Modification of the analysis of parathyroid hormone-related protein in milk and concentrations of this protein in commercial milk and milk products in Japan¹

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ABSTRACT

Parathyroid hormone-related protein (PTHrP), which causes hypercalcemia associated with malignant tumors, is known to be present in milk. Gene expression of PTHrP in the mammary gland increases markedly during parturition and with the onset of lactation. Even when circulating PTHrP levels are extremely low or below the detection limit, milk PTHrP levels are remarkably high. Parathyroid hormone-related protein derived from the mammary gland is assumed to play a role in maintaining the maternal calcium homeostasis and calcium transport from blood to milk. In previous studies that determined the PTHrP concentrations in milk, the pretreatments and diluent composition were not standardized. Here, we investigated the effect of various pretreatment procedures and diluent constitutions and the consequent PTHrP concentrations in commercial milk and milk products in Japan. Significant differences were found in PTHrP concentrations in raw milk samples subjected to different combinations of pretreatments (mixing, centrifugation, acidification, and heating) and diluents (0 pM standard solution of PTHrP, plasma treated with protease inhibitors, and original diluent). We measured the PTHrP concentrations in normal liquid milk, processed milk, milk drinks, formulated milk powders, and skim milk powder by using the appropriate combination of pretreatment (acidification) and diluent (plasma treated with protease inhibitors). The PTHrP concentration in normal liquid milk, processed milk, and skim milk powder was as high as that in raw milk (>5 nM), whereas that in milk drinks differed considerably. The PTHrP concentration in infant formulas (<2 nM) was lower than that

in the other milk products. These results indicate that a certain amount of PTHrP is ingested when milk and milk products are consumed.

Key words: cattle, milk, parathyroid hormone-related protein

INTRODUCTION

Twenty years ago, Budayr et al. (1989) reported that milk contains a large amount of parathyroid hormone-related protein (PTHrP). Parathyroid hormone-related protein was initially identified as the most common cause of malignant hypercalcemia. This protein was found to be normally present in various tissues in animals, including the mammary gland. Expression of the PTHrP gene in the mammary gland markedly increases during parturition and with the onset of lactation (Thiede, 1994). Even when circulating PTHrP levels are extremely low or below the detection limit, milk PTHrP levels remain remarkably high in humans, rats, and cows (Thiede, 1994; Seki et al., 1997; Cross et al., 1998). The concentration of PTHrP in bovine milk was reported to be approximately 3.3 nM (Thurston et al., 1990), which is more than 100 times the concentration of PTHrP in the plasma of patients with malignant hypercalcemia. During embryonic mammary development, PTHrP has a critical role in nipple formation and branching morphogenesis (Wysolmerski and Stewart, 1998). An experiment involving the conditional ablation of PTHrP expression in the mouse mammary gland showed that the mammary gland secretes PTHrP into the circulation during lactation, and this protein promotes calcium resorption from the bone and kidney to maintain maternal Ca homeostasis (VanHouten et al., 2003). Barlet et al. (1992) demonstrated that the infusion of PTHrP into the jugular vein in goat induced significant increases in the levels of Ca, P, and Mg in milk, indicating that PTHrP stimulates mineral transport in the mammary gland. Law et al. (1991) demonstrated that milk PTHrP levels showed a positive correlation with milk Ca levels in cows. In contrast, no

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significant correlation was observed in rats (Yamamoto et al., 1992). In the mammary gland, alternative splicing of PTHrP generates 3 initial translation products, namely, PTHrP [1–139], PTHrP [1–141], and PTHrP [1–173] (Andersson et al., 1997). Intact PTHrP undergoes extensive posttranslational processing because it has many lysine- and arginine-rich regions, which react easily with protease, especially in PTHrP [88–108]. Different regions of PTHrP are assumed to have different physiological functions. The amino terminal of PTHrP, PTHrP [1–36], functions like parathyroid hormone (PTH) through classical PTH/PTHrP receptor. The middle region of PTHrP is thought to be involved in placental Ca transport. The carboxy terminal of PTHrP, PTHrP [107–139], appears to inhibit osteoclastic bone resorption (Wysolmerski and Stewart, 1998). Although 20- and 21-kDa species of PTHrP have been identified in milk (Ratcliffe et al., 1990; Thurston et al., 1990), the other forms of PTHrP present in milk and circulation remain unknown. Moreover, the physiological roles of PTHrP when present in high concentrations in milk remain unclear. Many studies have reported high PTHrP concentrations in milk; however, the pretreatments and diluent compositions used differ among these studies. Although the specific function of PTHrP in milk is presently unclear, it is important to determine the accurate concentration of PTHrP in milk and milk products, because humans might be ingesting appreciable quantities of this protein. Therefore, we compared the concentration of PTHrP in milk samples that were subjected to various pretreatments and dilutions by using diluents of different compositions; we also analyzed the PTHrP concentration in commercial Japanese milk and milk products.

