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Effect of β-lactoglobulin on plasma retinol and triglyceride concentrations, and fatty acid composition in calves

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Summary. β-Lactoglobulin (β-lg) is the main protein of ruminant milk whey. Although β-lg can bind *in vitro* to a variety of hydrophobic substrates, mainly retinol and long-chain fatty acids, its physiological function is still unknown. In Exp. 1, we investigated the effect of β-lg on the plasma retinol concentration in preruminant calves. Holstein male calves ($n = 20$) were fed Holstein whole milk at 40 g/kg body weight (BW) plus vitamin A acetate (500,000 i.u.) with or without β-lg (0·4 g/kg BW). The plasma retinol concentration of 10-d-old calves was greater ($P < 0·05$) in the β-lg-fed group than in the control group during the period from 8 to 12 h and at 24 h after the feeding. The postprandial change of plasma retinol in 40-d-old calves fed milk with β-lg was higher ($P < 0·05$) than that in the control calves only at 12 h after the feeding. In Exp. 2, Holstein male calves ($n = 18$) were used to investigate the effect of β-lg on plasma triglyceride concentration and fatty acid composition. Calves were fed Holstein whole milk at 40 g/kg BW plus milk fat prepared from whole milk at 2 g/kg BW with or without β-lg (0·4 g/kg BW). Plasma triglyceride concentration at age 10 d was higher ($P < 0·05$) in the β-lg-fed group than in the controls during the periods from 1 to 2 h and from 7 to 11 h after the feeding. At age 40 d, plasma triglyceride in the β-lg-fed group was higher ($P < 0·05$) than in the control group only at 9 h. Ratios of palmitic, stearic, and oleic acids to total plasma lipids were higher ($P < 0·05$) in the calves fed β-lg milk than in the control calves at age 10 d. These results suggest that β-lg enhances the intestinal uptake of retinol, triglyceride, and long-chain fatty acids in preruminant calves.

Keywords: β-Lactoglobulin, calves, retinol, triglyceride, fatty acid.

β-Lactoglobulin (β-lg) is a major globular protein in the milk of ruminants (Pervaiz & Brew, 1985). Its concentration varies throughout lactation; it is higher in colostrum immediately after calving (11·4 ± 1·0 g/l), and becomes stable during the 2nd week postpartum (2·9 ± 0·2 g/l) (Hodate *et al.* 1978). β-Lg has also been detected in the milk of non-ruminants, such as horses, pigs, and dogs; however, the

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milk of humans and rodents is devoid of β-lg (Perez & Calvo, 1995). A certain degree of homology of the primary (Godovac-Zimmermann *et al.* 1985) and the three-dimensional structures (Cowan *et al.* 1993) of bovine β-lg and human retinol-binding protein (RBP) has been reported. Interestingly, it was demonstrated that bovine β-lg could bind *in vitro* to a variety of hydrophobic substances, mainly retinol, triglyceride, and long-chain fatty acids (Perez & Calvo, 1995). Moreover, a recent *in vitro* study indicated that β-lg participated in the digestion of milk lipids during the neonatal period by enhancing the activity of pregastric lipase in saliva (Perez *et al.* 1992). Based on these findings, it has been suggested that bovine β-lg may play a role in the transport of milk lipids in the intestine of neonatal calves (Perez & Calvo, 1995).

Therefore, in the present study, we examined the effect of dietary bovine β-lg on the plasma concentrations of retinol and triglyceride, and on the fatty acid composition of plasma lipid in preruminant calves, and clarified the physiological function of β-lg *in vivo*.

MATERIALS AND METHODS

Calves and feeding

Thirty-eight male Holstein calves were used from birth to 6 weeks of age. All calves were removed from their dams after birth and were given colostrum within 3 h. They were housed individually in pens and had *ad libitum* access to fresh water. The calves were fed warm (38–40 °C) Holstein whole milk twice daily at 09.00 and 16.00. The daily volume of milk was fixed at 100 g/kg (morning, 40; evening, 60) body weight (BW) of the calves at the beginning of every week of the experimental period. The calves were cared for according to the Guide for the Care and Use of Agricultural Animals in Agricultural Research of the Tohoku National Agricultural Experimental Station.

