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Retinol binding protein 4 in dairy cows: its presence in colostrum and alteration in plasma during fasting, inflammation, and the peripartum period

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Retinol-binding protein 4 (RBP4) is a plasma protein involved in retinol transportation, and recent evidence in rodents suggests that RBP4 is also a metabolic regulator that modifies insulin sensitivity. To assess how RBP4 levels are regulated in ruminants, we determined the RBP4 concentrations in bovine plasma and milk using Western blot analysis. Plasma RBP4 levels in non-pregnant non-lactating (control) cows were around 45 μg/ml, which were sustained during 60-h fasting, but decreased significantly 4 h after lipopolysaccharide (LPS) administration. Basal plasma retinol concentration was around 30 μg/dl, but this decreased to approximately one-third and one-half of these values during fasting and 8 h after LPS challenge, respectively. Plasma RBP4 and retinol levels in cows 3–6 d before parturition were comparable to those of the controls. However, on the day of parturition both were significantly decreased and had returned to basal levels by two weeks after calving. Interestingly, RBP4 was clearly detected in colostrum (16·4 ± 5·6 μg/ml) but was only faintly detected in milk from cows at 7 d and 15 d after calving. Retinol concentrations in colostrum were almost 10-fold higher than those in plasma, while those in milk were comparable to those in plasma. These results suggest that RBP4 and retinol levels are independently regulated under physiological and pathophysiological conditions and that RBP4, like retinol, is transferred from maternal stores to calves through colostrum.

Keywords: Colostrum, cattle, RBP4, retinol, vitamin A.

Vitamin A (retinol) is essential for a variety of physiological processes, including vision, immune functions, reproduction, embryonic development as well as cellular growth and differentiation (Stephensen, 2001; Blomhoff & Blomhoff, 2006).

Retinol represents the most abundant retinoid in bovine blood (Van Merris et al. 2004) and its levels in steers are maintained for at least 145 d even if the animals are fed a diet with no supplemental vitamin A (Gorocica-Buenfil et al. 2008). However, plasma retinol and β-carotene levels in heifers and cows decrease at the end of gestation, reach their lowest values around parturition and increase again during the first week of lactation (Johnston & Chew, 1984; Goff & Stabel, 1990; Lindberg et al. 1999; Kumagai et al. 2000; Kumagai et al. 2001; Nonnecke et al. 2001; Goff et al. 2002; Van Merris et al. 2004; Debier et al. 2005; Rezamand et al. 2007). The decrease in plasma retinol concentration is probably due to the transfer of large amounts of retinol and its derivatives into colostrum (Goff et al. 2002), resulting in the accumulation of retinoids in colostrum rather than in milk (Debier et al. 2005). In addition, plasma retinol concentrations decrease immediately after intramammary injection of *Escherichia coli* (Van Merris et al. 2004) suggesting that vitamin A metabolism is also affected by the acute phase reactions that occur during coliform mastitis. Indeed, the plasma retinol levels in post-partum cows with mastitis are significantly lower than those of cows without mastitis (Johnston & Chew, 1984), and there is a significant delay in the recovery of the plasma β-carotene concentration in post-partum cows with a subclinical intramammary infection (Rezamand et al. 2007).

In newborn calves, plasma retinol levels at birth are low and increase markedly after colostrum intake (Blum et al. 1997; Kumagai et al. 2001; Debier et al. 2005; Puvogel

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et al. 2008). As colostrum provokes drastic morphological and functional changes in the gastrointestinal tract (GIT) in neonatal calves (Blum, 2006) and vitamin A plays a role in maintaining gut integrity (Thurnham et al. 2000; Quadro et al. 2000; Carroll & Forsberg, 2007) the retinol in colostrum is likely to contribute to the maturation of the GIT together with non-nutrient factors such as IGF-1 (Blum, 2006).

Retinoids are small hydrophobic compounds and therefore associate *in vivo* with soluble proteins. Retinol-binding protein 4 (RBP4) is the protein responsible for plasma retinol transport and binds with retinol in a 1 : 1 ratio (Noy, 2000). Recently, it has been reported that RBP4 induces insulin resistance in mice (Yang et al. 2005) and that the serum RBP4 levels in humans correlate with the magnitude of insulin resistance in subjects with impaired glucose tolerance or type 2 diabetes (Graham et al. 2006). More recently, STRA6 was identified as a cell surface receptor for RBP4 (Kawaguchi et al. 2007), indicating that RBP4 is a novel biologically active factor.