MATERIALS AND METHODS

Pretreatments and Diluents for Raw Milk

Raw milk was collected from 5 lactating Holstein dairy cows during their regular milking routine and immediately transported to the laboratory on ice. The raw milk samples were subjected to 4 different pretreatments, and the pretreated samples were diluted with 3 different diluents. The PTHrP concentration was measured using an immunoradiometric assay (IRMA) kit (Mitsubishi Kagaku Iatron, Tokyo, Japan) previously validated for the measurement of bovine PTHrP concentrations (Onda et al., 2006). The raw milk samples were subjected to the following 4 pretreatments: vortexing (hereafter referred to as mixing) and then centrifuging at $15,000 \times g$ for 20 min at 4°C (hereafter referred to as centrifugation), adding 100 μ L of 2 M acetic acid to 1 mL of the samples, and vortexing and centrifuging

the resultant solution (hereafter referred to as acidification). The other raw milk samples were heated at 65°C for 5 min and then subjected to acidification and centrifugation (hereafter referred to as heating). The supernatants obtained on centrifugation were collected by carefully avoiding the floating lipid layer, and the sediments thus obtained were used for the PTHrP assay. A 0 pM standard solution of PTHrP (hereafter referred to as the 0 pM diluent; contains no PTHrP) was provided with the kit. The plasma used for dilution was treated with 0.01 M EDTA-2Na and 500 kallikrein inhibitor units (KIU)/mL aprotinin (Trasylol, Bayer, Leverkusen, Germany), hereafter referred to as the plasma diluent. The original diluent consisted of 0.01 M EDTA-2Na, 0.01% sodium azide, 5% horse serum, 0.1% Triton X-100, 0.5% BSA, and 500 KIU/mL aprotinin in PBS. The pH of this diluent was adjusted to 7.4 by adding NaOH; thus, our diluent differed from the diluents used in a previous report (Ratcliffe et al., 1992), and is hereafter referred to as the original diluent. All the chemicals, except for aprotinin, were purchased from Kanto Kagaku (Tokyo, Japan). To ensure that the PTHrP concentrations in the milk samples were within the detectable range for the PTHrP assay (1 to 100 pM), the milk samples were diluted to 1:400 with each of the 3 diluents.

Dose–Response Curve

The human PTHrP [1–87] provided in the kit was diluted with the 0 pM and plasma diluents. One raw milk sample (pretreated by acidification) and maltose-binding protein-linked recombinant bovine PTHrP [1–141] (produced using an *Escherichia coli* system; Onda et al., 2006) were diluted to approximately 100 pM with the plasma diluent. All the samples were diluted in 2-fold graded dilutions starting from 100 pM, and the PTHrP concentrations were analyzed using the abovementioned IRMA kit, with human PTHrP [1–87] as the standard.

Commercial Milk and Milk Products

We analyzed the PTHrP concentrations in commercial milk and milk products, which were classified according to the amended portions of the ministerial ordinance on Food Sanitation Law Enforcement Regulations and the ordinance concerning the compositional standards for milk and milk products in Japan (2001). We measured the PTHrP concentrations in 6 normal liquid milk samples, 3 processed milk samples, 4 milk drinks, 5 formulated milk powders, and 1 skim milk powder sample (Table 1). The samples were obtained from different retail suppliers. All the samples of formu-

lated milk powder were prepared using infant formulas. The formulated milk powder and skim milk powder samples were reconstituted according to the instructions provided on the package. The samples were pretreated by acidification and diluted to 1:200 with the plasma diluent, as described in the previous section. The same IRMA kit was used to evaluate each commercial milk and milk product sample in triplicate.

