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Bone Calcium Turnover, Formation, and Resorption in Bromocriptine- and Prolactin-Treated Lactating Rats

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To evaluate the effect of endogenous prolactin (PRL) on bone metabolism, we studied bone calcium turnover by the ^{45}Ca kinetic method and bone formation and resorption by bone histomorphometry and biochemical markers in 13-wk-old lactating Wistar rats. For 1 wk, the animals received daily administration of 0.9% NaCl (control) intraperitoneally, 6 mg of bromocriptine/kg of body wt intraperitoneally, or 6 mg of bromocriptine/kg of body wt plus 2.5 mg of ovine PRL/kg of body wt subcutaneously. Bromocriptine, a dopaminergic inhibitor of endogenous PRL secretion, significantly decreased calcium ion deposit rate and calcium resorption rate in femur, tibia, vertebrae 5 and 6, and sternum by 20–42%. By contrast, calcium resorption rate of the vertebrae and the sternum of the PRL-treated group was higher than that of controls, whereas the tibia and sternum exhibited a greater net loss of calcium. The suppression of bone calcium turnover in the bromocriptine-treated group was further supported by a significant decrease in the urinary deoxypyridinoline, a biochemical index of bone resorption, and the histomorphometric data, which showed changes indicative of suppressed bone resorption and formation. The histomorphometric data from the PRL-treated group were not different from those of the control group with the exception of an increase in the longitudinal growth rate. The results suggested a role of endogenous PRL in the stimulation of bone turnover during lactation.

Key Words: Bone formation; bone resorption; bromocriptine; histomorphometry; lactation; prolactin.

Introduction

Pregnancy and lactation impose a substantial drain on maternal calcium stores to satisfy an increased need for fetal mineralization and milk production, respectively. In rats, the amount of calcium loss to pups during lactation could be equivalent to 40% of the maternal skeleton *(1)* and may result in lower plasma calcium concentrations *(2)*. Bone loss was accompanied by an increased bone formation rate *(3)*. A significant decrease in bone mineral density (BMD) was also reported in pregnant and lactating women *(4–6)*, indicating an accelerated bone turnover *(7)*. Calcium metabolism is normally regulated by the parathyroid hormone (PTH)–vitamin D endocrine system. During pregnancy and lactation, adaptive changes to prepare the maternal body for the high calcium demand include elevated levels of $1,25(OH)_2D_3$ *(8,9)* and increased intestinal absorption of calcium *(10)*. However, Halloran and DeLuca *(11)* and Boass et al. *(12)* reported enhanced calcium absorption in vitamin D–deficient pregnant and lactating rats, suggesting that other hormones besides $1,25(OH)_2D_3$, may be responsible for such adaptation during the reproductive states.

Because of elevated serum levels of prolactin (PRL) during pregnancy and lactation *(1,13)*, and its reported stimulatory effects on the intestinal calcium absorption *(14,15)*, it was possible that PRL may play a role in this adaptation. We have previously shown an acute stimulatory effect of a pharmacologic dose of 0.2 mg of PRL/kg of body wt administered intraperitoneally on intestinal passive *(16,17)* and active calcium transport *(18)* in sexually mature Wistar rats. Furthermore, we have demonstrated a physiologic significance of endogenous PRL in the regulation of calcium metabolism. Endogenous PRL was found to increase food intake in pregnant and lactating rats, and to increase fractional calcium absorption in pregnant rats, resulting in increased calcium retention *(19)*. Recently, by comparing control and bromocriptine-treated rats in which endogenous secretion of PRL was inhibited, we demonstrated a physiologic role of PRL in the stimulation of calcium absorption and maintenance of bone calcium content in 3-wk-old rats during 9-wk of growth and development *(20)*.

Thus, it appears that endogenous PRL secreted either at a higher rate during pregnancy and lactation or at a much lower rate in young rats has a significant role in the maintenance of the intestinal calcium absorption, presumably to supply the much-needed calcium for fetal bone and milk calcium during pregnancy and lactation, and also for growth and development in young animals.

