels of free fatty acids (FFA), β -hydroxybutyrate (BHBA), alanine, insulin, glucagon and glucose in ewes before the treatment and on the third day of treatment with phlorizin (phlo) or phlo + alanine (phlo + ala)*

(Least square means with their standard errors for six ewes)

	Day before treatment		Day 3 of treatment		SE	Effect of day: P =
	Phlo	Phlo + ala	Phlo	Phlo + ala		
FFA (mmol/l)	0.14	0.12	0.27	0.18	0.01	0.009
BHBA (mmol/l)	0.60	0.56	0.82	0.75	0.01	< 0.001
Alanine (mmol/l)	0.202	0.195	0.136	0.150	0.003	< 0.001
Insulin (pg/ml)	875	918	777	976	40	0.89
Glucagon (pg/ml)	85.7	83.4	73.1	80.5	1.2	0.023
Glucose (mmol/l)	4.2	4.3	4.1	4.2	0.04	0.30

* Each value represents the mean of the samplings at 08.00, 14.00 and 20.00 hours. For details of procedures, see pp. 806–807.

sheep which received only phlorizin (Table 2). The plasma level of FFA peaked 2 h after the phlorizin treatment in both phlo + ala sheep and phlo sheep but the average level was about 50 % higher in phlo sheep (Fig. 1 and Table 2). The insulin level declined after the phlorizin injection in both phlo and phlo + ala sheep and reached its lowest level at 12.00 hours (Fig. 1). The average plasma insulin level was higher in the phlo + ala sheep than in the phlo sheep on the third day of treatment (Table 2). Plasma glucagon showed a peak 2 h after the phlo + ala treatment while there was a lack of glucagon response when the sheep received only phlorizin (Fig. 1). There was no significant difference in the average glucagon concentration on the third day of treatment between phlo and phlo + ala treated sheep.

In Fig. 2 the relationship between the plasma concentrations of alanine and BHBA are shown for the phlo sheep. The negative correlation was significant ($P < 0.05$) for two of the six sheep. When the data from the six animals were put together the correlation was significant ($P < 0.001$).

The semi-quantitative registrations of glucose in urine indicated that the concentration ranged from 30–60 mmol/l during phlo and phlo + ala treatments. No glucose in urine could be detected during the baseline measurements.

Table 2. Plasma levels of free fatty acids (FFA), β -hydroxybutyrate (BHBA), alanine, insulin, glucagon and glucose in ewes treated with phlorizin (phlo) or phlo + alanine (phlo + ala) 60–72 h after the initial treatment (day 3 of treatment)*

(Least square means with their standard errors for six ewes)

	Phlo	Phlo + ala	SE	Effect of treatment: P =
FFA (mmol/l)	0.29	0.18	0.01	< 0.001
BHBA (mmol/l)	0.89	0.81	0.01	0.08
Alanine (mmol/l)	0.144	0.235	0.026	0.02
Insulin (pg/ml)	704	917	19	0.002
Glucagon (pg/ml)	76.5	88.7	2.4	0.14
Glucose (mmol/l)	4.1	4.2	0.01	< 0.001

* Each value represents the mean of samplings every second hour from 08.00 to 20.00 hours. For details of procedures, see pp. 806–807.

