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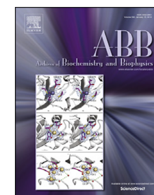

# Intestinal mucosal changes and upregulated calcium transporter and FGF-23 expression during lactation: Contribution of lactogenic hormone prolactin

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ABSTRACT

As the principal lactogenic hormone, prolactin (PRL) not only induces lactogenesis but also enhances intestinal calcium absorption to supply calcium for milk production. How the intestinal epithelium responses to PRL is poorly understood, but it is hypothesized to increase mucosal absorptive surface area and calcium transporter expression. Herein, lactating rats were found to have greater duodenal, jejunal and ileal villous heights as well as cecal crypt depths than age-matched nulliparous rats. Morphometric analyses in the duodenum and cecum showed that their mucosal adaptations were diminished by bromocriptine, an inhibitor of pituitary PRL release. PRL also upregulated calcium transporter expression (e.g., TRPV6 and $PMCA_{1b}$) in the duodenum of lactating rats. Since excessive calcium absorption could be detrimental to lactating rats, local negative regulator of calcium absorption, e.g., fibroblast growth factor (FGF)-23, should be increased. Immunohistochemistry confirmed the upregulation of FGF-23 protein expression in the duodenal and cecal mucosae of lactating rats, consistent with the enhanced FGF-23 mRNA expression in Caco-2 cells. Bromocriptine abolished this lactation-induced FGF-23 expression. Additionally, FGF-23 could negate PRL-stimulated calcium transport across Caco-2 monolayer. In conclusion, PRL was responsible for the lactation-induced mucosal adaptations, which were associated with compensatory increase in FGF-23 expression probably to prevent calcium hyperabsorption.
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## 1. Introduction

Besides being the salient lactogenic hormone during lactation, prolactin (PRL) has been recognized as a calcium-regulating hormone that helps supply calcium for milk production, while 1,25-dihydroxyvitamin $D_3$ [$1,25(OH)_2D_3$] becomes less important during this reproductive period [1,2]. To achieve its calciotropic action, PRL has been postulated to act directly on the proximal intestine—the principal site for calcium entry into the body—to increase the intestinal absorptive surface, expression of calcium transporters, or the combination of both. However, direct evidence to support the physiological significance of PRL action on intestinal absorptive surface, particularly in the large intestine, was scant since previous investigations were mostly performed in hyperprolactinemic nulliparous rodents rather than in lactating animals.

Among the small and large intestinal segments, duodenum and cecum are segments with the highest calcium absorption rate per surface area [3–5]. It is possible that PRL enhances the expression of transporters responsible for calcium hyperabsorption in these segments. Generally, the transcellular calcium transport is mediated by apical calcium channels (e.g., TRPV6), cytoplasmic calcium-binding proteins (e.g., calbindin-$D_{9k}$), and basolateral calcium transporters (e.g., $PMCA_{1b}$ and NCX1), whereas calcium transport across the paracellular space is regulated by permselectivity of tight junction, or more precisely the protein components of tight junction (e.g., ZO-1 and claudin-12) [1,6,7]. TRPV6 expression has been

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shown to increase in lactating mice [8], but whether PRL contributed to its upregulation remained elusive.

Since excessive calcium absorption could cause side effects, such as hypercalcemia, increased cardiovascular accident, and kidney stone [9], we further postulated that PRL release during lactation also triggered certain mechanisms, for example, the release of factor(s) to counteract calcium hyperabsorption. One of the factors can be FGF-23 that is an endocrine member of the fibroblast growth factor family. FGF-23 is known as a phosphaturic hormone that increases urinary excretion of phosphate. Recently, we discovered that FGF-23 directly and completely abolished the $1,25(OH)_2D_3$-enhanced duodenal calcium flux in nulliparous mice [10,11], suggesting that FGF-23 could be the negative regulator of intestinal calcium absorption. In other words, FGF-23 is probably a part of feedback loop, which consists of at least two components, i.e., (*i*) calciotropic hormones [e.g., $1,25(OH)_2D_3$ or PRL] for stimulation, and (*ii*) FGF-23 for inhibition of calcium absorption [12]. Under normal conditions, circulating FGF-23 is mostly secreted from osteoblasts and osteocytes in bone [13]; however, it was recently shown that the intestinal enterocytes themselves could express FGF-23 [10]. It was thus postulated that calcium hyperabsorption in lactation could be prevented by PRL-stimulated local production of FGF-23 that, in turn, suppressed calcium absorption.

Therefore, the objectives of the present study were to determine changes in the intestinal mucosal, the duodenal calcium transporter mRNA expression, and FGF-23 protein expression in the intestine of lactating rats. Bromocriptine, a potent inhibitor of pituitary PRL secretion was administered to some lactating rats in order to demonstrate the contribution of PRL to the intestinal mucosal adaptations.