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Table 1
Total Calcium Contents of Bones in Lactating Rats on d 12 (Basal Group) and on d 19 of Lactation (Sample Group)[a]

	Calcium content (mmol/g dry wt)			
		Sample group (d 19)		
Bone	Basal group (d 12) (n = 9)	Control (n = 9)	Bromo (n = 9)	PRL (n = 9)
Femur	6.12 ± 0.08	5.87 ± 0.05	5.92 ± 0.07	5.84 ± 0.05
Tibia	6.31 ± 0.10	5.90 ± 0.15	5.99 ± 0.07	5.62 ± 0.10[b]
Vertebrae	5.97 ± 0.11	5.73 ± 0.08	5.81 ± 0.09	5.68 ± 0.08
Sternum	4.54 ± 0.04	4.77 ± 0.07	4.83 ± 0.10	4.48 ± 0.10[b]

[a] The sample group was divided into the saline-treated group (control), bromocriptine-treated group (Bromo), and bromocriptine plus PRL-treated group (PRL).
[b] $p < 0.05$ compared with control.

Table 2
Total ^{45}Ca Contents of Bones in Lactating Rats on d 12 (Basal Group) and d 19 of Lactation (Sample Group)[a]

	^{45}Ca content (% administered dose/g dry wt)			
		Sample group (d 19)		
Bone	Basal group (d 12) (n = 9)	Control (n = 9)	Bromo (n = 9)	PRL (n = 9)
Femur	0.91 ± 0.09	0.38 ± 0.03	0.48 ± 0.04[b]	0.31 ± 0.02
Tibia	1.02 ± 0.11	0.41 ± 0.03	0.54 ± 0.03[b]	0.34 ± 0.02
Vertebrae	0.97 ± 0.07	0.37 ± 0.01	0.62 ± 0.04[b]	0.28 ± 0.02[b]
Sternum	0.99 ± 0.05	0.41 ± 0.03	0.62 ± 0.04[b]	0.30 ± 0.02[b]

[a] The sample group was divided into the saline-treated group (control), bromocriptine-treated group (Bromo), and bromocriptine plus PRL-treated group (PRL).
[b] $p < 0.01$ compared with control.

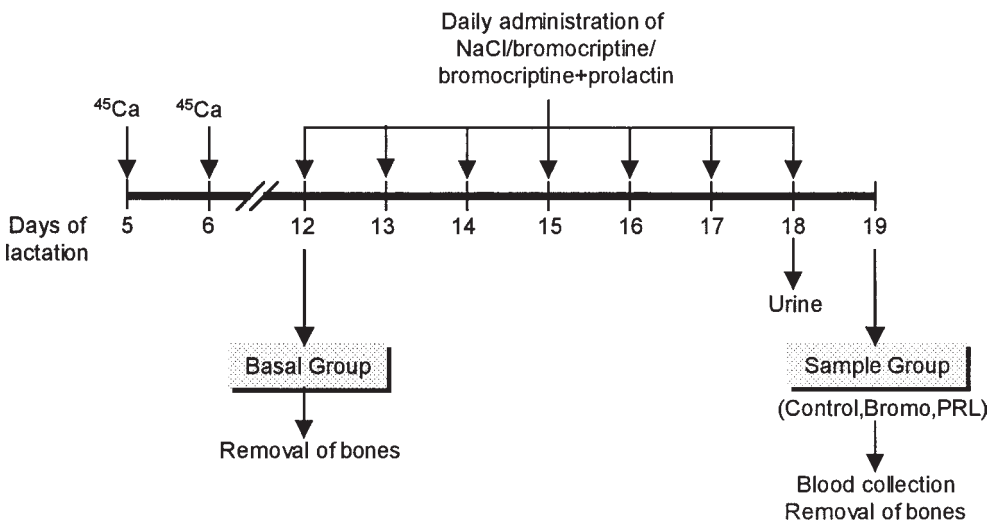

Fig. 1. Experimental protocol. Animals of the basal group were sacrificed 6 d after the second ip injection of 1.25 mM $CaCl_2$ + ^{45}Ca. The sample groups received the same dose, and 6 d later received the first daily ip injection of 0.9% NaCl (control), 6 mg of bromocriptine/kg of body wt (Bromo) or 6 mg of bromocriptine/kg of body wt plus 2.5 mg of PRL/kg of body wt (PRL).

