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Original article

The effect of supplemental propionate on insulin responsiveness to glucose and tissue responsiveness to insulin in relation to feeding in sheep

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Abstract – Two glucose clamp techniques were performed for 6 h starting 2 h before the initiation of feeding to investigate the effect of dietary propionate supplementation on insulin responsiveness to glucose and tissue responsiveness to insulin in relation to feeding in rams. The rams were fed alfalfa hay without (Cont diet) and with 10 mmol·kg $BW^{-1}\cdot d^{-1}$ of calcium propionate (Prop diet) for 4 weeks in randomized order. With the hyperglycemic clamp, the ratio of plasma insulin increment to glucose infusion rate did not differ between the diets, but for the Prop diet the ratio was less during the pre-feeding period. With the hyperinsulinemic euglycemic clamp, the glucose infusion rate was lower ($P < 0.05$) for the Prop diet than the Cont diet, and increased ($P < 0.05$) after feeding. In rams supplemented propionate tissue responsiveness to insulin was reduced. Propionate supplementation may either impair glucose utilization in response to insulin infusion or enhance glucose production from propionate. © Inra/Elsevier, Paris

insulin / glucose clamp / propionate / feeding / sheep

Résumé – Effet d'un supplément de propianate sur la réponse insulinique au glucose et sur la réaction tissulaire à l'insuline en rapport avec la prise alimentaire chez le mouton. Deux techniques de clamp glycémique ont été utilisées pendant 6 h, à partir de 2 h avant le début de la prise d'aliments pour étudier l'effet d'un supplément alimentaire de propionate sur la réaction insulinique et la réaction tissulaire à l'insuline en rapport avec l'alimentation chez des béliers. Les animaux étaient nourris avec du foin de luzerne sans (régime Cont) et avec (régime Prop) 10 mmol·kg$PV^{-1}\cdot j^{-1}$ de propionate de calcium pendant 4 semaines dans un ordre aléatoire. Avec le clamp hyperglycémique, le rapport de l'argumentation de l'insuline plasmatique au taux d'infusion de glucose ne différait pas entre les régimes, mais pour le régime Prop, le rapport était plus

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faible pendant la période précédant la prise alimentaire. Avec le clamp hyperinsulinémique, la vitesse d'infusion de glucose était plus faible ($p < 0,05$) pour le régime Prop que pour le régime Cont, et augmentait ($p < 0,05$) après la prise d'aliments. La supplémentation en propionate pourrait inhiber l'utilisation du glucose en réponse à l'infusion d'insuline ou augmenter la production de glucose à partir du propionate. (© Inra/Elsevier, Paris.)

insuline / clamp glycémique / propionate / régime / mouton

1. INTRODUCTION

In ruminants, volatile fatty acids (VFA) are produced through the fermentation of dietary carbohydrates by microorganisms in the rumen. They supply 50 to 70 % of the digestible energy of the host animal. Propionate, a VFA, is the major precursor of gluconeogenesis and stimulates the release of insulin, a key hormone controlling nutrient metabolism and energy partitioning [2, 5, 11]. The VFA molar ratio in the rumen has previously been changed by switching from low to high concentrate diets in lambs [10]. The proportion of propionate in the rumen and the concentrations of blood propionate and plasma insulin have been observed to increase in ruminants fed higher concentrate diets [1, 6, 24, 26]. Insulin responsiveness to glucose and tissue responsiveness to insulin, assessed by glucose clamp techniques, were greater for a high concentrate diet than a high roughage diet, and were enhanced during feeding [17, 18]. Therefore, they may be influenced by propionate availability in ruminants. Little information, however, has been reported as to whether they are influenced by supplemental propionate. In the present experiment, insulin responsiveness to glucose and tissue responsiveness to insulin were measured using the hyperglycemic and hyperinsulinemic euglycemic clamp techniques over the feeding cycle of rams fed a diet with or without supplemental propionate. The glucose clamp technique has the advantage of being able to prevent the hypoglycemia induced by insulin infusion by using a concomitant glucose infusion.