Experimental protocol

Experiment 1. Holstein calves ($n = 20$) were used to investigate the effect of dietary bovine β-lg on the plasma retinol concentration. Highly purified bovine β-lg was a generous gift from Meiji Milk Products Co. (Higashimurayama, Tokyo, Japan). On the experimental days, at age 10 and 40 d (mean BW ± SEM, 43·2 ± 2·0 kg and 62·2 ± 1·5 kg, respectively), the calves were offered Holstein whole milk at 40 g/kg BW with β-lg (β-lg group; $n = 10$) or without β-lg (control group; $n = 10$) at 09.00. β-lg at 0·04 g/kg BW was dissolved in the whole milk, and Vitamin A acetate (500,000 i.u.) was mixed with the whole milk used for the experiment. On experimental days, a sterile jugular vein catheter was inserted into the calves at 07.00, and the calves were then allowed to rest for 2 h. The catheter was maintained by the flushing of saline solution containing heparin (10 units/ml).

Experiment 2. Holstein calves ($n = 18$) were used to investigate the effects of dietary bovine β-lg on the plasma triglyceride concentration and fatty acid composition of plasma lipid. On experimental days at age 10 d (45.5 ± 1.3 kg) and 40 d (62.9 ± 2.2 kg), the calves were offered whole milk at 40 g/kg BW with (β-lg group; $n = 9$) or without (control group; $n = 9$) β-lg (0·04 g/kg BW) at 09.00 h. The whole milk was supplemented with milk fat that was obtained by centrifugation (1000 ***g***, 4 °C, 30 min) of whole milk corresponding to 20 g/kg BW. The fat-supplemented milk (final fat content 47–50 g/l) was prepared on the day before the experiment, and was continuously stirred at 4 °C until use. The protocol on the experimental days was the same one as described for Experiment 1.

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Blood sampling and analysis

Blood samples (4 ml) were collected via the jugular vein catheter into heparinized test tubes just before the feeding (0), at 0·5 and 1 h, then hourly until 15 h, and finally 24 h after the feeding. The blood samples were centrifuged immediately (1000 ***g***, 4 °C 25 min) and plasma was harvested and stored at −20 °C until analysed. Plasma retinol was determined by using HPLC (McCormik *et al.* 1978). Plasma triglyceride was measured with a commercial kit (Wako Pure Chemical Industries, Osaka, Japan) using a Hitachi 7070 autoanalyzer (Hitachi Ltd., Tokyo, Japan). Total plasma lipids were extracted (Folch *et al.* 1957) and transesterified with boron trifluoride in anhydrous methanol (Hyun *et al.* 1965). Fatty acid methyl esters were analysed by gas-liquid chromatography (GC) in a Hewlett-Packard chromatograph model 6890 fitted with a frame ionization detector, using a column (30 m × 0·32 mm i.d.) coated with Carbowax (J&W Scientific, Folsom, CA). The oven temperature was maintained at 50 °C during sample injection, and was increased at 30 deg C/min to 175 °C and then at 1 deg C/min to 220 °C where it was held for 20 min. Injector and detector temperature were maintained at 260 and 300 °C, respectively. Helium was used as the carrier gas at 1 ml/min. Fatty acid methyl esters were identified by comparing their retention times with those of individually purified standards and quantified with heptadecanoic acid methyl ester as the internal standard.

Statistical analysis

Analysis was performed using the STATVIEW program for Macintosh (Abicus, CA, USA). In both experiments, all data were subjected to a two-way repeated-measures ANOVA with the following terms in the statistical model: treatment, time, treatment × time. If a significant effect of treatment or treatment × time was observed ($P < 0·05$), means were separated by Fisher's PLSD *post hoc* test. All data are expressed as mean ± SEM.

RESULTS

Experiment 1.

Plasma retinol concentrations at age 10 d were increased by feeding in both groups (Fig. 1*a*). In the calves given β-lg, the mean plasma retinal concentration at 8 h increased ($P < 0·05$) to 191 % of the pre-feeding level (18·5 ± 0·9 μg/dl). In the control calves, the plasma retinal increased up to 155 % of the pre-feeding level (18·6 ± 0·7 μg/dl) at 8 h ($P < 0·05$). As a result, the plasma retinol concentration was significantly higher ($P < 0·05$) in the β-lg-fed group than in the control group over the post-feeding period from 8 h (35·3 ± 1·8 and 28·8 ± 0·9 μg/dl respectively) until 12 h (33·7 ± 2·1 and 27·6 ± 0·8 μg/dl respectively). Thereafter, the plasma retinol concentration in the control group returned to the pre-feeding level by 24 h after the feeding (19·8 ± 0·9 μg/dl). In contrast, the plasma concentration of retinol in the β-lg-fed group at 24 h was higher ($P < 0·05$) than the pre-feeding level and the level at 24 h in the control group (24·1 ± 1·4 μg/dl).