Bone is the largest reservoir of calcium in the body and has a major role in supplying calcium to the extracellular fluid compartment for maintenance of serum calcium. We previously found that daily sc injection of 2.5 mg of PRL/kg of body wt for 13 d significantly increased bone formation and tibia calcium content in growing rats *(21)* and increased bone turnover in weaned and sexually mature rats *(22)*. In lactating rats, the calcium requirement for milk production cannot be met entirely by increased calcium intake. Thus, it was pertinent to evaluate the role of PRL in the regulation of bone calcium turnover in lactation especially when as much as 19% of the calcium transferred to milk is derived from the maternal skeleton *(23)*. In the present study, by using both the calcium kinetic method and histomorphometric analyses, we found evidence of the physiologic role of PRL in the stimulation of bone calcium turnover in lactating rats.

Results

Kinetic Studies

Tables 1 and 2 and Fig. 1 depict data from the ^{45}Ca kinetic study in the control, bromocriptine-treated, and bromocriptine plus PRL–treated lactating rats. Total calcium content and total ^{45}Ca content in femur, tibia, vertebrae 5 and 6, and sternum of the basal and sample groups are shown in Tables 1 and 2, respectively. Bones of the basal group were harvested on d 12 of lactation and those of the sample groups on d 19.

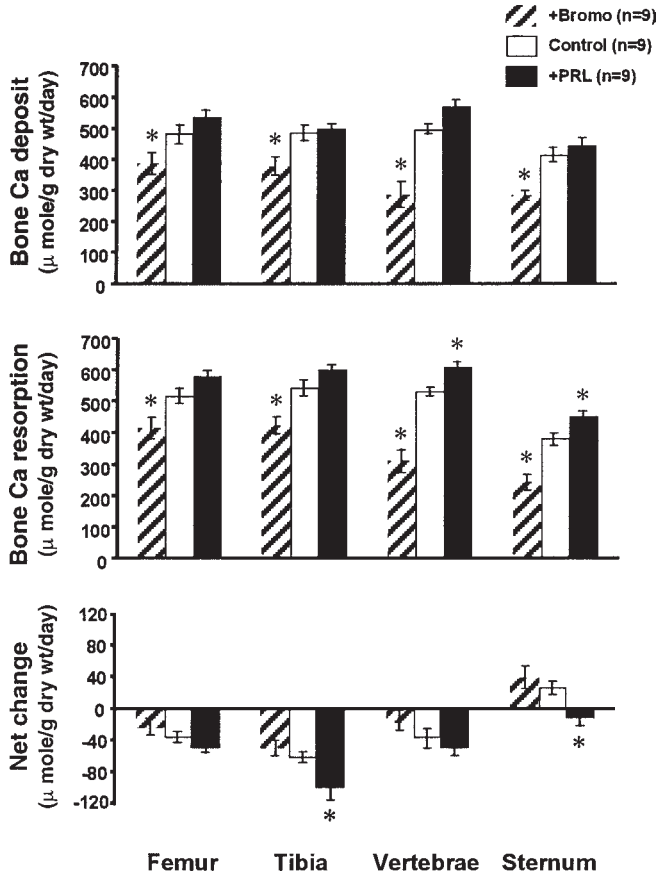

Fig. 2. Bone calcium deposit (μmol/[g dry wt·d]), bone calcium resorption (μmol/[g dry wt·d]), and net change (μmole/[g dry wt·d]) of femur, tibia, lumbar vertebrae 5 to 6, and sternum of lactating rats that received daily injections of 0.9% NaCl (control), 6 mg of bromocriptine/kg of body wt (+Bromo), or 6 mg of bromocriptine/kg of body wt plus 2.5 mg of PRL/kg of body wt (+PRL) for 7 consecutive d. *$p < 0.05$ compared with control.