2. MATERIALS AND METHODS

2.1. Animals and diets

Five shorn Suffolk rams were used, aged 2 years old and weighing 47 ± 1 kg. More than 3 months before the experiment, they were surgically prepared under anesthesia with a skin loop enclosing the left carotid artery. The rams were kept in individual crates in an animal room at temperatures of 21 ± 2 °C. The basal diet was alfalfa hay cubes (12.8 % moisture, 12.4 % crude protein, 3.3 % ether extract, 23.5 % crude fiber, 10.1 % crude ash, and 37.9 % nitrogen-free extract). This diet was estimated to contain 2.0 Mcal of metabolizable energy per kg and 9 % digestible crude protein [14]. The dietary treatments consisted of 2 % BW (body weight) of the basal diet alone and 2 % BW of the basal diet plus 10 mmol·kg BW^{-1}·d^{-1} of calcium propionate (Cont diet and Prop diet, respectively). The Prop diet was estimated to contain 18 % greater metabolizable energy than the Cont diet. The experiment consisted of two experimental periods in which either the Prop and Cont diets were fed. Three rams were fed the Cont diet for 4 weeks during the first period, and then they were fed the Prop diet for 4 weeks during the second period. The other two rams were fed the diets in the reverse order. Therefore, each sheep received both diets over the two experimental periods. The rams were fed the diet once daily at 1300 hours. Water was freely available. A polyvinyl catheter for infusion was inserted into the jugular vein more than 1 week before the experiment started. An arterial catheter for blood sampling was placed in the arterial loop 2 h before each glucose clamp experiment. The catheters were filled with a sterile solution of 3.8 % trisodium citrate. All blood samples were obtained from the catheters without noticeable stress to the rams. All infu-

sions were sterilized by autoclaving (121 °C, 20 min).

2.2. Hyperglycemic clamp

The hyperglycemic clamp was used to determine insulin responsiveness to glucose [17]. The glucose solution was prepared at 20 % (wt/vol). The preinfusion blood glucose concentrations were determined three times at 10-min intervals before starting the glucose infusion. Glucose was infused through the infusion catheter using a multichannel peristaltic pump (Model AC-2120, Atto Co. Ltd., Japan). The glucose infusion was started 2 h before the initiation of feeding and continued for 6 h. The infusion was designed to increase blood glucose concentrations at a rate of 1 $mg \cdot dL^{-1} \cdot min^{-1}$ during the initial 50 min, and to clamp levels at 50 $mg \cdot dL^{-1}$ above preinfusion values thereafter. Blood samples (1 mL) were taken from the sampling catheter every 5 min and blood glucose concentrations were determined by a glucose analyzer within 1 min after collecting each blood sample. Immediately after the determination of the blood glucose concentration, the glucose infusion rate was adjusted to maintain the desired hyperglycemia. Blood samples taken at 10-min intervals (3 mL) were transferred to centrifuge tubes which contained 30 units of heparin sodium and were stored in ice-water until centrifugation. Additional blood samples (5 mL) were taken at 1-h intervals for VFA determination. The amount of glucose infused was recorded every 10 min throughout the glucose infusion period.

2.3. Hyperinsulinemic euglycemic clamp

The hyperinsulinemic euglycemic clamp was used to determine tissue responsiveness to insulin [17] and was carried out 4 days after the hyperglycemic clamp experiment. Insulin (400 U/L, Actrapid monocomponent porcine insulin, Novo Industri, Denmark) was dissolved in a sterile solution of 0.9 % sodium chloride and 2.5 % potassium chloride. The insulin infusion was started 2 h before the initiation of feeding, and continued for 6 h at a constant rate of 6.0 $mU \cdot kg\ BW^{-1} \cdot min^{-1}$ into the infusion catheter using the multichannel peristaltic pump. The glucose solution was infused through the same infusion catheter using another peristaltic pump at variable rates to maintain the preinfusion blood glucose concentration. The same procedures as described in the hyperglycemic clamp experiment were used for the determination of the blood glucose concentrations and glucose infusion rates. Blood samples taken at 1-h intervals (3 mL) were transferred to centrifuge tubes which contained heparin sodium and were stored in ice-water until centrifugation. Additional blood samples (5 mL) were taken at 1-h intervals for VFA determination. After finishing the insulin infusion, the glucose solution was continued for a minimum of 1 h while the blood glucose concentrations were monitored to prevent hypoglycemia.