Statistical Analysis

Concentrations of PTHrP in raw milk resulting from the combinations of different pretreatments and diluents were subjected to 2-factor factorial ANOVA by using statistical software (Statcel2, OMS Publishing, Saitama, Japan). When the concentration level of a group was significant ($P < 0.01$), the means of each pretreatment and each diluent were compared using Tukey's multiple comparison test. The results are expressed as means and standard errors.

RESULTS

Pretreatments and Diluents for Raw Milk

Statistical analysis using 2-factor ANOVA showed no interaction between the pretreatments and diluents (Table 2). Tukey's multiple comparison test revealed that the PTHrP concentration in the samples used for mixing was significantly higher than that in the samples subjected to the other 3 pretreatments, and acidification and heating did not significantly affect the PTHrP concentration (Figure 1A). Milk samples diluted with the original diluent showed the highest mean PTHrP concentration, whereas those diluted with the plasma diluent showed the lowest PTHrP concentration (Figure 1B). The combination of mixing and the original diluent yielded the highest PTHrP concentration [mean (SE)]: 28.4 (4.8) nM, whereas the combination of centrifugation and the plasma diluent yielded the lowest concentration: 2.6 (0.6) nM. In the subsequent experiments, we used acidification as the pretreatment method and plasma as the diluent for the samples for the following reasons. With regard to the pretreatment, the existence of lipids and proteins in samples may interfere with the mixing and centrifugation; moreover, acidification of the samples is easier than heating. With regard to the diluent, the amount of 0 pM diluent provided with the kit is not enough for analyzing the PTHrP concentration in milk, and the original diluent may have a nonspecific background of PTHrP. The PTHrP concentration in acidified raw milk samples that were diluted with the plasma diluent was 6.0 (0.6) nM.

Table 1. Japanese milk and milk products analyzed in this experiment

Ingredient classification	Sample ¹	SNF (%)	Fat (%)	Source	Pasteurization	Type
Milk						
	Normal liquid milk					
	a	>8.3	>3.5	Raw milk (100%)	130°C, 2 s	
	b	>8.3	>3.5	Raw milk (100%)	130°C, 2 s	
	c	>8.3	>3.5	Raw milk (100%)	130°C, 2 s	
	d	>8.3	>3.5	Raw milk (100%)	65°C, 30 min	
	e	>8.3	>3.5	Raw milk (100%)	130°C, 2 s	
	f	>8.3	>3.5	Raw milk (100%)	130°C, 2 s	
Processed milk	g	10.0	1.0	Raw milk (<50%), skim milk powder	130°C, 2 s	Low fat
	h	9.0	0.8	Raw milk (<50%), skim milk powder	130°C, 2 s	Low fat
	i	9.5	0.1	Concentrated skim milk	130°C, 2 s	No fat
Milk products						
	Milk drinks					
	j	8.7	4.3	Normal liquid milk, milk product, etc.		High fat
	k	8.6	3.0	Normal liquid milk, milk product, etc.		Calcium accretion
	l	9.0	1.5	Milk product, milk protein, etc.		Low fat
	m	8.0	0.6	Milk product, calcium carbonate, etc.		Low fat, calcium accretion
Formulated milk powder	n	3.6 ²	3.6 ²	Milk product, milk protein, lactose, etc.		Infant formula
	o	3.6 ²	3.6 ²	Milk product, whey protein, skim milk powder, etc.		Infant formula
	p	3.5 ²	3.5 ²	Milk product, whey protein, etc.		Infant formula
	q	3.5 ²	3.5 ²	Milk product, whey protein, lactose, etc.		Infant formula
Skim milk powder	r	3.5 ²	3.5 ²	Milk product, whey protein, lactose, etc.		Infant formula
	s	<0.1 ²	<0.1 ²	Skim milk powder		Infant formula

¹Sample letters correspond with those on x-axis of Figure 3.

²Values were calculated on the basis of the information provided in the ingredient labels on the products used to prepare the infant formulas and skim milk drinks.

