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Influence of Dietary Salinomycin on Feeding-induced Variations of Glucose Kinetics and Blood Volatile Fatty Acids and Insulin Concentrations in Sheep Fed a High-roughage Diet

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ABSTRACT : This study was conducted to determine effects of salinomycin (SL) on feeding-induced changes in glucose kinetics and blood VFA concentrations in sheep fed a high-roughage diet. Four sheep were fed the diet with or without 20 mg/kg diet of SL once daily for 21 d. Glucose entry and utilization rates were determined during the prefeeding and 3 h postfeeding periods, using a [$^{13}C_6$]glucose dilution method and non-steady state equations. Ruminal characteristics and concentrations of blood VFA, plasma glucose and insulin were also measured during the same periods. A feeding-induced increase in ruminal total VFA concentration tended to be inhibited ($p<0.10$) with SL, although ruminal pH was unaffected ($p>0.10$) with SL or by feeding. Salinomycin decreased ($p<0.05$) acetate proportion and increased ($p<0.05$) propionate proportion in the rumen, but did not modify these changes in response to feeding ($p>0.10$). A feeding-induced increase in blood acetate concentration was attenuated ($p<0.05$) with SL. Salinomycin tended to increase ($p<0.10$) blood propionate concentration without modifying its response patterns to feeding ($p>0.10$). Plasma concentrations of glucose or insulin were unaffected ($p>0.10$) with SL. Salinomycin tended to enhance ($p<0.10$) glucose entry and utilization rates. Feeding also enhanced ($p<0.01$) both rates, whereas their interactive effect was not detected ($p>0.10$). We conclude that SL possibly enhances whole body glucose entry and utilization with an increase in blood propionate concentration in sheep given a high-roughage diet, although SL does not appear to affect their responses to feeding. (**Key Words :** Feeding, Glucose Metabolism, Insulin, Salinomycin, Sheep, Volatile Fatty Acid)

INTRODUCTION

Salinomycin (SL), an ionophore antibiotic, improves feed efficiency and enhances ruminal propionate molar proportion and/or concentration in ruminants fed a high-roughage diet (Bagley et al., 1988; Reffett-Stabel et al., 1989; Terashima et al., 1990). These typical effects of SL on ruminal characteristics suggest that dietary SL would enhance whole body glucose kinetics in ruminants, because propionate is the most important precursor for gluconeogenesis and a stimulus for insulin secretion (Bergman, 1990), and glucose production originating from propionate is poorly suppressed with insulin (Brockman, 1990). Some workers have reported that monensin supplementation increased glucose kinetics in cattle fed a high-roughage diet frequently (Van Maanen et al., 1978; Arieli et al., 2001).

Feeding, once or twice daily, changes plasma glucose and insulin concentrations (Armentano et al., 1984; Sano et al., 1994) and blood concentrations of VFA (Sano et al., 1994) in ruminants. These results suggest that feeding may change glucose kinetics in ruminants given such a feeding regimen. However, previous results are inconsistent showing that glucose kinetics were enhanced (Armentano et al., 1984) or remained unchanged (Van der Walt, 1978; Sano et al., 1999) with time after feeding. Furthermore, SL supplementation may also affect glucose kinetics during the early period after feeding in ruminants, because Terashima et al. (1990) have shown an enhancement in plasma insulin concentration during the 6 h postfeeding period by SL supplementation in steers fed a high-roughage diet.

The present study was designed to investigate the effects of SL supplementation on responses of whole body glucose kinetics and concentrations of blood VFA and plasma insulin to feeding in sheep offered a high-roughage diet once daily.