2.4. Analyses

Blood glucose concentrations were determined using an automated glucose analyzer (Model GLU-1, Erma Optical Works, Japan) based on the glucose oxidase method. Blood VFA concentrations were determined by gas chromatography (Model 5890, Hewlett-Packard Co., USA) after steam distillation [20]. The residual blood samples were centrifuged in the cold (4 500 *g*, 10 min, 4 °C), plasma was stored at −20 °C until assayed for insulin. Plasma insulin concentrations were determined by a RIA kit (IRI 'Eiken', Eiken Chemical, Japan) based on the double antibody RIA method. The kit contained anti-insulin guinea pig serum and insulin iodine (^{125}I). Ovine insulin (Sigma Chem. Co., USA) was used for standard. Intra- and interassay CV were 5.7 and 9.0 %, respectively.

2.5. Calculations

Results are expressed as means and SE. The feeding cycle was divided into three periods, which consisted of the pre-feeding (60–0 min before feeding), early meal-feeding (0–90 min after feeding) and post-absorptive (90–240 min after feeding) periods. The mean values for glucose infusion rates and mean plasma insulin increments over the preinfusion level during each feeding cycle period of glucose clamp were termed GIR and MPII, respectively [18]. Insulin responsiveness to glucose was

expressed as the MPII/GIR ratio for the hyperglycemic clamp. Tissue responsiveness to insulin was expressed as the GIR for the hyperinsulinemic euglycemic clamp.

Data were analyzed with the GLM procedure of SAS [21]. A split-plot design was used to test for the effects of diet and time in relation to feeding. The main plot was diet and the sub plot was time. A one-way analysis of variance was used to compare the data of the Cont and Prop diets. A two-way analysis of variance for time and sheep, and the Scheffe test were used to compare between each feeding period. The repeated statement was used to analyze time × diet interaction. Results were considered significant at the $P < 0.05$ level.

3. RESULTS

3.1. Responses to feeding

Rams were strongly stimulated to eat immediately after feeding in both dietary treatments. Rams fed the Cont diet normally consumed their food ration within 1 h after feeding (49 ± 9 min), whereas rams fed the Prop diet needed a longer time to consume all the ration (105 ± 16 min). During the pre-feeding period, the concentration of blood glucose was higher ($P < 0.01$) and the concentrations of total VFA, acetate and propionate were lower ($P < 0.05$) for the Prop diet than the Cont diet (*table I*). The concentrations of plasma insulin, and blood *i*-butyrate, *n*-butyrate, *i*-valerate and *n*-valerate did not differ between the dietary treatments. Blood propionate concentrations for the Cont diet increased ($P < 0.01$) gradually after the initiation of feeding, while those for the Prop diet increased ($P < 0.01$) more than with the Cont diet (*figure 1*). These reached a peak at 1 h after feeding (101 ± 30 $\mu mol \cdot L^{-1}$), and then returned gradually to levels similar to the Cont diet. Blood acetate, *i*-butyrate, *n*-butyrate, and *i*-valerate increased ($P < 0.05$) after the initiation of feeding in both treatments. The increments of blood acetate and *n*-butyrate in response to feeding tended to be lower for the Prop diet than the Cont diet. Blood *n*-valerate did not change after feeding.

3.2. Hyperglycemic clamp

Blood glucose concentrations continued to increase ($P < 0.01$) during the first hour of the glucose infusion period (*figure 2*). The blood glucose concentrations for the Cont diet remained virtually constant thereafter. The blood glucose increments did not differ between the dietary treatments, but the increments for the Prop diet were more variable than the Cont diet (*table II*). The GIR increased ($P < 0.05$) during the early meal-feeding period, and then decreased to values lower than the pre-feeding values during the post-absorptive period in both dietary treatments. The GIR seemed to be more influenced by feeding for the Prop diet than the Cont diet, although the interaction of diet and time relation to feeding was not significant ($P = 0.11$). Plasma insulin concentrations increased ($P < 0.01$) from the pre-infusion values in relation to the glucose infusion rate in both dietary treatments (*figure 2*). The first peak of plasma insulin concentrations was observed approximately 1 h after the initiation of glucose infusion, corresponding to 1 h before the initiation of feeding, for both dietary treatments. The peak values were higher for the Cont diet than the Prop diet, but differences were not significant. During the pre-feeding period, the MPII and the MPII/GIR ratio tended to be lower for the Prop diet than the Cont diet (*table II*). Significant interactions of diet and time relation to feeding were observed for the MPII and MPII/GIR ratio ($P < 0.05$). Those for the Cont diet changed little after the initiation of feeding. The MPII/GIR ratio for the Prop diet was less ($P < 0.05$) during the pre-feeding period than the early meal-

