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Original article

Breast milk stimulates growth hormone secretion in infant mice, and phosphorus insufficiency disables this ability and causes dwarfism-like symptoms

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ABSTRACT

Introduction: Breast milk intake facilitates neonatal growth, and its effect is assumed to last long into the adulthood. We recently reported that dietary phosphorus insufficiency reduces the ability of breast milk to promote infant growth in mice. However, how phosphorus confers this ability to milk is still unclear.
Methods: To address this issue, we performed biochemical and physiological comparisons of milk secreted from C57BL/6J mice fed a low-phosphorus diet (LPD) or a normal-phosphorus control diet.
Results: Although serum phosphorus concentration was decreased, the body weight of mother mice was unaffected. By contrast, infant body weight was significantly reduced, and dwarfism-like symptoms were observed in adulthood. Quantitative analysis revealed that the serum concentration of growth hormone (GH) was substantially reduced, and concomitantly insulin-like growth factor 1 and fibroblast growth factor 23 were decreased. Immunohistochemical analysis revealed ectopic fat accumulation in the livers of infant mice along with increased blood cholesterol level. Moreover, electron microscopy indicated fragility of the outer membrane of milk droplets.
Conclusions: Our results suggest that phosphorus is essential for the formation of milk droplets, which function as a stimulator of growth factor secretion in infant offspring.

1. Introduction

Body growth depends on the transport of a variety of nutrients between tissues and their subsequent retention inside cells. In mammals, maternal nutrient transfer via the placenta and umbilical cord in the fetus controls fetal growth, whereas the supply from mothers to nursing babies via milk, regulates infant growth [1]. The importance of milk as a major nutrient source to infants is well established; however, the effect of milk consumption on growth is still under debate.

Milk contains highly concentrated nutrients including proteins and minerals such as calcium, potassium, and phosphorus [2]. Milk also contains regulators of body growth by stimulating secretion of endocrine hormones in infants [3]. In addition, dietary consumption of cow's milk increases blood concentration of insulin-like growth factor 1 (IGF-1), an important factor in body growth during development [4].

Phosphate is the second most abundant mineral nutrient in the mammalian body, and the regulation of phosphate metabolism has both clinical and biological significance [5–7]. Phosphate imbalance can lead to a wide range of disorders; for example, excessive

Abbreviations: FGF23, fibroblast growth factor 23; GH, growth hormone; IGF-1, insulin-like growth factor 1; LPD, low-phosphorus diet; NPD, normal phosphorus diet.

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phosphorus intake causes hypocalcemia and hyperphosphatemia. However, the effect of dietary insufficiency is unclear. We recently reported that dietary phosphorus insufficiency affected the ability of milk to promote infant growth in mice, particularly causing bony malformation [8]. Because the crucial role of milk in infant growth is still controversial, mother mice fed a low-phosphorus diet (LPD) appears to be a good model for analyzing the milk function. Here, we explored the molecular mechanism underlying infant growth via milk in mice.

2. Materials and methods

2.1. Components of minerals in the diet

The normal phosphorus diet (NPD) included the following minerals per total weight of the diet (weight/weight) (w/w): KH_2PO_4 (1.7%), $CaHPO_4 \cdot 2H_2O$ (1.5%), $MgSO_4 \cdot 7H_2O$ (0.8%), NaCl (0.6%), and $FeC_6H_5O_7 \cdot 5H_2O$ (0.2%). LPD included the following minerals per total weight of the diet (w/w): KH_2PO_4 (0%), $CaHPO_4 \cdot 2H_2O$ (0%), $MgSO_4 \cdot 7H_2O$ (0.8%), NaCl (0.6%), and $FeC_6H_5O_7 \cdot 5H_2O$ (0.2%). LPD contained 18.5% of the phosphorus of NPD.

2.2. Mouse diet and housing

C57BL/6J mice were purchased from Japan SLC Inc., Shizuoka, Japan. After weaning, mice were divided into two groups and fed either LPD or NPD. On maturity at 8 weeks old, mice were bred within each group to produce the next generation. Until the end of their weaning period, the next generation of mice was fed with milk generated by mothers continuously that fed with either LPD or NPD. The mice were housed in specific pathogen-free controlled conditions. Food and water were available *ad libitum*. The procedures for animal experiments were performed in accordance with the principles and guidelines of the Care and Use of Laboratory Animals of the National Institute for Child Health and Development. The animal committee approved all experiments performed in the present study.