## 2. Materials and methods

### 2.1. Animals

Day 15–16 pregnant and 8-week-old nulliparous Sprague–Dawley rats (*Rattus norvegicus*) were obtained from the National Laboratory Animal Center, Salaya, Nakhon Pathom, Thailand. After delivery (day 0), the litter size was adjusted to eight pups per dam. They were housed in standard stainless steel cages under 12:12 h light–dark cycle (light on at 0600) with average illuminance of 200 lux in the controlled temperature room at 22–25 °C. All rats were fed standard chow containing 1% wt/wt calcium, 0.9% wt/wt phosphorus, and 4000 IU/kg vitamin D (CP, Bangkok, Thailand) and reverse osmosis (RO) water ad libitum. This study has been approved by the Animal Care and Use Committee of the Faculty of Science, Mahidol University, Thailand.

### 2.2. Cell culture

Intestinal epithelium-like Caco-2 cells (American Type Culture Collection no. HTB-37) were grown in Dulbecco's modified Eagle medium (Sigma) supplemented with 15% fetal bovine serum, 1% L-glutamine, 1% nonessential amino acid, 100 U/mL penicillin-streptomycin, and 0.25 μl/mL amphotericin B (Sigma). Cells were propagated in a 75-$cm^2$ T flask (Corning, NY, USA) under a humidified atmosphere containing 5% $CO_2$ at 37 °C and subcultured as described by ATCC. As for the transepithelial calcium flux study, Caco-2 cells were seeded at 250,000 cells/well on polyester Snapwells (12-mm diameter and 0.4-μm pore size; Corning), and maintained for 14 days in humidified 5% $CO_2$ at 37 °C.

### 2.3. Experimental design

To determine the roles of PRL on mucosal adaptation, histomorphometric analysis of the mucosa and investigation of calcium transport-related gene expression and localization of FGF-23 were performed in maternal intestine of lactating rats. Small and large intestines were obtained from day-8 lactating rats (Lact) and the age-matched control nulliparous (Control) rats. For bromocriptine-treated group (Lact + Bromo), day-1 lactating rats were daily injected with 4 mg/kg bromocriptine s.c. (catalog no. B2134; Sigma, St. Louis, MO, USA) for 7 days. This regimen of bromocriptine administration was previously reported to successfully abolish hyperprolactinemia (serum PRL level <50 ng/mL) in lactating rats [14], while other groups were injected with vehicle (normal saline). After 7 days of injection, rats were euthanized, and the intestine was collected for quantitative real-time PCR (qRT-PCR) and histological preparation. In a separate experiment, we also compared FGF-23 mRNA levels in the duodenal mucosal cells of day-8 vs. day-21 lactating rats, the latter of which were on the last day of lactation (total lactating period was 21 days) and thus had lower calcium demand than day-8 rats (early lactation with high calcium demand). Moreover, to investigate the direct effect of PRL on FGF-23, and FGF-23-related transcripts, Caco-2 cells were treated with 100 and 200 ng/mL recombinant human PRL (rhPRL; catalog no. 682-PL; R&D Systems, Minneapolis, MN, USA) for 120 h before total RNA extraction and PCR study. Finally, PRL-exposed Caco-2 monolayers were treated with FGF-23 to demonstrate its inhibitory effect on calcium transport.

### 2.4. Duodenal collection and total RNA extraction

Under general anesthesia (50 mg/kg i.p. pentobarbitone sodium; Ceva Santé Animale, Libourne, France), a midline abdominal incision was made. The 5-cm duodenal segments were removed and cut longitudinally to expose the mucosal surface. Thereafter, tissues were cleaned with ice-cold sterile normal saline and scraped with ice-cold glass slides for mucosal cell collection [15]. Total RNA was extracted by using TRIzol reagent (Invitrogen, Carlsbad, USA) as previously described [14]. Total RNA concentration was determined by NanoDrop-2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA) and the 260/280-nm ratio was read to determine purity of total RNA (ranged 1.8–2.0).

### 2.5. Quantitative real-time PCR (qRT-PCR)

One microgram of total RNA from duodenal mucosal scraping or Caco-2 cells was reverse-transcribed to cDNA by iScript cDNA synthesis kit (Bio-rad, Hercules, CA, USA) using a conventional thermal cycler (model MyCycler; Bio-rad). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to check the consistency of reverse transcription (percent coefficient of variation <5%). Human and rat PCR primers used in qRT-PCR study are shown in Table 1 qRT-PCR and melting curve analyses were operated by Bio-rad MiniOpticon system with SsoFast EvaGreen Supermix (Bio-rad). Amplification reaction was performed for 45 cycles at 95 °C for 5 s and 53–61 °C annealing temperature for 10 s. PCR products were also visualized on 2% agarose gel stained with 1 μg/mL ethidium bromide (Sigma) under a UV transilluminator (Alpha Innotech, San Leandro, USA). The mRNA level was normalized by GAPDH expression.

### 2.6. Tissue preparation for histomorphometric analysis

After a midline abdominal incision was performed, small and large intestinal segments, i.e., duodenum (5 cm), jejunum (10 cm), ileum (8 cm), cecum (4 cm), proximal colon (8 cm), and distal colon (8 cm) were removed. Tissues were washed with ice-cold sterile normal saline, fixed at 4 °C in 4% paraformaldehyde for 12 h,

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