Considering the total calcium contents in bone, although there were no significant differences between the basal group and the sample control, there was a tendency for control rats to lose bone calcium during lactation. A week of bromocriptine administration to the sample group had no effect on bone calcium content while PRL administration significantly decreased calcium contents in the tibia and sternum. Regarding the ^{45}Ca contents, as expected, they decreased from d 12 to 19 in all four bones because ^{45}Ca was continuously resorbed from bone. However, as seen in Table 2, in the bromocriptine-treated group, the ^{45}Ca contents on day 19 were significantly higher than those of the control, indicating a lower rate of calcium resorption. On the other hand, the vertebrae and sternum of the PRL-treated group had significantly lower ^{45}Ca contents compared with the corresponding control.

Figure 2 depicts calcium turnover rates in four bones, presented as amounts of calcium deposit, calcium resorption, and net changes. As expected, lactating rats exhibited a net loss of calcium from femur, tibia, and vertebrae, as a result of an excess of calcium resorption over calcium deposit. Bromocriptine, which inhibited the endogenous secretion of PRL, suppressed the rates of both deposit and resorption of bone calcium. The net changes in bone calcium contents showed net calcium loss for femur, tibia and vertebrae. As for the sternum, the calcium deposit rate was greater than calcium resorption rate, resulting in a net gain in calcium. Administration of PRL resulted in a significantly greater loss of calcium in the tibia and reversed net calcium gain to net calcium loss in the sternum.

Table 3
Bone Alkaline Phosphatase
of Lactating Rats That Received Daily Administration
of 0.9% NaCl (control), 6 mg of Bromocriptine/kg
of body wt (Bromo), or 6 mg of Bromocriptine/kg of body wt
Plus 2.5 mg of PRL/kg of body wt (PRL)[a]

	Alkaline phosphatase (U/g wet wt)			
	Femur	Tibia	Vertebrae	Sternum
Control ($n = 9$)	13.56 ± 0.87	14.60 ± 1.29	34.64 ± 2.14	27.27 ± 1.33
Bromo ($n = 9$)	13.24 ± 0.87	11.72 ± 0.85	31.09 ± 1.33	25.20 ± 1.85
PRL ($n = 9$)	16.76 ± 1.02[a]	12.13 ± 0.58	38.65 ± 1.29	30.54 ± 2.67

[a] $p < 0.05$, compared with control.

Biochemical Markers of Bone Turnover

Table 3 shows the activity of bone alkaline phosphatase, which is an index of bone formation. Although the levels of alkaline phosphatase in all four bones in bromocriptine-treated lactating rats appeared to be lower than those of controls, the differences were not statistically significant. In the PRL-treated group, only the femur showed a significantly higher value of alkaline phosphatase. However, the activity of urinary deoxypyridinoline, an index of whole-body bone resorption, was markedly reduced in the bromocriptine-treated group: 161.44 ± 13.65 vs 295.45 ± 15.51 nmol/mmol of creatinine ($p < 0.05$) in bromocriptine-treated and control groups, respectively. PRL supplementation returned the enzyme activity to the control value. The urinary deoxypyridinoline data were consistent with the findings from the kinetic study that showed suppression of bone resorption in the bromocriptine-treated lactating rats.