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MATERIALS AND METHODS

Animals and management

Four adult Corriedale wethers (2 yr, initial average BW 48.9±4.9 kg) were surgically prepared under anesthesia with a skin loop enclosing the left carotid artery. Two of the sheep also had an established rumen fistula. Animals were kept individually in metabolic cages at room temperature. A basal diet consisted of 800 g/kg of orchardgrass hay (moisture 7.6, CP 16.9, ether extract 2.1, NDF 40.7, crude ash 9.0, nitrogen-free extract 38.8%, ME 8.03 MJ/kg) and 200 g/kg of a commercial concentrate (Blue, Showa Sangyo Co., Japan; moisture 11.2, CP 16.3, ether extract 2.8, NDF 32.5, crude ash 6.4, nitrogen-free extract 57.2%, ME 10.17 MJ/kg). The diet was supplemented with or without 20 mg/kg diet of SL mixed into the concentrate, and fed to the sheep once daily at 16:00 at the amount corresponding to 1.3 times ME for maintenance (National Research Council, 1985). Animals had free access to water and mineral blocks. Two sheep (one with and the other without the rumen fistula) were fed the diet with SL and the others two the diet without SL for 21 d. The treatments were then switched in each animal and continued for 21 d. The switch of treatment allowed all four sheep to be subjected to both of the treatments with and without SL. Glucose clamp experiments were also conducted during 18 to 21 d of each treatment as reported earlier (Fujita et al., 2000). Surgery, management, and blood sampling were carried out according to the guidelines established by the Animal Care Committee of Iwate University.

Blood metabolites and insulin, and ruminal characteristics

The responses of concentrations of blood VFA, plasma glucose and insulin to feeding were examined on day 14 of each treatment. A catheter for blood sampling was inserted into the skin loop of the carotid artery 2 h before the experiments, and then flushed and filled with a sterile solution of trisodium citrate (38 g/L). Blood samples (5 ml) for glucose and insulin determinations were obtained from the arterial catheter immediately before (0 min) and at 5, 10, 15, 30, 45, 60, 90, 120 and 180 min after feeding. Additional blood samples (5 ml) were taken for VFA determination at 0, 30, 60, 90, 120 and 180 min after feeding. The blood samples were heparinized immediately and stored in crushed ice until centrifugation. Plasma was separated from blood by centrifuging at 8,000 g for 10 min at 4°C, and stored at -20°C until analyzed for plasma glucose and insulin concentrations. The blood samples (5 ml) for VFA determination were deproteinized by adding 5 mL of sodium tungstate solution (100 g/L) and 5 ml of 0.34 M H_2SO_4, and then the supernatants obtained were stored at -20°C until the analysis.

In the two animals with the rumen fistula, rumen fluids (50 ml) were collected via the rumen fistula immediately after blood sampling at 0, 30, 60, 90, 120 and 180 min after feeding. The pH of the samples was immediately measured with a pH meter (HM-10P, Toa Electronics Ltd., Japan) and then stored at 4 °C after adding a few drops of saturated $HgCl_2$ solution. The samples were centrifuged at 700 g for 15 min at 4°C, and then the supernatants obtained were stored at -20°C until VFA analysis.

Glucose kinetics

On day 16 of each treatment, the response of whole body glucose kinetics to feeding was examined in a stable isotope dilution experiment. A catheter for infusion was inserted into a jugular vein a day prior to the experiment, and another for blood sampling was inserted into the skin loop of the carotid artery 2 h before the experiment. The catheters were flushed and filled with a sterile solution of trisodium citrate (38 g/L). [$^{13}C_6$]Glucose (D-glucose-$^{13}C_6$, 99 atm% ^{13}C, Isotec Inc., USA) dissolved in sterile saline (9 g/L sodium chloride solution) was injected as a priming dose of 300 μg/kg BW over 1 min via the jugular catheter 5 h before feeding, and then it was continuously infused at a rate of 3 μg/kg BW/min over 8 h via the same catheter using a peristaltic pump (AC-2120, Atto Co. Ltd., Japan). Blood samples (5 ml) were obtained from the arterial catheter before infusion (a background sample) and at 30-min intervals from 3 through 8 h after the initiation of infusion (from 2 h before through 3 h after feeding). The samples were heparinized immediately and stored in crushed ice until centrifugation. Animals were fed immediately after the blood sampling at 5 h after the initiation of infusion. Plasma was separated from blood by centrifuging at 8,000 g for 10 min at 4°C, and stored at -20°C until analysis of [$^{13}C_6$]glucose enrichment.