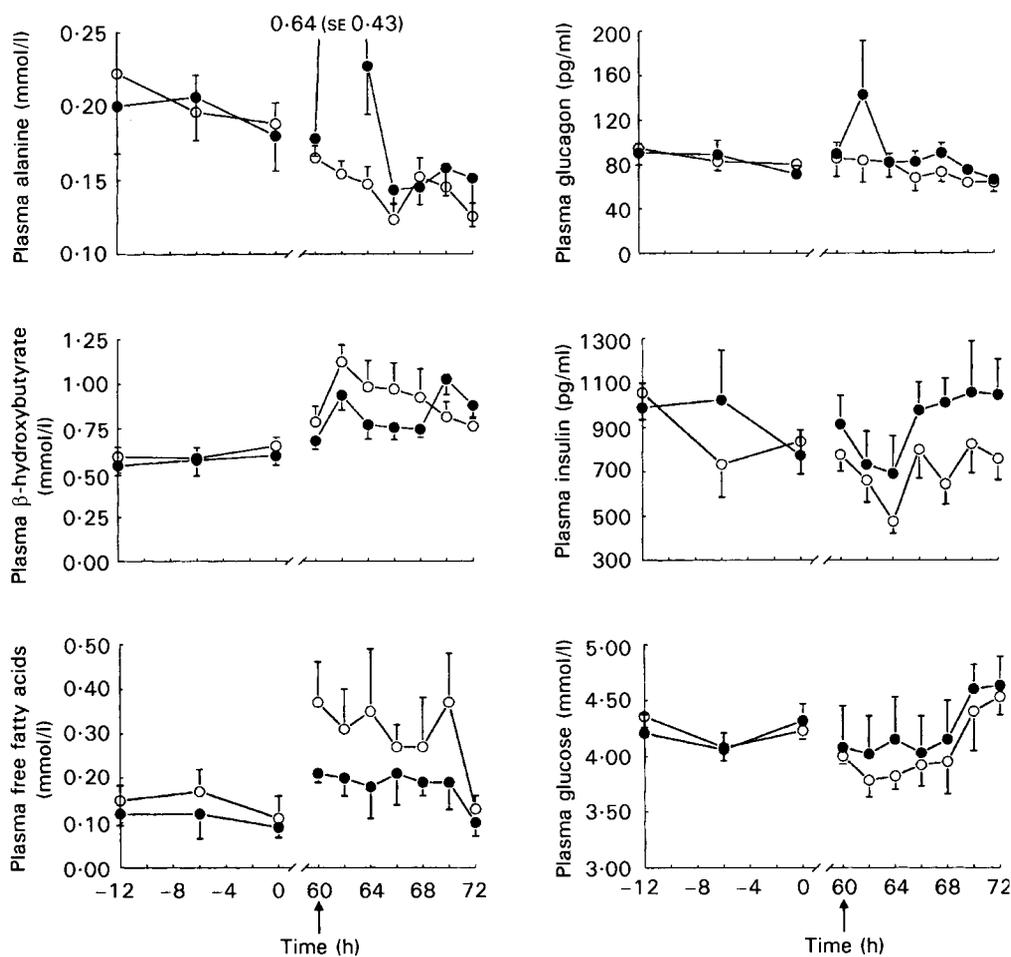


Fig. 1. Plasma levels of alanine, β -hydroxybutyrate, free fatty acids, glucagon, insulin and glucose before and during the third day of phlorizin treatment. (○), Ewes treated only with phlorizin, (●), ewes treated with phlorizin and peroral drenches with alanine. (\uparrow), Time of phlorizin injection or phlorizin injection + alanine drenching in the two treatments respectively. All values are means with their standard errors for six animals.

DISCUSSION

Phlorizin treatment

One aim of the present study was to investigate how lactating ewes responded to a glucose drain. The phlorizin-induced glucose drain across the kidneys was not quantitatively determined in this study. Assuming a daily urine production of 3 litres with observed glucose levels in spot samples ranging from 30 to 60 mmol/l, the glucose loss would be 20–30 g/d. If the sheep produced 2 litres milk/d with 37 g lactose/l and assuming that 70% of the glucose taken up by the mammary gland was used for lactose synthesis, the glucose needed for lactation can be estimated to be about 100 g/d. The requirement for maintenance is assumed to be about 80 g/d based on studies of irreversible glucose loss in wethers (Egan *et al.* 1983). Thus, the phlorizin-induced glucose loss can be estimated to increase the glucose requirement by about 15%.

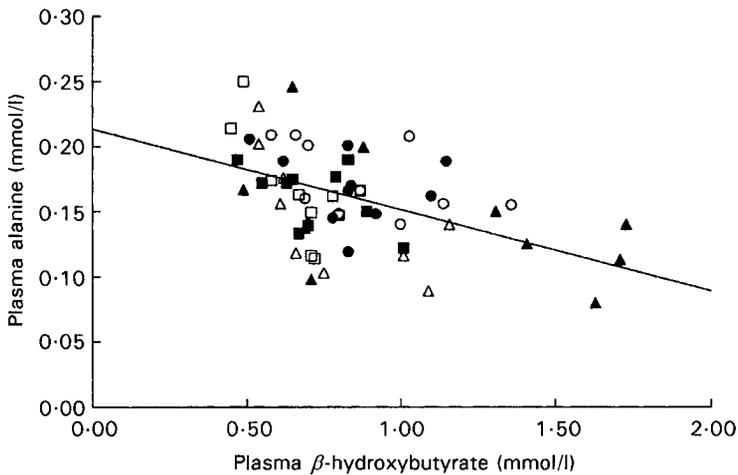


Fig 2. The plasma concentration of alanine v. the concentration of β -hydroxybutyrate in ewes treated with phlorizin. Each symbol represents one ewe. There are ten observations for each ewe which includes values from three baseline observations and seven observations during the third day of treatment. The line represents the regression based on the observations from all six ewes.