It was reported that the mean serum RBP4 and retinol concentrations in cows 4 weeks before calving were 42 μg/ml (~2 μM) and 53 μg/dl (~1·8 μM), respectively. RBP4 levels decreased one week before parturition and recovered to control levels at one week after calving (Lindberg et al. 1999) suggesting the possibility that RBP4, like retinol, is transferred from maternal stores to calves through colostrum and milk. However, a similar decrease in plasma RBP4 levels was found only in cows with subclinical intramammary infections, but not in uninfected cows (Rezamand et al. 2007), suggesting the decrease might be due to suppression of hepatic RBP4 synthesis during acute-phase reactions, as demonstrated in rats (Rosales et al. 1996).

In the present study, we measured the RBP4 and retinol concentrations in the plasma, colostrum and milk of cows to assess how plasma RBP4 and retinol levels are regulated in ruminants under physiological and pathophysiological conditions. Moreover, owing to the lack of such reports in any mammalian species, the study was undertaken to describe RBP4 levels in colostrum and/or milk.

Materials and Methods

Blood sampling and collection of colostrum and milk

All experimental procedures were performed in accordance with the guidelines outlined by the Animal Care and Use Committee of Hokkaido University.

In Experiment I, five non-pregnant non-lactating Holstein cows weighing 450–550 kg were used to examine the effects of fasting on plasma RBP4 and retinol levels. They were housed in individual stalls with free access to water and trace mineral blocks and fed a mixture of forage (orchard-grass hay, alfalfa hay cubes and corn silage) and concentrates. At the start of the experiment, feed was completely withdrawn, and food deprivation was maintained for 60 h, when the metabolic effects of starvation in cows were clearly observed (Baird et al. 1972). Water was provided throughout the study. Blood was collected from the jugular vein at 0, 12, 24, 36, 48 and 60 h after the onset of food deprivation. Plasma was separated by centrifugation and stored at −30 °C until use.

In Experiment II, six non-pregnant non-lactating Holstein cows were housed as described above and used to examine the effects of lipopolysaccharide (LPS) on plasma RBP4 and retinol levels. On the day of the experiment, the cows were divided into two groups (groups A and B, three per group). Group A received an intravenous injection of LPS (*Escherichia coli* 055: B5, Difco, Detroit MI, USA) solution dissolved in saline at a dose 500 ng/kg, while group B was injected with saline as a control. Blood was collected at 0, 2, 4, 6 and 8 h after the injection, and the plasma was stored at −30 °C.

In Experiment III, six pregnant Holstein cows were housed as described above. Blood and milk (colostrum) samples were collected 3–6 d before calving, on the day of parturition and 1 and 2 weeks after calving. These samples were stored at −30 °C.

Western blot analysis of RBP4 in plasma, colostrum and milk

Bovine RBP4 was purified as previously described (Miyamoto et al. 1989) and anti-sera were obtained by immunizing purified RBP4 to rabbits. Plasma was diluted 20-times with distilled water, and the whole milk and colostrum were undiluted and diluted 10-times with distilled water, respectively. These samples (7·5 μl for plasma and 24 μl for milk) and increasing concentrations of purified RBP4 were separated simultaneously by SDS-PAGE (13% gel) under reducing conditions. Thereafter, the proteins were electroblotted onto a PVDF membrane (Immobilon™, Millipore, Bedford MA, USA). The membrane was blocked for 1 h at room temperature in 5% (w/v) skimmed milk in a buffer [20 mM-Tris–HCl (pH 7·5), 0·15 M-NaCl and 0·1% Tween 20] and then incubated with the anti-RBP4 sera (1 : 2000) overnight at 4 °C. The membrane was washed five times with the wash buffer and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1 : 4000, Zymed Laboratories Inc., South San Francisco CA, USA) for 1 h at room temperature. Visualization was performed using an enhanced chemiluminescence detection system (Millipore) according to the manufacturer's instructions. The intensities of the immunoreactive bands were analysed densitometrically using the NIH Image program. RBP4 concentration was measured by comparing the intensity of the bands with those of purified RBP4. As shown in Fig. 1, the band intensity of purified bovine RBP4 was closely correlated with the protein amount, at least from 10 ng to 80 ng of RBP4. Purified RBP4, added to bovine plasma and milk, was recovered at 96·2 ± 0·4% and 95·4 ± 0·8%, respectively. The intra- and inter-assay variations in