Chemical and isotope analysis

Plasma glucose concentration was determined by a glucose oxidase method (Huggett and Nixon, 1957). Plasma insulin concentration was assayed with a kit based on a double antibody radioimmunoassay method (IRI 'Eiken', Eiken Chemical, Japan). Blood concentrations of VFA were determined by GLC (Model 5890, Hewlett-Packard Co., USA) after extraction from the supernatants, which were obtained from the deproteinization described above, by steam distillation (Sano et al., 1989). The total VFA concentration in rumen fluid was determined in a steam distillate by titrating with 0.1 M NaOH. The titrated distillate was dried, and then the molar percentages of individual VFA were measured by GLC.

Plasma [$^{13}C_6$]glucose enrichment (mol% excess) was measured by the method of Tserng and Kalhan (1983) with the following modifications. Plasma (0.5 ml) was

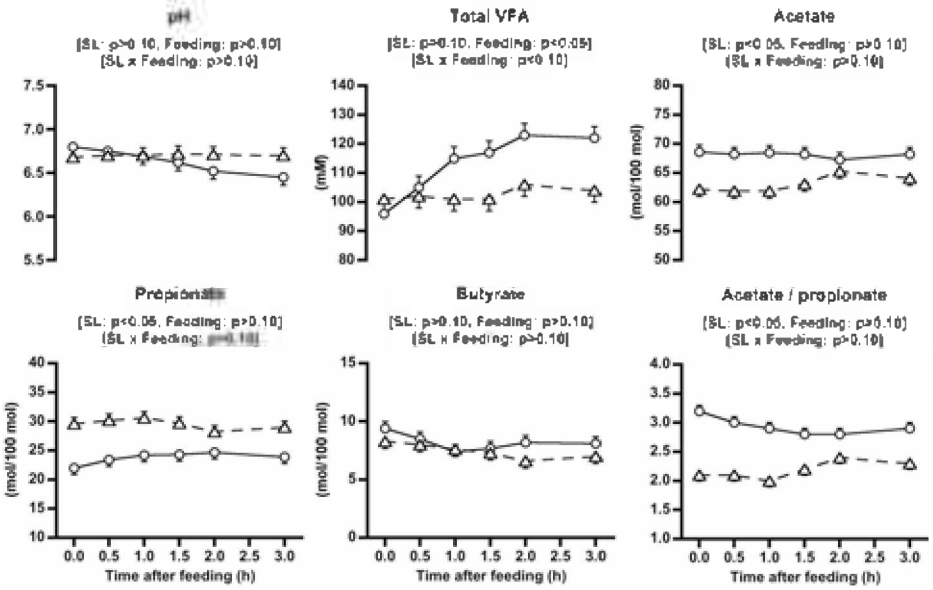

Figure 1. Effects of salinomycin (SL) on changes in ruminal characteristics with time after feeding in sheep fed a high-roughage diet once daily. ○: without SL, △: with 20 mg/kg diet of SL. Data represent means and SEM of two animals. The p-values of main effects of SL, the time after feeding (Feeding) and an interaction between SL and the time after feeding (SL×feeding) are shown in brackets.