At age 40 d, postprandial changes of the plasma retinol concentration in both groups resembled the feeding-induced changes observed in the calves at 10 d (Fig. 1*b*). However, the difference between the groups was significant only at 12 h after the feeding (β-lg-fed group: 33·3 ± 2·7 μg/dl; control group: 25·9 ± 1·6 μg/dl; $P < 0·05$). In addition, the concentration of plasma retinol at 24 h in the β-lg-fed group (24·9 ± 2·7 μg/dl) was not significantly different from the pre-feeding level (20·5 ± 1·5 μg/dl).

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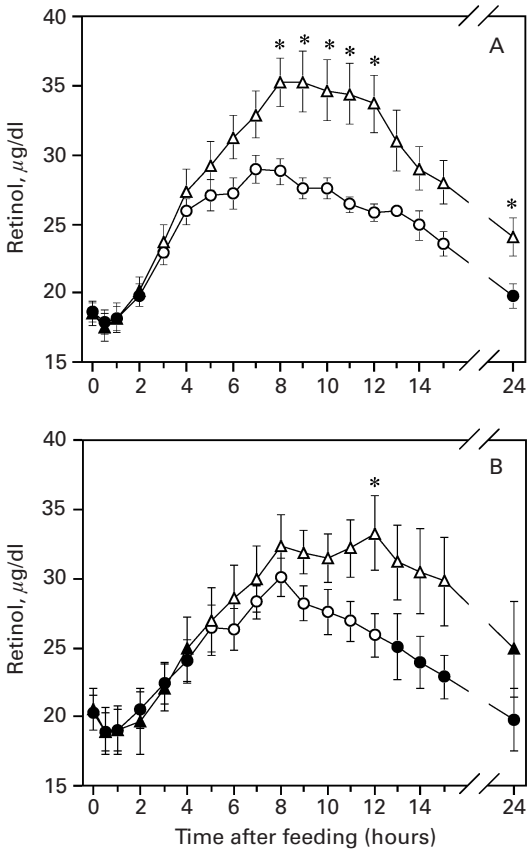

Fig. 1. Postprandial changes of the plasma retinol concentration in calves fed whole milk with (▲) or without (●, control) β-lg at 10 (A) and 40 days (B) of age. Data points are means ± SEM. $n = 10$ for each treatment. Asterisks indicate a significant difference ($P < 0{\cdot}05$) from controls. Open symbols indicate a significant difference ($P < 0{\cdot}05$) vs zero time within the same treatment group.

Experiment 2.

The plasma concentrations of triglyceride before the feeding at age 10 d were similar in the two groups (β-lg-fed: 12·6 ± 1·1 μg/dl; control: 12·4 ± 1·4; Fig. 2*a*). The feeding caused approximately biphasic increases of plasma triglyceride at 0·5 and 6 h after the feeding in both groups. The postprandial changes of the plasma triglyceride concentration were greater ($P < 0{\cdot}05$) in the β-lg-fed group than in the control group during the period from 1 h (β-lg-fed: 20·9 ± 2·5 mg/dl; control: 13·0 ± 0·9 mg/dl) to 2 h (β-lg-fed: 15·6 ± 2·0 mg/dl; control: 10·3 ± 1·1 mg/dl) and from 7 h (β-lg-fed: 29·8 ± 2·1 mg/dl; control: 19·2 ± 2·7 mg/dl) to 11 h (β-lg-fed: 18·2 ± 1·4 mg/dl; control: 12·4 ± 1·5 mg/dl) after the feeding.

In the calves at age 40 d, postprandial changes of the plasma triglyceride concentration in both groups were similar to those in the calves at age 10 d (Fig. 2*b*). However, the plasma concentration of triglyceride was higher ($P < 0{\cdot}05$) in the calves given β-lg (34·3 ± 3·5 mg/dl) than in the control calves (15·8 ± 1·9 mg/dl) only at 9 h after the feeding.