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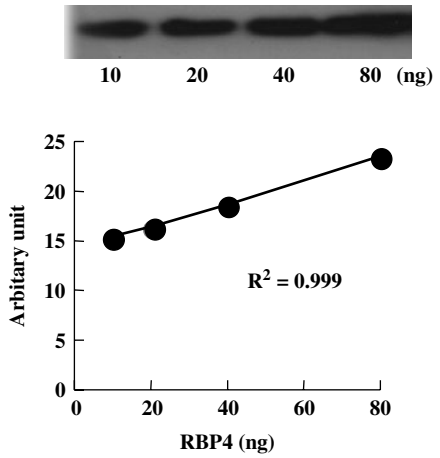

Fig. 1. Quantitative analysis of RBP4 by Western blot analysis. Representative blot of the increasing amounts of purified RBP4 and a standard curve obtained by densitometric analysis of the bands are shown.

plasma RBP4 determination for five independent experiments were less than 5·1% and 2·4%, respectively.

Spectrophotometric measurement of retinol in plasma, colostrum and milk

Retinol concentrations in plasma, colostrum and milk were determined by modifying the method described by Suzuki & Katoh (1990). In brief, 50 µl of ethanol and 150 µl of hexane were added to 50 µl of plasma, colostrum, or milk, and the hexane phase was recovered after 40-min mixing and 10-min centrifugation at 6500 ***g***. Retinol concentrations were calculated based on the absorbance of hexane extracts at 325 nm and 453 nm using the equations described (Suzuki & Katoh, 1990). Recovery of retinol added to milk was 97·8±0·4%.

Statistical analysis

Results are expressed as means±SEM. Statistical analysis was performed using one-way repeated measures ANOVA for Experiments I and III and two-way repeated measures ANOVA for Experiment II, and Fisher's post hoc test, with $P<0·05$ being considered statistically significant.

Results

Changes in plasma RBP4 levels during fasting and after LPS challenge

Basal plasma RBP4 levels in the two experimental groups were almost the same (41·8±1·9 µg/ml in Experiment I and 46·1±4·6 µg/ml in Experiment II) and were sustained during the 60-h fasting (Fig. 2A) and after saline administration (Fig. 3A). However, RBP4 levels were significantly reduced 4 h after LPS administration and decreased for at least 8 h after the injection (Fig. 3A). Basal plasma retinol concentrations were 36·2±3·2 µg/dl (Experiment I) and 25·5±1·8 µg/dl (Experiment II). During fasting, the concentration progressively decreased to 13·1±2·7 µg/dl at 60 h (Fig. 2B), and the retinol/RBP4 molar ratio fell from 0·65±0·05 to 0·23±0·05 (Fig. 2C). Plasma retinol concentration was also significantly decreased at 2 h and had decreased by almost 50% by 8 h after the LPS injection

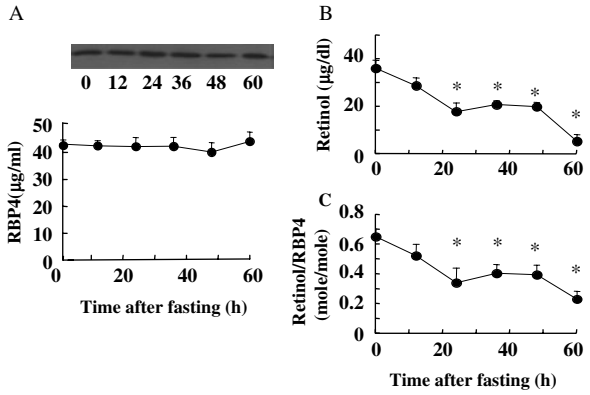

Fig. 2. Effects of fasting on plasma RBP4 and retinol levels in cows. Cows ($n=5$) were housed without feeding for 60 h. Blood was collected at 0, 12, 24, 36, 48 and 60 h after the onset of food deprivation, and isolated plasma samples were stored. Plasma concentrations of RBP4 (A) and retinol (B) were quantified by Western blot analysis and a spectrophotometric method, respectively. (C) The molar ratio of retinol to RBP4 was calculated. Values are expressed as means±SEM. *: $P<0·05$ *v.* 0 h.