deproteinized by mixing with 1.5 ml of sulfosalicylic acid solution (30 g/L). After centrifugation, the supernatant was applied to a tandem column consisting of 0.5 ml cation exchange resin (Dowex 50 W×8, hydrogen form) and 1 ml anion exchange resin (Dowex 1×8, acetate form), and then the column was washed with distilled water (4×1 ml). The resulting eluent was placed in a glass screw-capped vial and evaporated to dryness at 100°C under a stream of dry air. The glucose in the dried residue was derivatized to its aldononitrilpentacetate derivative as described by Tserng and Kalhan (1983), and then its enrichment of [$^{13}C_6$]glucose was determined by an electron impact ionization-selective ion monitoring method with a gas chromatograph-mass spectrometer (G-3000 gas chromatograph - M-2000 mass spectrometer, Hitachi Ltd., Japan). Ions m/z 328 (natural glucose) and 334 ([$^{13}C_6$]glucose) were monitored, and peak area abundance was calculated as (m/z 334)/(m/z 328+m/z 334). Standard solutions of known enrichments, ranging from 0.025 to 0.500 mol% excess, were analyzed every time. The enrichments of samples were obtained by comparing their peak area abundances with those of the standard solutions.

Calculations for evaluation of feeding-induced variation

Because the time intervals in sampling during the 1 h after feeding were different between ruminal characteristics, concentrations of plasma glucose, insulin and blood VFA, these measurements were standardized to the same time interval as the glucose kinetics. The standardization was conducted by dividing the areas under the curves of these measurements by time intervals every 0.5 h from 0 through 2 h after feeding or every 1 h from 2 through 3 h after feeding. Thus, the calculated values had the same units as the original measurements, and were used as data in figures shown in the results section and for statistical analysis.

Glucose turnover rate (mg/kg BW/min) was calculated during the prefeeding period, which was considered to be a steady state, as follows (Tserng and Kalhan, 1983):

$$I \cdot (E_i/E_p - 1) \quad (1)$$

where I is the infusion rate of [$^{13}C_6$]glucose (mg/kg BW/min), and E_i and E_p are the enrichments of [$^{13}C_6$]glucose in the tracer solution and plasma (mol% excess), respectively. During the postfeeding period, the rates of entry (R_e) and utilization (R_u) of glucose (mg/kg BW/min) were separately calculated every 0.5 h using non-steady state equations (Cowan and Hetenyi, 1971).

$$R_e = (I - p \cdot V \cdot ((C_1+C_2)/2) \cdot ((E_2-E_1)/(T_2-T_1)/100)) \cdot (2 \cdot E_i/(E_2+E_1) - 1) \quad (2)$$

$$R_u = R_e - p \cdot V \cdot ((C_2-C_1)/(T_2-T_1)) \quad (3)$$

where C_1 and C_2 are plasma glucose concentrations (mg/ml), and E_1 and E_2 are the enrichments of plasma [$^{13}C_6$]glucose (mol% excess) at time T_1 and T_2 (min), respectively. V is a distribution volume of glucose and p is a pool fraction, and the respective values were assumed to be 179 ml/kg BW and 0.85 (Weekes et al., 1983).

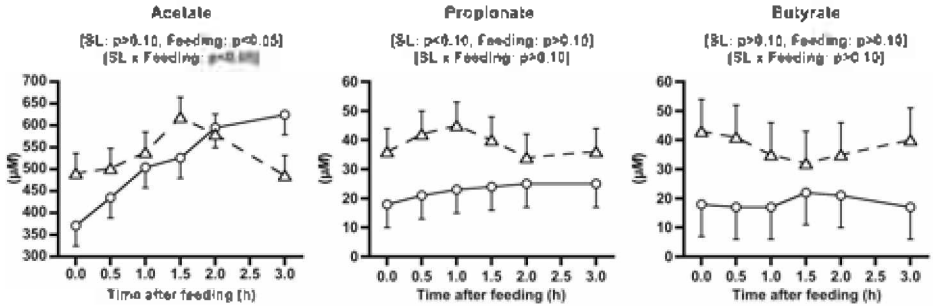

Figure 2. Effects of salinomycin (SL) on changes in blood concentrations of VFA with time after feeding in sheep fed a high-roughage diet once daily. ○: without SL, △: with 20 mg/kg diet of SL. Data represent means and SEM of four animals. The p-values of main effects of SL, the time after feeding (Feeding) and an interaction between SL and the time after feeding (SL×feeding) are shown in brackets.