The ratios of fatty acids to total plasma lipids at 0 and 9 h after the feeding are presented in Table 1. In calves at both age 10 d and 40 d, there were no differences in

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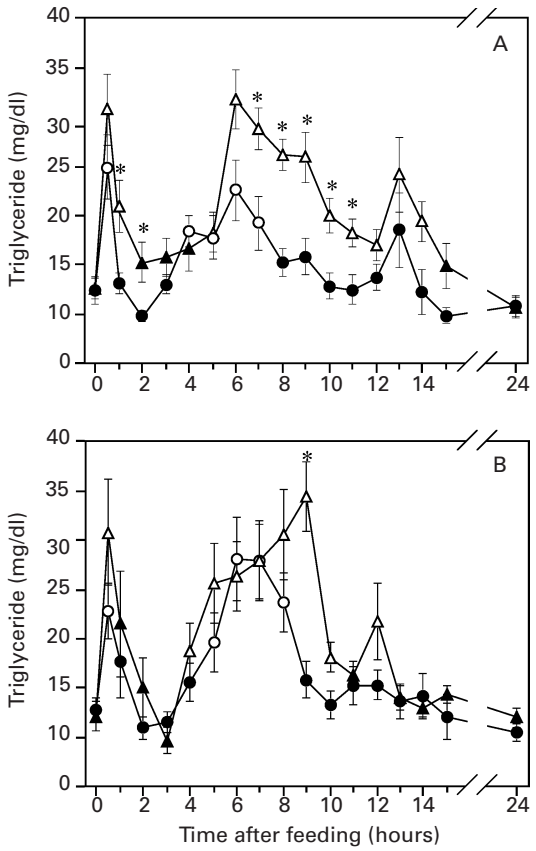

Fig. 2. Postprandial changes of the plasma triglyceride concentration in calves fed whole milk with (▲) or without (●, control) β-lg at 10 (A) and 40 days (B) of age. Data poinst are means ± SEM. $n = 9$ for each treatment. Asterisks indicate a significant difference ($P < 0{\cdot}05$) from controls. Open symbols indicate a significant difference ($P < 0{\cdot}05$) vs zero time within the same treatment group.

Table 1. *The ratio of fatty acids to total plasma lipids in 10-d and 40-d-old calves pre-feeding (0 h) and 9 h post feeding. Calves were fed whole mild (control group) or whole milk supplemented with 0·4 g β-lactoglobulin (β-lg)/kg bodyweight (β-lg-fed). For details see description of Experiment 2 in the materials and methods section*

(Values are means ± SEM, for n, calves per group = 9)

	10 days of age				40 days of age			
	0 h		9 h		0 h		9 h	
	Control	β-lg-fed	Control	β-lg-fed	Control	β-lg-fed	Control	β-lg-fed
C12:0	0·4 ± 0·06	0·3 ± 0·03[a]	0·5 ± 0·05	0·6 ± 0·03[b]	0·3 ± 0·04	0·4 ± 0·04	0·4 ± 0·04	0·6 ± 0·03
C14:0	2·8 ± 0·25	3·0 ± 0·20[a]	3·3 ± 0·24	3·7 ± 0·16[b]	2·8 ± 0·30	3·0 ± 0·30	3·2 ± 0·13	4·0 ± 0·21
C16:0	19·6 ± 0·26	19·7 ± 0·37[a]	20·1 ± 0·28	22·9 ± 0·39*[b]	18·2 ± 0·42	18·9 ± 0·19[a]	19·3 ± 0·20	21·3 ± 0·18*[b]
C16:1	3·1 ± 0·16	2·8 ± 0·13	3·1 ± 0·18	2·6 ± 0·12	2·5 ± 0·13	2·5 ± 0·08	2·4 ± 0·16	2·4 ± 0·06
C18:0	12·4 ± 0·40	12·4 ± 0·33[a]	12·3 ± 0·34	13·9 ± 0·29*[b]	12·6 ± 0·43	12·4 ± 0·19	12·7 ± 0·48	12·6 ± 0·11
C18:1	23·3 ± 0·44	22·6 ± 0·35[a]	23·3 ± 0·41	24·7 ± 0·34*[b]	22·3 ± 0·65	22·1 ± 0·44	22·7 ± 0·62	23·0 ± 0·54
C18:2	28·3 ± 0·78	28·5 ± 0·75[a]	27·1 ± 0·76	22·4 ± 0·73*[b]	29·8 ± 0·79	29·7 ± 0·65[a]	28·3 ± 0·65	25·9 ± 0·37*[b]
C18:3	3·1 ± 0·19	2·9 ± 0·13[a]	3·0 ± 0·17	2·3 ± 0·15[b]	2·9 ± 0·13	2·8 ± 0·07	2·8 ± 0·10	2·5 ± 0·02
Others	7·0 ± 0·39	7·8 ± 0·49	7·3 ± 0·33	6·9 ± 0·38	8·6 ± 0·29	8·2 ± 0·17	8·2 ± 0·35	7·7 ± 0·30