2.3. Measurement of body weight and height

Body weight and height were measured each day between 1200 and 1400 h and compared between the groups of mice fed LPD or NPD.

2.4. Collection of milk from mother mice

To measure cytokine levels, milk was directly collected from the nipples of mice with a teat cup attached to a mouth pipette (NATSUME SEISAKUSHO Co. Ltd. Tokyo, Japan). After collection, the samples were centrifuged for 10 min at 12,000×*g*, and the supernatants, termed whey, were frozen at −20 °C until use.

2.5. Measurement of growth hormone and IGF-1 levels in the serum and milk

Blood samples were drawn from hearts with a micropipette after overnight fasting, centrifuged for 10 min at 1000×*g*, separated, and frozen at −80 °C until use. Growth hormone (GH) levels in the serum were measured using a GH Rat/Mouse Growth Hormone ELISA kit (Merck Millipore, MA, USA) according to the manufacturer's instructions. IGF-1 was measured by an IGF-1 Mouse ELISA kit (Abcam, Massachusetts, USA) according to manufacturer's instructions.

2.6. Measurement of cholesterol

Total cholesterol was measured by a cholesterol oxidase DAOS method (LabAssay Cholesterol, Wako Pure Chemical Industries, Ltd., Japan).

2.7. Histochemical analysis

To observe pathological symptoms in liver tissues, a histochemical analysis was performed as described previously [9]. Briefly, after mice were sacrificed, their livers were isolated and embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek Japan Co., Ltd. Japan), and 5-μm sections were prepared from frozen tissues. The sections were stained with Oil red O (lipid stain).

2.8. Statistical analysis

Comparisons were made using one-way analysis of variance following Scheffe's method, Mann–Whitney *U* test, or Fisher's exact test. Statistical significance was defined as $P < 0.05$. Results were expressed as mean ± SD.

2.9. Electron microscopy of milk

Milk samples were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4. After this fixation the samples were postfixed with 2% osumium tetroxide in 0.1 M cacodylate buffer. After dehydrated in ethanol solutions, the samples were infiltrated with propylene oxide (PO) and were put into a 70:30 mixture of PO and resin (Nisshin EM Co., Tokyo, Japan) for 1 h, then PO was volatilized overnight and the samples were transferred to a fresh 100% resin, and were polymerized at 60 °C for 48 h. The polymerized resins were ultra-thin sectioned at 70 nm with a diamond knife using an ultramicrotome (Leica, Vienna, Austria).

2.10. Observation and imaging

The grids were observed by a transmission electron microscope (JEM-1400Plus; JEOL Ltd., Tokyo, Japan) at an acceleration voltage of 80 kV. Digital images (2048 × 2048 pixels) were taken with a CCD camera (VELETA; Olympus Soft Imaging Solutions GmbH, Münster, Germany).

3. Results

3.1. Phosphorus-deficient dietary intake

To study the influence of phosphorus insufficiency on homeostasis, mice were fed LPD, with 18.5% of the phosphorus in the NPD control diet. Since NPD and LPD were yellow and white in color, respectively, the two diets were easily distinguishable (Fig. 1a). Despite outward differences, the amounts consumed daily by male and female mice were similar between groups (Fig. 1b). As depicted in Fig. 1c, C57BL/6J male and female mice were divided into two groups and were considered “first generation” mice; they were fed either NPD or LPD. Mice delivered from first-generation mothers were considered as the “second generation” mice and were also fed either NPD or LPD.

First-generation mice were divided into two groups with equal body weight at 21 days after birth (8.9 ± 1.18 g for Group 1 [n = 25] and 8.9 ± 1.29 g for Group 2 [n = 26]) (Fig. 2a). There was no significant difference in body weight between first-generation mice fed LPD and those fed NPD from 4 to 11 weeks (4-week-old mice: 9.6 ± 1.80 g for LPD [n = 8] and 10.3 ± 1.44 g for NPD [n = 12]; 5-