Phlorizin did not cause a significant decrease in the plasma level of glucose. Egan *et al.* (1983) found that the level of glucose decreased by about 10% in wether sheep due to an intravenous infusion with a slightly lower dose of phlorizin than we used. Those non-producing animals had lower glucose requirements; thus they lost a larger proportion of glucose, about 30% of the requirement, which might explain the different results. The plasma level of FFA increased due to the phlorizin-induced glucose loss indicating an elevated rate of lipolysis. This finding agrees with previous studies on lactating sheep and dairy cows (Zander, 1976; Amaral-Phillips *et al.* 1993). It is reasonable to assume that the observed elevated level of BHBA was induced mainly by an increased amount of circulating FFA which could be hepatically converted to BHBA. On the other hand, phlorizin caused a significant drop in the plasma concentration of alanine. Since the feed consumption was not affected by the treatments it might be assumed that the same amount of alanine was absorbed from the gut during phlorizin treatment as it was before the treatment. Thus the phlorizin-induced decrease in plasma alanine could result from a decrease in muscle outflow of alanine, an increase in hepatic uptake, or both. We are not aware of any previously published studies showing any reducing effects of phlorizin on plasma alanine. However, it has previously been shown that intravenous infusion with BHBA decreases the plasma level of alanine concomitant with a drop in plasma glucose in man, sheep and dogs (Sherwin *et al.* 1975; Radcliffe *et al.* 1983; Umpleby *et al.* 1988). Furthermore, alanine was the only one of the fifteen amino acids examined which showed a significantly decreased plasma level in ketotic cows (Chi *et al.* 1973). It has been proposed that BHBA acts in the skeletal muscle by inhibiting glycolysis, resulting in reduced pyruvate production, which in turn reduces transamination of other amino acids to alanine. BHBA may therefore indirectly control hepatic gluconeogenesis and glucose output by limiting the availability of the gluconeogenic substrate alanine (Thompson & Wu, 1991; Umpleby *et al.* 1995). In the present study there was a negative correlation between BHBA and alanine in the sheep treated with phlorizin. We have recently observed a similar pattern with negative correlation between alanine and endogenous BHBA in high-yielding dairy

cows in their second week of lactation (M. E. Åkerlind, M. Emanuelson and K. J. Holtenius, unpublished results). BHBA might, apart from its function as an oxidative fuel, play an important role by limiting glucose oxidation and protein degradation in skeletal muscles during periods of negative energy balance also in ruminants.

The pancreatic hormones insulin and glucagon both decrease the plasma concentration of alanine in sheep (Brockman & Bergman, 1975; Brockman *et al.* 1975). In the present study insulin was not affected while glucagon showed a small but significant decrease due to the phlorizin treatment. Thus, the drop in alanine concentration could not be explained by elevated plasma insulin or glucagon levels. The phlorizin-induced decrease in glucagon was unexpected. Amaral-Phillips *et al.* (1993) reported a small increase in plasma glucagon level in lactating dairy cows given similar phlorizin doses (based on calculated metabolic body weights). In contrast to the sheep in the present study, those cows responded with a drop in the plasma concentration of glucose and insulin which would facilitate glucagon secretion (see Hedge *et al.* 1987).

Alanine supplementation to phlorizin-treated sheep

The plasma concentration of alanine increased due to the peroral alanine load, which indicates that a fraction of the alanine escaped metabolism in the rumen. However, probably mainly due to rapid metabolism, the plasma concentration had returned to the pre-supplementation level within about 6 h. It was shown previously that the plasma level of BHBA decreased in phlorizin-treated sheep after an intravenous infusion with 1 g alanine/kg body weight (Weik & Zander, 1975). Alanine is thought to inhibit ketogenesis by entering the tricarboxylic acid cycle as oxaloacetate to increase oxidation of acetyl CoA (Zammit, 1981). In the present study we observed that alanine supplementation tended to decrease the plasma concentration of BHBA. It is possible that the stronger response in the study of Weik & Zander (1975) was caused by the much higher dose of alanine given. Heifers that received a mesenteric vein infusion with alanine responded with a significant depression of hepatic ketogenesis but with no alteration in the plasma concentration of BHBA (Reynolds & Tyrrell, 1991). Thus it can not be excluded that alanine had an inhibiting effect on liver ketogenesis also in the present study, but this was not mirrored by a significantly decreased plasma level of BHBA.

It was shown in *in vitro* studies that alanine had an inhibiting effect on the FFA release from rat adipocytes and it was suggested that alanine also plays an important role *in vivo* as a regulator of peripheral FFA mobilization (Giudicelli *et al.* 1976). Furthermore, both intravenously and orally administered alanine decreased the plasma level of FFA in man (Rossini *et al.* 1975; Funovics *et al.* 1981). These results might explain our observation that the average plasma concentration of FFA was 40% lower in ewes supplemented with alanine than in those not supplemented with alanine. Thus it appears as if the lipolysis induced by phlorizin was partially blocked by alanine. This effect remained after alanine had returned to the same level as before the alanine load.

Extensive mobilization of body reserves of fat and the release of FFA into the bloodstream is a prerequisite for many production diseases including ketosis (Breukink & Wensing, 1992). However, most therapies for ruminant ketosis induce supra-physiological plasma levels of glucose and/or insulin (Herdt & Emery, 1992). It is possible that oral loads of alanine could be one way of decreasing the production of ketone bodies without inducing high levels of glucose and insulin.

In summary, phlorizin-induced glucose drainage caused an increase in the plasma concentrations of FFA and BHBA and a concomitant decrease in the alanine concentration.

A peroral alanine drench led to a substantial, but short-lasting, rise in the concentration of alanine in plasma. The alanine administration decreased FFA and also tended to decrease BHBA in the plasma and induced a small, but significant, rise in the plasma levels of glucose and insulin. It is suggested that alanine supplementation might be used in order to reduce the ketone body production in ketotic ruminants.

The authors are grateful to Mrs Anna Stepinska for excellent technical assistance. This work was supported by Nordiska Djurskyddsforeningen.

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