* Mean value of β-lg-fed group was significantly different from the corresponding value for the control group, $P < 0{\cdot}05$

[a,b] Means with different superscripts within the same row are significantly different, $P < 0{\cdot}05$.

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the ratios of fatty acids to total plasma lipids between the β-lg and control groups at 0 h. At age 10 d, the ratios of palmitic, stearic and oleic acids to total plasma lipids in the β-lg-fed calves were higher (22·9, 13·9, and 24·7 %, respectively; $P < 0{\cdot}05$) than those in the control calves (20·1, 12·3 and 23·3 % respectively) 9 h after feeding. The post-feeding increase (0 h cf. 9 h) in the ratio of palmitic acid to total plasma lipids, in the calves at age 40 d, was greater in the β-lg-fed group (2·4 % increase) than the control group (1·1 % increase). In contrast to those changes, in the calves at both age 10 and 40 d, the ratio of linoleic acid (C18:2) to total plasma lipids in the β-lg-fed group was lower ($P < 0{\cdot}05$) than that in the control group at 9 h after the feeding.

DISCUSSION

Many past studies have indicated the existence of various hydrophobic ligand-binding sites on β-lg (Godovac-Zimmermann *et al.* 1985; Perez & Calvo, 1995). Indeed, it is known that bovine β-lg can bind to retinol, triglyceride, and long-chain fatty acids *in vitro* (Perez & Calvo, 1995). However, in spite of these findings that β-lg interacts strongly with hydrophobic molecules, the exact physiological role of β-lg is unknown. The most significant findings in the present study were increases of the circulating plasma retinol and triglyceride concentrations in response to the oral administration of bovine β-lg compared with the control (not administered β-lg) in preruminant calves.

A certain degree of homology of the primary (Godovac-Zimmermann *et al.* 1985) and the three-dimensional structures (Cowan *et al.* 1993) of bovine β-lg and human RBP has been reported. Therefore, it has been postulated that β-lg belongs to a superfamily of proteins showing strong interactions with small hydrophobic ligands, which include RBP and intracellular RBP (Perez & Calvo, 1995). Because of these findings and the known function of RBP in delivery of retinol to tissues (Papiz *et al.* 1986), it has been suggested that β-lg may play a role in the delivery of retinol to the intestinal absorptive cells after hydrolysis of esterified retinol (the form of vitamin A in milk) in the intestine (Papiz *et al.* 1986; Pervaiz & Brew, 1985). In an *in vitro* study using suckling rats, uptake of retinol bound to bovine β-lg was higher than that of free retinol in both the jejunum and the ileum (Said *et al.* 1989). Moreover, it has also been reported that specific receptors for the β-lg -retinol complex exist in the intestines of neonatal calves (Perez *et al.* 1989). The β-lg-retinol complex binds specifically to purified microvilli prepared from the intestines of 1-week-old calves (Papiz *et al.* 1986). This specific binding was observed only in the small intestine and was not observed in 6-month-old animals (Papiz *et al.* 1986). In the present study, the concentration of plasma retinol in calves fed the diet supplemented with β-lg was higher than that in the control calves at both 10 and 40 d of age. This suggests that β-lg enhances the intestinal uptake of retinol. However, under our experimental conditions, it is unclear whether specific receptors for the β-lg-retinol complex equally enhance the retinol uptake in the intestines of calves at both 10 and 40 d of age. The mechanism by which β-lg increases retinol uptake in preruminant calves at various ages remains to be elucidated.