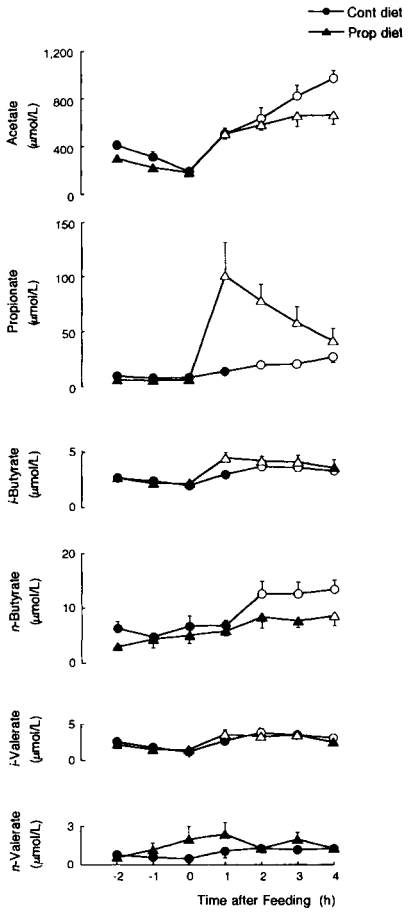

Figure 1. The concentrations of blood VFA in response to feeding in rams fed alfalfa hay cubes supplemented with and without calcium propionate (Prop diet and Cont diet, respectively). Data are expressed as mean and SE. Open symbols indicate differences ($P < 0.05$) from the mean pre-feeding values.

Table I. The concentrations of plasma insulin, and blood glucose and VFA during the pre-feeding period in rams fed alfalfa hay cubes supplemented with and without calcium propionate[a].

	Cont diet[b]		Prop diet		Significance[c]
	Mean	SE	Mean	SE	
No. of rams	5		5		
Insulin, $\mu U \cdot mL^{-1}$	8	1	8	1	NS
Glucose, $mg \cdot dL^{-1}$	40	1	42	1	**
Total VFA, $\mu mol \cdot L^{-1}$	324	29	243	16	*
Acetate, $\mu mol \cdot L^{-1}$	305	28	228	15	*
Propionate, $\mu mol \cdot L^{-1}$	9	1	6	1	*
i-Butyrate, $\mu mol \cdot L^{-1}$	2	0.3	2	0.3	NS
n-Butyrate, $\mu mol \cdot L^{-1}$	6	0.5	4	1	NS
i-Valerate, $\mu mol \cdot L^{-1}$	2	0.2	2	0.2	NS
n-Valerate, $\mu mol \cdot L^{-1}$	1	0.2	1	0.5	NS

[a] The pooled data for the hyperglycemic clamp and the hyperinsulinemic euglycemic clamp are presented;
[b] Cont diet = the basal diet; Prop diet = the basal diet plus 10 $mmol \cdot kg\ BW^{-1} \cdot d^{-1}$ of calcium propionate.
[c] ** $P < 0.01$; * $P < 0.05$; NS = not significant.