Figure 1. Differences in the concentrations of parathyroid hormone-related protein (PTHrP) among raw milk samples subjected to different pretreatments, namely, mixing, centrifugation (CFG), acidification (Acid), and heating (A), and diluted with different diluents, namely, 0 pM standard solution of PTHrP (0 pM diluent), plasma treated with protease inhibitors (plasma diluent), and original diluent (B). ^{a-c}Statistically significant differences ($P < 0.05$), as determined by Tukey's multiple comparison test. Data are presented as the mean (SE). Details are provided in the Materials and Methods section.

Dose–Response Curve

Two-fold graded dilutions of all the samples starting from 100 pM were verified by comparing their dilution curves with the standard reference curve for human PTHrP [1–87] diluted with 0 pM diluent (Figure 2).

Commercial Milk and Milk Products

The PTHrP concentrations in normal liquid milk, processed milk, and skim milk powder were high—almost equal to those in raw milk. The PTHrP concentrations in the milk drinks greatly varied and those

Figure 2. Dose–response curves for raw milk (●), human parathyroid hormone-related protein (PTHrP) [1–87] (▲), and maltose-binding protein (MBP)-bovine PTHrP [1–141] (▼), which were diluted in 2-fold graded dilutions with plasma treated with 0.01 M EDTA-2Na and 500 KIU (kallikrein inhibitor units)/mL of aprotinin. The normal standard curve represents human PTHrP [1–87] (100 pM; □), which was diluted with the 0 pM standard solution of PTHrP that was provided in the commercial kit for preparing 2-fold graded dilutions of the samples. CPM = counts per minute.

in infant formulas were lower than in the other milk products (Figure 3).

DISCUSSION

Presently, the PTHrP concentration in blood and milk is determined by radioimmunoassay or 2-site IRMA. The IRMA kit used in this experiment contained rabbit anti-human PTHrP [50–83] as the indicator antibody, mouse anti-human PTHrP [1–34] as the solid-phase antibody, and ¹²⁵I-labeled anti-rabbit γ -globulin antibody as the tracer. This kit enables the detection of the bioactive PTHrP fragment, including the sequence from the amino terminal to the middle region, and of longer PTHrP species (Fukumoto et al., 1992); therefore, this kit is suitable for analyzing PTHrP in milk because milk reportedly contains PTHrP [1–108] and PTHrP [1–141] (Law et al., 1991).

In this experiment, before measuring the PTHrP concentration, we treated the milk samples by using various procedures as follows. Raw milk was used as a sample (VanHouten et al., 2003) and was initially heated (Uemura et al., 1997). After heating, the sample was subjected to acidification to remove most of the ca-

Table 2. Results of 2-factor factorial ANOVA for the treatment of milk with diluent

Factor	Sum of squared deviation	df	Mean square	P-value
Treatment	1,478.9	3	493	<0.01
Diluent	1,754.93	2	877.47	<0.01
Interaction	223.5	6	37.25	0.27

Figure 3. Concentrations of parathyroid hormone-related protein (PTHrP) in commercial Japanese milk and milk products. Data are presented as the mean (SE).

sein (Goff et al., 1991; Kocabagli et al., 1995) and then centrifuged to exclude the lipid layer (Seki et al., 1997; Cross et al., 1998). Cross et al. (1998) reported that the total PTHrP concentrations in raw milk samples after centrifugation at 15,000 rpm for 30 min were in the range of 77 to 84%. They reported that this decrease in PTHrP concentration after centrifugation was because of the interference with the lipid content of milk. Our results showed that the PTHrP concentration in the mixed samples decreased to 27.8% after centrifugation and was 72.8% after acidification. Lipids, which non-specifically increase the PTHrP concentration of milk, were detected by the Gerber method only in the mixed samples. The protein concentration in the mixed samples was higher than that in the samples subjected to the other 3 pretreatments, as determined by the Lowry method (data not shown). By subjecting equal volumes of the sample to SDS-PAGE, we confirmed that the casein fractions had been completely removed after acidification and heating (data not shown). No significant difference in the PTHrP concentrations was observed between the acidified and heated samples; therefore, we selected acidification as the pretreatment procedure in this study. With regard to the diluent, the 0 pM diluent