Statistical analysis

Statistical analysis was conducted using the MIXED procedure of the SAS (SAS Institute Inc., 1996). The data (n = 4) for ME intake were analyzed by a one-way analysis of variance. The data for ruminal characteristics (n = 2), blood and plasma components (n = 4), and the entry and utilization rates of glucose (n = 4) were analyzed by a split-plot design with repeated measures for the time after feeding. A main-plot was SL treatment and sub-plots were the time after feeding and an interaction between SL treatment and the time after feeding (a SL×feeding interaction). Autoregressive order one was used as a covariance structure for repeated measures. A comparison between entry and utilization rate of glucose at each time after feeding was carried out using a two-way analysis of variance. The difference in least square means with Tukey adjustment was used to compare between levels in the main effects. Significance of results was taken at $p<0.05$ and a tendency at $0.05 \leq p<0.10$.

RESULTS

Salinomycin did not affect ME intake (187 without SL vs. 178 with SL, SEM 6 kJ/kg BW, $p>0.10$).

Ruminal characteristics

The pH of ruminal fluid was unaffected by SL supplementation ($p>0.10$, Figure 1), feeding ($p>0.10$) or a SL×feeding interaction ($p>0.10$). Ruminal total VFA concentration was not affected by SL ($p>0.10$), but altered by feeding ($p<0.05$). A trend for a SL×feeding interaction was also observed ($p<0.10$) in ruminal total VFA concentration. Ruminal total VFA concentration without SL tended to increase gradually from before through 2 h after feeding (from 96 to 123, SEM 5 mM, $p<0.10$) and then reached a plateau ($p>0.10$); in contrast, the concentration with SL did not change with time after feeding (in the range of 101 to 106, SEM 3 mM, $p>0.10$). Salinomycin decreased ruminal proportion of acetate (68.1 without SL vs. 63.1 with SL, SEM 0.8 mol/100 mol, $p<0.05$), but increased that of propionate (23.8 without SL vs. 29.5 with SL, SEM 0.9 mol/100 mol, $p<0.05$), and thus decreased the ratio of acetate to propionate (2.9 without SL vs. 2.2 with SL, SEM 0.1, $p<0.05$). Ruminal butyrate proportion was not affected ($p>0.10$) by SL. Feeding did not significantly influence ($p>0.10$) proportions of any ruminal VFA, and also no significant SL×feeding interaction was observed ($p>0.10$) on these proportions.

Blood VFA, plasma glucose and insulin concentrations

Blood acetate concentration was not affected by SL ($p>0.10$, Figure 2), but was influenced by feeding ($p<0.05$). A significant SL×feeding interaction was also found ($p<0.05$) on blood acetate concentration. Blood acetate concentration without SL increased gradually from before through 3 h after feeding (from 371 to 625, SEM 25 μM, $p<0.05$), but that with SL did not change greatly with time after feeding (in the range of 491 to 619, SEM 60 μM, $p>0.10$). Blood propionate concentration tended to be increased with SL (23 without SL vs. 39 with SL, SEM 5 μM, $p<0.10$), but did not change greatly with time after feeding ($p>0.10$). Salinomycin or feeding did not affect ($p>0.10$) blood butyrate concentration. Blood propionate or butyrate concentrations were not subjected ($p>0.10$) to SL× feeding interactions.