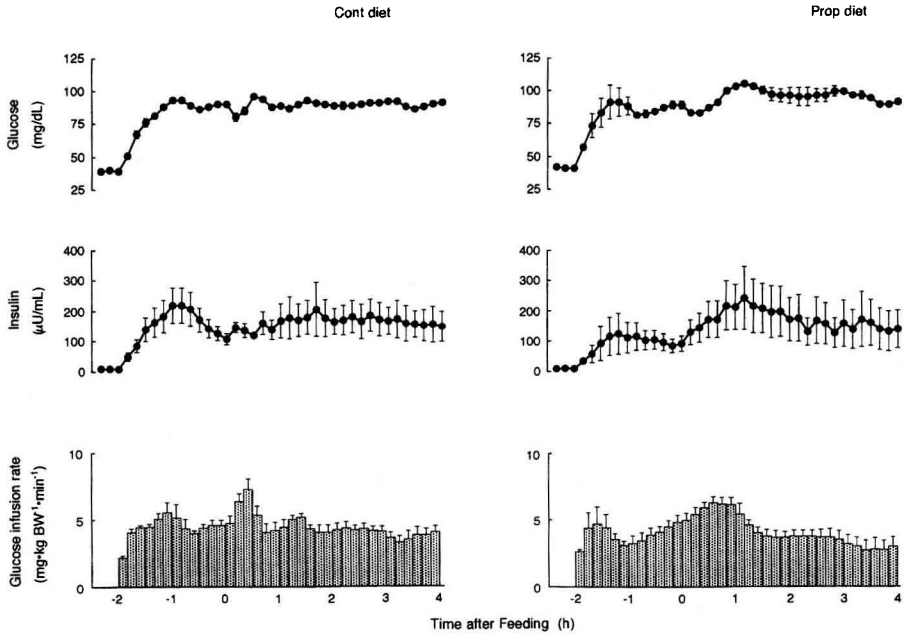

Figure 2. The concentrations of blood glucose and plasma insulin and glucose infusion rate during the hyperglycemic clamp in rams fed alfalfa hay cubes supplemented with and without calcium propionate (Prop diet and Cont diet, respectively). Data are expressed as mean and SE.

Table II. The concentrations of blood glucose and plasma insulin and glucose infusion rates during the hyperglycemic clamp in rams fed alfalfa hay cubes supplemented with and without calcium propionate.

	Cont diet[a]		Prop diet		Significance[b]	
	Mean	SE	Mean	SE	Diet[c]	Diet × Time
No. of rams	5		5			
Glucose increments, mg·dL^{-1}						
Pre-feeding	50	1	45[g]	2		
Early meal-feeding	50	1	53[f]	1	NS	*
Post-absorptive	51	0.4	54[f]	3		
GIR[d], mg·kg BW^{-1}·min^{-1}						
Pre-feeding[g]	4.5	0.4	4.0	0.5		
Early meal-feeding[f]	5.2	0.5	5.4	0.5	NS	NS
Post-absorptive[g]	4.0	0.4	3.4	0.5		
MPII[e], μU·mL^{-1}						
Pre-feeding	160	40	91	31		
Early meal-feeding	140	34	170	64	NS	*
Post-absorptive	159	55	150	70		
MPII/ GIR ratio						
Pre-feeding	35	8	21[g]	7		
Early meal-feeding	27	6	29[fg]	10	NS	*
Post-absorptive	37	9	37[f]	14		

[a] Cont diet = the basal diet; Prop diet = the basal diet plus 10 mmol·kg BW^{-1}·d^{-1} of calcium propionate; [b] * $P < 0.05$; NS = not significant; [c] Diet = Cont and Prop diets; Diet × Time = Diet and time relation to feeding interaction; [d] GIR = mean glucose infusion rate during each period; [e] MPII = mean plasma insulin increment during each period; [f,g] $P < 0.05$ between time relation to feeding.

the pre-feeding period than the early meal-feeding and post-absorptive periods.

3.3. Hyperinsulinemic euglycemic clamp

Plasma insulin concentrations increased ($P < 0.01$) markedly from the preinfusion values during the first hour of insulin infusion (*figure 3*). Plasma insulin concentrations at the pre-feeding period were lower than those at the early meal-feeding and post-absorptive periods (*table III*). Blood glucose concentrations were successfully clamped at the preinfusion values during insulin infusion by concomitant glucose infusion in both dietary treatments. The GIR was less ($P < 0.05$) for the Prop diet than the Cont diet. The GIR increased ($P < 0.05$) during the early meal-feeding period, and then returned during the post-absorptive period to the prefeeding values in both dietary treatments. No significant interaction between diet and time in relation to feeding was observed.