Previous studies have shown that the absorption of dietary fat in suckling calves is a two-phase process (Bazin & Brisson, 1976). The first phase consists of the rapid hydrolysis of a portion of the triglyceride in the abomasum under the action of pregastric lipase produced in saliva (Roy, 1974). In the second phase, there is a considerable increase in fat flow through the pylorus associated with the disintegration of the curd at around 6 h after feeding (Roy, 1974). In the present

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study, there was a resemblance between the patterns of the postprandial change of the plasma triglyceride levels in both the control and β-lg -fed groups. In the first phase, the level of plasma triglyceride in the calves fed the diet supplemented with β-lg was high between 0.5 and 2 h following feeding as compared with the control calves at age 10 d. The concentration of plasma triglyceride in the calves at age 40 d was also high during this time period after feeding in the β-lg group; however, there was no significant difference in plasma triglyceride levels between the β-lg-fed and the control groups. Pregastric lipase action in calves is stimulated by suckling, and continues in the abomasum for at least 2 h after feeding (Edwards-Webb & Thompson, 1977). Recent observations *in vitro* suggest that the activity of ruminant pregastric lipase is increased by the presence of β-lg (Perez *et al*. 1992). This lipase activity is higher in newborn animals and decreases markedly in older animals (Moreau *et al*. 1988). Therefore, our *in vivo* data support the hypothesis that β-lg could participate in the digestion of milk fat during the neonatal period by enhancing the activity of pregastric lipase.

In the second phase of the present study, the concentrations of plasma triglyceride in calves at 10 d of age were significantly higher in those given the β-lg diet than in the control calves during the period from 7 to 11 h after feeding. In a previous study, we clearly showed that preruminant calves fed whey protein concentrate (WPC) had significantly higher concentrations of plasma triglyceride than control calves (Kushibiki *et al*. 1997). Because β-lg is a major protein in WPC, this response may be partly dependent upon the higher β-lg content of a milk diet (Kushibiki *et al*. 1997). On the other hand, β-lg of ruminants isolated from milk by non-denaturing methods contains several bound lipids, mainly triglyceride and long-chain fatty acids (Perez *et al*. 1989). The predominant fatty acids bound to β-lg of cows and sheep are palmitic, oleic, and stearic acids, which account for 63–65 % of the total bound lipids (Perez *et al*. 1989, 1993). In contrast, very little lauric (C12:0), palmitoleic (C16:1), linoleic (C18:2), and linolenic acids (C18:3) are bound to β-lg (Diaz de Villegas *et al*. 1987; Perez *et al*. 1989). In the present study, the ratios of palmitic, stearic, and oleic acids to plasma lipid in calves fed the basal milk with β-lg were increased at 10 d of age. Our *in vivo* data were consistent with the osbervations in previous *in vitro* studies (Perez *et al*. 1989, 1993). In an *in vitro* incubation system, the binding affinity of fatty acids to bovine β-lg was shown to decrease in the order of palmitic, stearic, and oleic acids (Spector & Fletcher, 1970). In this study, the ratio of palmitic acid in the β-lg group alone was increased by the feeding at both 10 and 40 d of age. Our results also suggest that the affinity of palmitic acid for β-lg is higher than those of stearic and oleic acids. In an *in vivo* study using calves, β-lg was very poorly hydrolyzed after the consumption of a milk diet (Yvon *et al*. 1984), because the binding of fatty acids to β-lg increases its conformational stability to enzymatic degradation (Puyol *et al*. 1993). The mechanism by which the long-chain fatty acids bound to β-lg are preferentially absorbed in the intestine is not completely understood. However, it has been determined that β-lg shows a degree of homology with intracellular lipid-binding proteins such as Z-protein (Pervaiz & Brew, 1985). In addition, the hypothesis that some of the components of fat globules (e.g., triglyceride and long-chain fatty acids) are bound by β-lg to the gut wall, allowing them to transfer to intracellular lipid-binding proteins within the gut villi, was proposed recently (Lawrence, 1996).

In conclusion, the present study provides the first *in vivo* evidence that β-lg participates in the lipid metabolism of preruminant calves. Our results suggest that β-lg participates in the transport of retinol in the intestine and also plays a role in

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the digestion and/or intestinal absorption of long-chain fatty acids and triglyceride in neonatal calves.

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