Salinomycin did not affect plasma concentrations of glucose ($p>0.10$, Figure 3) or insulin ($p>0.10$), but feeding influenced these concentrations ($p<0.01$). Plasma glucose concentrations increased slightly from before through 0.5 h after feeding (from 68.0 to 71.9 without SL vs. from 67.7 to 69.2 with SL, SEM 2.8 mg/dl), and then returned to values close to prefeeding. Plasma insulin concentrations also increased from before through 0.5 h after feeding (from 14.4 to 18.6 without SL vs. from 19.4 to 24.3 with SL, SEM

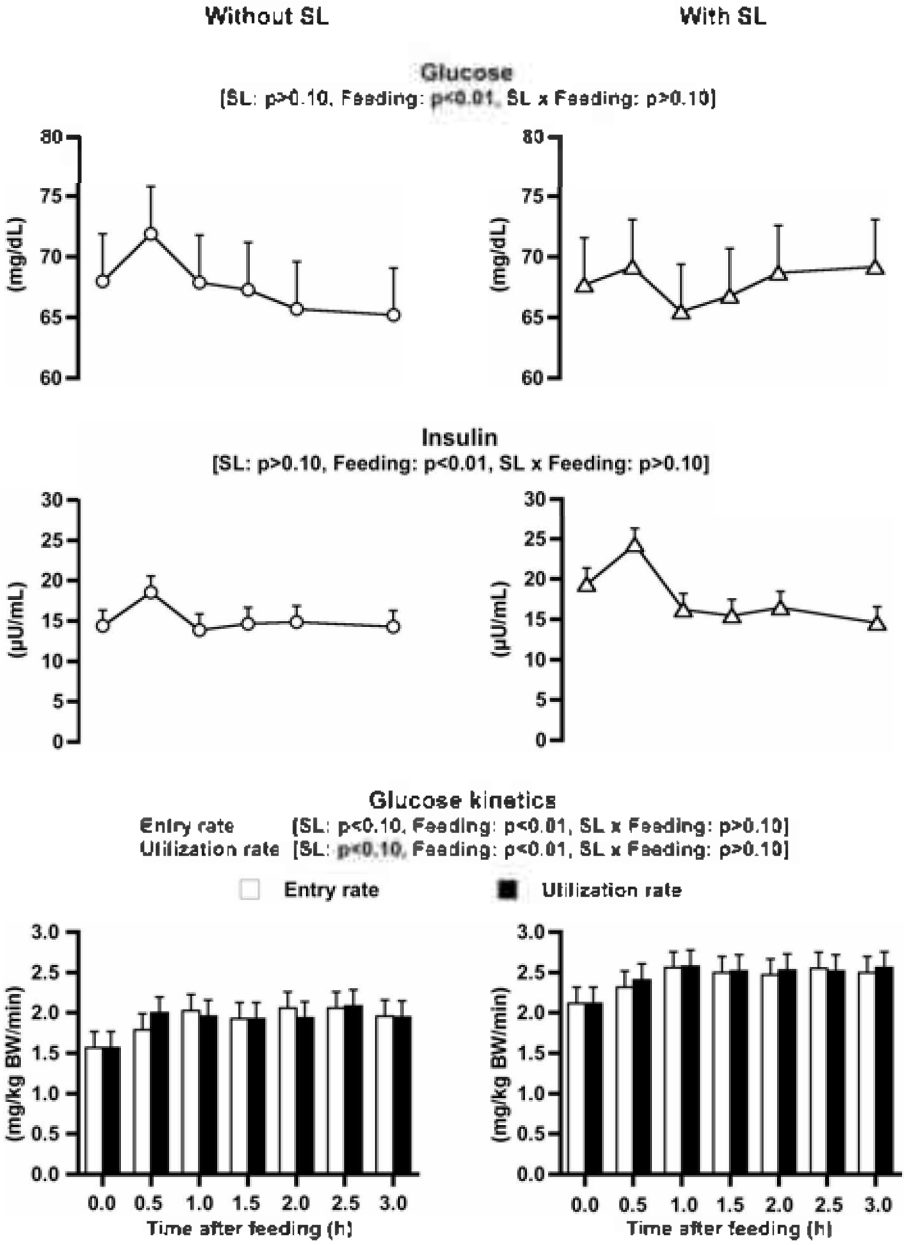

Figure 3. Effects of salinomycin (SL) on changes in plasma glucose and insulin concentrations, and rates of entry and utilization of glucose with time after feeding in sheep fed a high-roughage diet once daily. ○: without SL, △: with 20 mg/kg diet of SL. Data represent means and SEM of four animals. The p-values of main effects of SL, the time after feeding (Feeding) and an interaction between SL and the time after feeding (SL×feeding) are shown in brackets.