provided in the commercial kit is specifically for analyzing PTHrP in plasma samples that are treated with protease inhibitors. Moreover, only a limited volume of the 0 pM diluent is provided with the kit. However, because milk contains a large amount of PTHrP, substantial dilution is essential. Phosphate-buffered saline, cell culture media (minimal Eagle's medium, Dulbecco's minimal Eagle's medium, and medium 199), and a plasma substitute (6% hydroxyethylated starch) were not used as diluents because they have a nonspecific background of PTHrP (maximum amount of diluent that can be used <2.9 pM; data not shown). This was also the case with the original diluent; therefore, we selected the plasma diluent, in which PTHrP was not detected, for use in our study. We confirmed that the dose-response curve obtained after dilution with the plasma diluent was identical to that obtained with the 0 pM diluent.

Despite the use of the same 5 raw milk samples and the same IRMA procedure for testing all samples, the highest PTHrP concentration was 10 times greater than the lowest PTHrP concentration. The cause of this wide difference in concentration is unclear. The mixed samples had high protein and lipid contents, and the

original diluent may have nonspecifically increased the PTHrP concentration. Therefore, when comparing the concentrations of PTHrP obtained in this experiment with those reported in other papers, we considered not only the measurement methods but also the pretreatments and diluents used for the milk samples.

Unlike the case in other countries, in Japan, ultra-high-temperature heating (120–150°C for 1–5 s) is the most widely used method for milk pasteurization. To the best of our knowledge, the concentration of PTHrP in Japanese commercial milk has been reported in only one study (Otsubo et al., 1990). In the present experiment, we used 2-site IRMA to confirm the results of the bovine PTHrP assay and to determine the assay conditions. Although different analyses, pretreatments, and diluents have been used in different studies, the results of our experiment showed the same tendency as those of previous studies: PTHrP concentrations in normal liquid milk and processed milk are similar to those in raw milk (Ratcliffe et al., 1990; Law et al., 1991), whereas infant formulas generally show lower PTHrP concentrations (Budayr et al., 1989; Otsubo et al., 1990). The addition of a protease inhibitor is essential and routine during the measurement of plasma PTHrP concentrations (Pandian et al., 1992). However, a protease inhibitor is not added to raw milk samples when measuring PTHrP concentrations; instead, these samples are immediately frozen until analysis. Because of this, the properties of PTHrP observed in milk might differ from those observed in plasma. The raw milk samples analyzed in this experiment were not frozen but were immediately used for the measurement of PTHrP. Normal liquid milk and processed milk are usually pasteurized and packaged separately; these commercial milk samples contain a large amount of PTHrP. Skim milk powder contains high levels of PTHrP because this product is obtained by removing fat and moisture from raw milk or normal liquid milk. Although processed milk contains raw milk, which is rich in PTHrP, the proportion of raw milk to processed milk is low (<50%). Nevertheless, the PTHrP concentration of processed milk is high because it contains either skim milk powder or concentrated skim milk. The PTHrP concentration varied greatly among milk drinks, and we speculate that the PTHrP concentration in these drinks depends on the preexisting level of normal liquid milk or milk products in each drink. All the 5 infant formulas were found to have low PTHrP concentrations (<2 nM). We confirmed that 3 of the 5 infant formulas tested contained skim milk powder as a milk product. The low level of PTHrP in infant formulas is probably because of 1) the low PTHrP level in skim milk powder and milk products or 2) the degeneration of the immunoreactive structure of PTHrP during the purification

of milk nutrients and peptidization, which is performed to reduce allergenic activity.

Some researchers have reported that milk PTHrP is not essential for growth and Ca homeostasis in pups. This was confirmed by studies in which antibody treatment (Kukreja et al., 1991) and conditional knockout mice (VanHouten et al., 2003) were used to obtain milk that did not contain PTHrP. Although a 10-fold difference was found between the highest and lowest PTHrP concentration, the nanomolar-order concentration of PTHrP in milk and milk products was much higher than that in the plasma of patients with hypercalcemia of malignancy. Thus, a certain amount of ingested PTHrP reaches the digestive tract in humans after the consumption of milk and milk products. Further studies are required to clarify the importance and functions of PTHrP as one of the bioactive components of milk.

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