4. DISCUSSION

In the present experiment, the level of calcium propionate supplementation (10 mmol·kg $BW^{-1}·d^{-1}$) was the same as that used previously in sheep [15, 16]. The amount of supplementary propionate was of similar magnitude to levels of propionate production in the rumen of sheep [8] and the highest concentrations of blood propionate (101 ± 30 $\mu mol·L^{-1}$) were lower than values reported in sheep receiving a high-concentrate diet [6]. However, the dose of propionate supplementation used in this study did not necessarily act within the physiological range, because

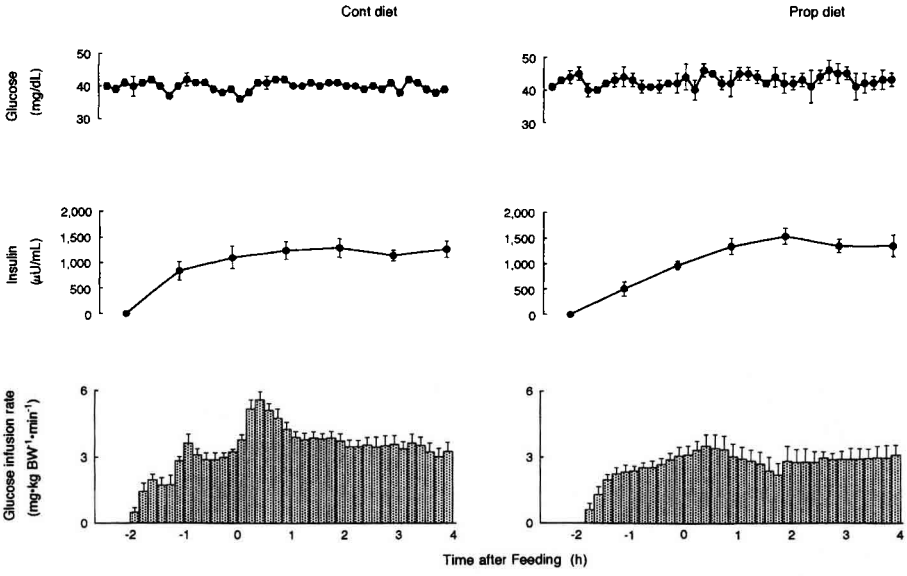

Figure 3. The concentrations of blood glucose and plasma insulin and glucose infusion rate during the hyperinsulinemic euglycemic clamp in rams fed alfalfa hay cubes supplemented with and without calcium propionate (Prop diet and Cont diet, respectively). Data are expressed as mean and SE.

Table III. The concentrations of blood glucose and plasma insulin and glucose infusion rates during the hyperinsulinemic euglycemic clamp in rams fed alfalfa hay cubes supplemented with and without calcium propionate.

	Cont diet[a]		Prop diet		Significance[b]	
	Mean	SE	Mean	SE	Diet[c]	Diet × Time
No. of rams	5		5			
Glucose increments, mg·dL^{-1}						
Pre-feeding	0	0.4	−1	0.4		
Early meal-feeding	0	1	0	2	NS	NS
Post-absorptive	0	0.2	0	1		
Insulin, μU·mL^{-1}						
Pre-feeding[f]	969	201	964	85		
Early meal-feeding[e]	1 169	173	1 135	80	NS	NS
Post-absorptive[e]	1 233	134	1 314	73		
GIR[d], mg·kg BW^{-1}·min^{-1}						
Pre-feeding[f]	3.1	0.2	2.7	0.3		
Early meal-feeding[e]	4.5	0.2	3.1	0.5	*	NS
Post-absorptive[ef]	3.5	0.3	2.8	0.4		

[a] Cont diet = the basal diet; Prop diet = the basal diet plus 10 mmol·kg BW^{-1}·d^{-1} of calcium propionate; [b] * $P < 0.05$; NS = not significant; [c] Diet = Cont and Prop diets; Diet × Time = Diet and time relation to feeding interaction; [d]GIR = mean glucose infusion rate during each period; [e,f] $P < 0.05$ between time relation to feeding.

the supplemental propionate must have been absorbed from the rumen faster than the normal rate.

Propionate is the major precursor for gluconeogenesis, and the contribution of propionate to glucose production changed with time in relation to feeding [4, 27]. Moreover, intraruminal and intravenous infusions of propionate have been shown to increase plasma insulin concentrations in ruminants [2, 5, 11]. Therefore, supplemental propionate seemed to influence glucose metabolism in the whole-body and its endocrine control. In the hyperglycemic clamp, the MPII/GIR ratio during the pre-feeding period tended to be lower for the Prop diet than the Cont diet. This may suggest that insulin secretory responses to secretogogues other than propionate were reduced in rams fed diets supplemented propionate.