1.4 μU/ml), and thereafter decreased to values close to prefeeding. Plasma glucose or insulin concentrations were not subjected ($p>0.10$) to a SL×feeding interaction.

Glucose kinetics

Salinomycin tended to increase both rates of glucose entry (1.91 without SL vs. 2.43 with SL. SEM 0.17 mg/kg BW/min. $p<0.10$. Figure 3) and utilization (1.91 without SL vs. 2.46 with SL. SEM 0.16 mg/kg BW/min. $p<0.10$). Feeding also changed ($p<0.01$) both rates. The entry rate increased from before through 1 h after feeding (from 1.57 to 2.03 without SL vs. from 2.12 to 2.41 with SL. SEM 0.20 mg/kg BW/min), and the utilization rate increased from before through 0.5 h after feeding (from 1.57 to 2.00 without SL vs. from 2.12 to 2.58 with SL. SEM 0.20 mg/kg BW/min); thereafter each rate was held at these high values. The entry rate was not different from the utilization rate at any time after feeding in any SL treatment ($p>0.10$). Rates of glucose entry or utilization were not subjected ($p>0.10$) to a SL×feeding interaction.

DISCUSSION

Ruminal characteristics

Although careful interpretation of the results on ruminal characteristics is needed because of the very small number of animals used (two animals per SL treatment), we chose to include this because we feel it indicates that animals did receive SL and there was an SL response in the rumen. We consider that SL supplementation exerted its typical effects on ruminal metabolism in the present study, since changes in pH, proportions of acetate and propionate and the ratio of acetate to propionate with SL agree with previous results (Bagley et al., 1988; Reffett-Stabel et al., 1989; Terashima et al., 1990). Monensin, one of the ionophore antibiotics, also has shown an increase in propionate and no change in acetate percentages in an *in vitro* rumen fermentation study of wheat straw (Singh and De, 2005). A lack of SL×feeding interaction in these measurements indicates that SL may not modify responses of proportions of the main three VFA in rumen fluid to feeding when a high-roughage diet is fed. In contrast, a trend for a significant SL×feeding interaction on ruminal total VFA concentration, indicating a tendency for an increased concentration with time after feeding without SL but not with SL, suggests that SL may decrease the fermentation rate of roughage during the early period after feeding. This may be supported by results in grazing steers (Bagley et al., 1988) and steers fed corn silage (Reffett-Stable et al., 1989), which have shown a decrease in ruminal total VFA concentration with SL.

Blood VFA concentrations

The results for concentrations of blood VFA can be more reliably interpreted than those for ruminal characteristics, because the data were obtained from four animals per SL treatment. Different responses of blood acetate concentration to feeding between with- and without-SL treatments indicate that SL would inhibit an increase in ruminal acetate production after feeding. In ruminants, blood acetate mostly originates from acetate produced in the digestive tract, mainly the rumen (Pethick et al., 1981), and acetate is metabolized less by the portal-drained viscera and the liver than propionate and butyrate (Bergman, 1990). This suggestion is supported by the result of Armentano and Young (1983), who have reported that 33 mg/kg of monensin supplementation decreased ruminal acetate production in steers fed 700 g/kg of alfalfa hay and 300 g/kg of corn.

A trend for an increase in blood propionate concentration with SL suggests a possible increase in propionate availability in the body with SL, resulting from increased ruminal propionate production, since some researchers have reported increased ruminal propionate production by monensin supplementation (Prange et al., 1978; Van Maanen et al., 1978; Rogers and Davis, 1982; Armentano and Young, 1983). However, the trend in blood propionate concentration disagrees with the result of Sano et al. (1994), who have shown that blood propionate concentration during a 4 h postfeeding period was not affected by 20 mg/d of SL supplementation in sheep fed once daily a diet containing 750 g/kg diet of alfalfa hay and 250 g/kg diet of concentrate. This inconsistency may be attributed to the extent of change in ruminal metabolism due to a difference in the amount of supplemented SL, because the amount was higher in the present study (20 mg/kg diet) than in the study of Sano et al. (1994) (12 mg/kg diet).