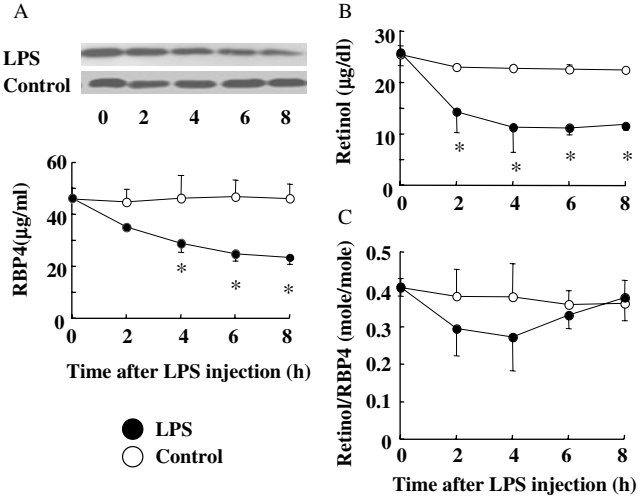

Fig. 3. Effect of LPS administration on plasma RBP4 and retinol levels in cows. Cows ($n=6$) were injected intravenously with either LPS (500 ng/kg, $n=3$) or saline ($n=3$) as a control. Blood samples were collected at 0, 2, 4, 6 and 8 h after the injection. Plasma concentrations of RBP4 (A) and retinol (B) and their molar ratio (C) are shown. Values are expressed as means±SEM. *: $P<0·05$ *v.* control.

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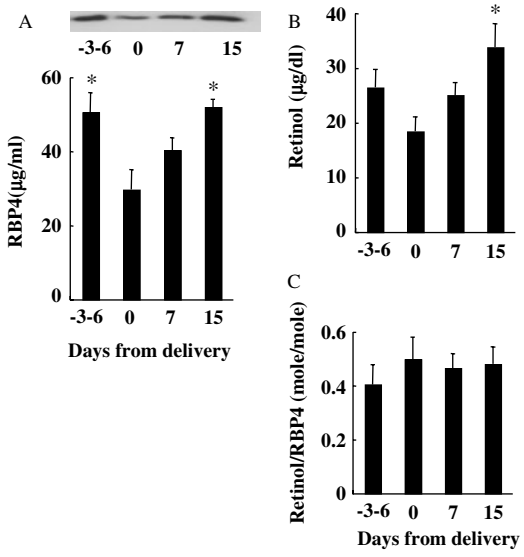

Fig. 4. Changes in plasma RBP4 and retinol levels in cows before and after parturition. Blood was collected from 6 cows before and after parturition. Plasma concentrations of RBP4 (A) and retinol (B) and their molar ratio (C) are shown. Values are expressed as means ± SEM. *: $P < 0{\cdot}05$ *v.* day 0.

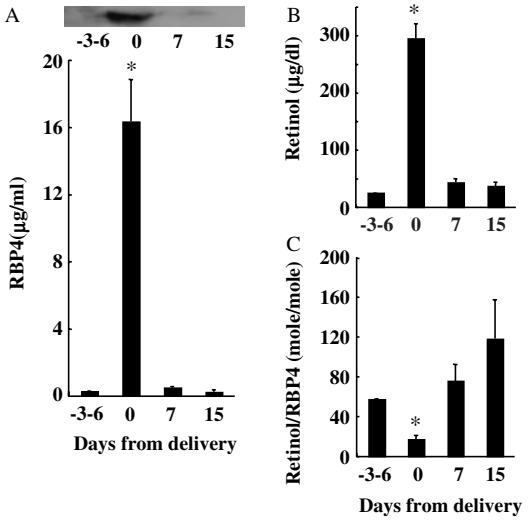

Fig. 5. Changes in RBP4 and retinol levels in colostrum and milk. Milk was collected from cows before ($n=2$) and after ($n=6$) parturition. Concentrations of RBP4 (A) and retinol (B) in colostrum and milk were measured by Western blot analysis and a spectrophotometric method, respectively. (C) The molar ratio of retinol to RBP4 is also shown. Values are expressed as means ± SEM. *: $P < 0{\cdot}05$ *v.* day 7 and day 15.

(Fig. 3B). In contrast to the fasting experiment, the retinol/RBP4 ratio was not changed significantly after LPS injection, although there was a tendency for it to decrease in the LPS-treated cows 2–4 h after the injection (Fig. 3C).

Changes in plasma RBP4 and retinol levels in cows before and after parturition

Plasma RBP4 levels at 3–6 d before parturition were 50·7 ± 5·3 μg/ml, which were comparable to those of non-pregnant, non-lactating cows in Experiments I and II. However, on the day of parturition, RBP4 levels were significantly decreased (29·8 ± 5·4 μg/ml) but they had returned to basal levels by 2 weeks after parturition (52·1 ± 2·1 μg/ml) (Fig. 4A). Plasma retinol levels before parturition were 26·6 ± 3·3 μg/dl, which were comparable to those of non-lactating cows. Similarly to RBP4, the retinol levels were significantly decreased on the day of parturition (18·5 ± 2·7 μg/dl) and had returned to basal levels by 15 d after parturition (33·9 ± 4·3 μg/dl) (Fig. 4B). There was no significant difference among the retinol/RBP4 ratios obtained before and after parturition.