Tissue responsiveness of glucose metabolism to insulin, indicated as the GIR of the hyperinsulinemic euglycemic clamp, was impaired by propionate supplementation. This is in good agreement with the data reported by Sano et al. [16] who observed that the plasma glucose disappearance rate following the intravenous glucose tolerance test was reduced in sheep supplemented with the same amount of calcium propionate. This may suggest that propionate supplementation impaired the increase in glucose utilization induced by insulin infusion. Insulin inhibits gluconeogenesis as well as accelerating glucose utilization, and intravenous insulin infusion results in decreasing blood glu-

cose concentrations. In the hyperinsulinemic euglycemic clamp, glucose solution was concomitantly infused to maintain euglycemia. Therefore, the GIR represents the sum of the enhanced glucose utilization rate and the reduced glucose entry rate induced by insulin infusion, assuming a constant glucose distribution volume. The reduced GIR for the Prop diet may be related to impaired glucose utilization, enhanced gluconeogenesis from propionate, or their combination in response to insulin infusion. Because the blood glucose utilization rate with labelled glucose was not measured in this study, the exact cause could not be explained. Van Houtert et al. [28] reported that propionate supplementation in growing lambs increased the net flux of glucose. Moreover, gluconeogenesis from propionate was not influenced by insulin [3]. This differed from the case of lactate, alanine and glycerol which were suppressed by insulin. Therefore, the possibility that the propionate convertion to glucose is enhanced with propionate supplementation as well as the existence of an impaired glucose utilization in response to insulin infusion should be considered. However, gene expression of phosphoenolpyruvate carboxykinase in the liver of goats was inhibited by insulin [13]. Insulin infusion reduces whole body protein degradation and concentrations of plasma free amino acids [7, 25]. Therefore, gluconeogenesis from propionate and glucogenic amino acids might not be enhanced during the hyperinsulinemic euglycemic clamp in rams fed the diet supplemented propionate compared with rams fed the basal diet.

Feeding enhances the insulin response to glucose, as assessed by the continuous glucose infusion, the intravenous glucose tolerance test and the glucose clamp [17, 19, 23]. However, in the present experiment using the hyperglycemic clamp, insulin responsiveness to glucose for the Cont diet did not change over the feeding cycle. The reason for the inconsistency could not be explained from the present experiment, but it may be possible that either insulin synthesis or insulin secretion from the pancreas failed to be enhanced in response to feeding owing to the relatively longer period of glucose infusion during the pre-feeding period (2 h) than was used in the above experiments. On the contrary, tissue responsiveness to insulin increased after feeding in the present study, in agreement with data reported previously [17]. Katz and Bergman [12] observed that in sheep glucose production and blood flow to the liver increased after feeding. Van der Walt [27] reported that in sheep fed lucerne hay twice daily the glucose entry rate remained almost constant over the course of the feeding cycle. Our observations may suggest that glucose utilization by insulin sensitive tissues increased after feeding. Moreover, in both glucose clamp experiments, it should be considered that the enhanced GIR during the early meal-feeding period is partly related to increases in glucose utilization by insulin independent tissues, because blood glucose concentrations usually decrease after feeding in ruminants [22].

Feed intake of rams did not differ between the two dietary treatments, but the time spent for eating was longer for the Prop diet than the Cont diet. Hikosaka et al. [9] showed that in sheep neither intravenous glucose nor insulin infusion for 2 h affected short-term feed intake. Sano et al. [19] also reported that in sheep the amount and rate of feed intake were only slightly influenced by two different levels of a 4-h intravenous glucose infusion. Therefore, in this experiment, feeding behavior may mainly have been influenced by propionate supplementation, not by infusions of glucose and insulin.

In conclusion, this study showed that tissue responsiveness to insulin in rams, as assessed by the glucose clamp tech-

nique, is reduced by dietary supplementation with propionate, which has insulinogenic properties. Moreover, it is enhanced during feeding regardless of the dietary treatment.

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