Salinomycin, feeding and their combination effects on glucose kinetics

The present results indicate that SL may increase both rates of glucose entry and utilization without affecting plasma glucose and insulin concentrations. A lack of change in plasma concentrations of glucose and insulin with SL is consistent with previous results in sheep (Sano et al., 1994), but not in cattle (Terasima et al., 1990) fed a high-roughage diet. Although there are no reports about the effect of SL on glucose kinetics in ruminants, tendencies for increases in rates of glucose entry and utilization are similar to previous results in cattle supplemented with another ionophore, monensin. Arieli et al. (2001) showed that monensin supplementation enhanced glucose kinetics in dairy cattle fed a diet containing 730 g/kg of roughage. Furthermore, Van Maanen et al. (1978) reported that monensin tended to increase the rates of entry and irreversible loss of glucose with increased ruminal propionate production, in steers fed a diet containing 700 g/kg of alfalfa hay and 300 g/kg of cracked corn. The tendency for increase in entry rate in the present study appears to be mainly attributed to increased propionate availability, as suggested from an increase in ruminal propionate proportion and a trend for an increase in blood propionate concentration with SL. The tendency for increase in glucose utilization rate may be the result of a metabolic adaptation in glucose utilization by the body tissue to increased gluconeogensis without being mediated by insulin, because the tendency was not accompanied by any increase in plasma glucose or insulin concentration and also does not appear to be associated with an enhancement in tissue responsiveness to insulin. Previous reports have shown that SL did not affect tissue responsiveness to insulin evaluated by a glucose clamp approach in sheep fed a high-roughage diet (Sano et al., 1994; Fujita et al., 2000).

Little, and inconsistent, information is available about the effect of feeding on glucose kinetics in ruminants. Following feeding, the glucose entry rate increased for 1 h.

the glucose utilization rate increased for 0.5 h and thereafter these rates remained at the higher values in the present study. The results are similar to those of Armentano et al. (1984). When [U-^{14}C]glucose was infused for 30 h in Holstein steers fed a diet containing 700 g/kg of hay and 30 g/kg of cracked corn twice daily, they found that the response of plasma glucose specific radioactivity to feeding showed a sinusoidal pattern with a cyclic period of 12 h and a maximum value at feeding time, suggesting an enhancement in glucose kinetics by feeding. However, glucose kinetics of sheep fed hay alone has not responded greatly to feeding (Van der Walt, 1978; Sano et al., 1999). A difference in the amount of ingested starch may be associated with the different responses in glucose kinetics to feeding between these studies, because starch is more rapidly fermented than fiber and an increase in starch ingestion may produce enhancements in propionate availability and insulin secretion.

Some researchers have shown that SL increased insulin secretory responses to feeding (Terashima et al., 1990) and to glucose injection (Ambo et al., 1989) in sheep fed a high-roughage diet. We therefore expected that SL would affect glucose kinetics more after feeding than before feeding owing to increasing propionate availability and insulin secretion. However, a lack of SL×feeding interaction was observed on the concentrations of blood propionate, plasma glucose and insulin, and the rates of glucose entry and utilization in the present study. The results suggest that SL may not modify the response patterns of insulin secretion or glucose kinetics to feeding.

In conclusion, SL may enhance whole body glucose kinetics without affecting plasma glucose and insulin concentrations, but possibly it does not modify the response pattern of the kinetics to feeding, in sheep fed a high-roughage diet once daily. The possible enhancement in glucose kinetics with SL may be associated with an increase in propionate availability.

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