Changes in RBP4 and retinol levels in colostrum and milk from cows before and after parturition

In the two milk samples obtained 3–6 d before parturition, RBP4 was hardly detected. However, in colostrum, RBP4 was clearly present at a concentration of 16·4 ± 5·6 μg/ml ($n=6$, Fig. 5A). RBP4 had almost disappeared from the milk of the cows at 7 d and 15 d after parturition. Retinol concentrations in milk (43·5 ± 6·9 μg/dl at 7 d after delivery) were comparable to plasma retinol concentrations, but those in colostrum were markedly higher (295·3 ± 5·9 μg/dl). The molar ratio of retinol to RBP4 in colostrum was almost 20 : 1.

Discussion

In the present study, we have demonstrated for the first time that considerable amounts of RBP4 are present in colostrum, but that it is scarce in milk. The increase in RBP4 levels in colostrum was accompanied by a comparable decrease in plasma RBP4 on the day of calving. The lack of alteration in plasma RBP4 levels during the 60-h fasting experiment suggests that its decrease on the day of calving may not be due to food availability or negative energy status during and shortly after parturition. The mild inflammation induced during the process of parturition may acutely decrease in plasma RBP4 as evidenced by the experiment with LPS. However, in bovine colostrum, a variety of non-nutrient bioactive compounds such as immunoglobulin G (IgG), growth hormone, prolactin, insulin and IGF-I are present, many of which are derived from blood (Barrington et al. 2001; Van de Perre, 2003; Taylor et al. 2004; Blum, 2006). In addition, since RBP4 was substantially lacking in milk, it is unlikely that RBP4 is synthesized in the mammary gland. Therefore, it is most likely that RBP4 is transferred from the blood to colostrum, resulting in a decrease its plasma levels.

In milk, β-lactoglobulin acts as a retinol carrier and thereby enhances intestinal retinol uptake in calves (Said

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et al. 1989; Perez & Calvo, 1995). As one molecule of RBP4 binds to one molecule of retinol (Noy, 2000) the presence of almost a 20-fold excess of retinol over RBP4 in colostrum suggests that β-lactoglobulin is the primary retinol carrier in colostrum as well as in milk. Thus, the physiological relevance of colostrum RBP4 as a retinol carrier is currently unknown, but it is plausible that RBP4 contributes to calf development as a metabolic regulator, as described in rodents (Yang et al. 2005). It is, however, unlikely that maternal RBP4 contributes to the circulating RBP4 level in calves, although some macromolecules in colostrum such as IgG are transported across the intestinal epithelium into the neonatal circulation (Barrington et al. 2001; Van de Perre, 2003; Blum, 2006). This is because RBP4 is detected in the plasma of calves shortly after birth (before being fed colostrum) and its levels are unchanged one day after colostrum feeding, although its levels are approximately 50% of those in dairy heifers and cows (Nonnecke et al. 2001).

Nonnecke et al. (2001) also showed a positive correlation between plasma retinol and RBP4 levels in pre-ruminant calves (from birth to 27 d of age) fed a milk replacement with different amounts of supplemental vitamin A. A similar relationship between circulating retinol and RBP4 levels is observed in children and infants (Shenai et al. 1990; Craft, 2001; Aeberli et al. 2007) suggesting that RBP4 is a surrogate marker for retinol. However, the apparent dissociation of the plasma retinol levels from the plasma RBP4 levels seen during the 60-h fasting indicates that their levels in the plasma of cows are regulated independently of each other. Moreover, there were rapid changes in both plasma retinol and RBP4 levels after LPS challenge, and both changes seemed to be independent. The decrease of plasma retinol caused by LPS challenge was confirmed by a similar finding showing a decrease in plasma retinol concentrations immediately after intramammary injection of *Esch. coli* (Van Merris et al. 2004) and may be attributed to an increase of vitamin A consumption in various tissues such as the lung during inflammation (Kanda et al. 1990). On the other hand, the decrease of plasma RBP4 induced by LPS injection was probably due to the inhibition of hepatic RBP4 synthesis, as observed in rats (Rosales et al. 1996). It is therefore suggested that plasma RBP4 levels in cows are regulated independently of retinol concentrations under these conditions.

In summary, RBP4 was found in abundance in cow colostrum, accompanied by a comparable decrease in plasma RBP4, suggesting that RBP4 is transported from the blood to colostrum. In addition, RBP4 and retinol levels are independently regulated under physiological and pathophysiological conditions.

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