Bone Histomorphometric Studies

Table 4 presents the histomorphometric data from tibial metaphyses and tibial shafts from control, bromocriptine-treated, and bromocriptine plus PRL–treated lactating rats. There were no differences in the body weights of the three groups (data not shown). Bromocriptine administration resulted in a 30% increase in the cancellous bone volume, which was accounted for by an increase in the trabecular

Table 4
Histomorphometric Data from Tibial Metaphysis and Tibial Shaft from Lactating Rats That Received Daily Administration of 0.9% NaCl (control), 6 mg of Bromocriptine/kg of body wt (Bromo), or 6 mg of Bromocriptine/kg of body wt Plus 2.5 mg of PRL/kg of body wt (PRL)

	Control (n = 7)	+Bromo (n = 7)	+PRL (n = 7)
Tibial metaphysis			
BV/TV (%)	13.23 ± 0.41	17.25 ± 1.04[b]	12.82 ± 0.83
dL.S/BS (%)	20.90 ± 2.10	5.47 ± 0.51[b]	24.50 ± 1.31
MAR (μm/d)	1.48 ± 0.08	0.36 ± 0.04[b]	1.60 ± 0.05
BFR/BS ($\mu m^3/[\mu m^2 \cdot d]$)	0.31 ± 0.04	0.02 ± 0.01[b]	0.30 ± 0.03
LGR (μm/d)	4.90 ± 0.15	0.886 ± 0.13[b]	7.98 ± 0.51[b]
Tb.Th (μm)	42.54 ± 3.40	49.58 ± 2.07	45.21 ± 2.27
Tb.N (per mm)	3.02 ± 0.25	3.79 ± 0.30[b]	2.69 ± 0.16
Tb.Sp (μm)	302.76 ± 26.61	224.20 ± 21.49[b]	336.57 ± 23.75
Ob.S/BS (%)	2.08 ± 0.18	0.65 ± 0.11[b]	1.72 ± 0.13
Oc.S/BS (%)	0.67 ± 0.03	0.49 ± 0.04[b]	0.72 ± 0.08
ES/BS (%)	5.09 ± 0.35	2.87 ± 0.35[b]	4.53 ± 0.37
Tibial shaft			
Cortical bone area (%)	75.43 ± 1.32	80.94 ± 1.06[b]	74.04 ± 1.12
Marrow area (%)	24.51 ± 1.32	19.06 ± 1.06[b]	25.56 ± 1.12
Periosteal dL.S/BS (%)	15.89 ± 1.46	12.66 ± 1.19	12.27 ± 0.78
Periosteal MAR (μm/d)	0.93 ± 0.04	0.31 ± 0.02[b]	0.81 ± 0.07
Periosteal bone formation rate ($\times 10^{-2}$ $\mu m^3/[\mu m^2 \cdot d]$)	11.67 ± 1.55	3.94 ± 0.45[b]	9.83 ± 0.83

[a]See text for definitions of abbreviations.
[b]$p < 0.05$ compared with control.

number and a decrease in trabecular separation. The double-labeled surface and mineral apposition rate were suppressed by approx 70%, resulting in a marked decrease in bone formation rate. There was also a marked reduction in the osteoblast surface. Administration of bromocriptine significantly suppressed all measures of the resorptive activities when compared to control lactating rats. The responses of the cortical bone formation in the tibial shaft to bromocriptine were not as marked as those seen in the tibial cancellous metaphysis. Periosteal bone apposition rate and formation rate were decreased by 67 and 73%, respectively, in the bromocriptine-treated group. Bromocriptine suppression of the percentage of cortical bone loss was reflected by a small but significant 7% increase in the cortical bone area and a 22% decrease in the percentage of marrow area without any change in total cross-sectional area. PRL supplementation had no effect on most parameters with the exception of the longitudinal growth rate of the tibial metaphysis, which was markedly stimulated.

Discussion

Lactation is accompanied by a substantial redistribution of calcium. Theoretically, to compensate for the calcium loss in milk, maternal adaptations could include increased intestinal calcium absorption, renal conservation of calcium, and increased resorption of calcium from the skeleton. The mechanisms of hormonal control of a temporary increase in bone turnover during lactation have not been elucidated, but resorption of bone does not appear to be mediated by PTH *(24–26)* $1,25(OH)_2D_3$ *(24,25)*, adrenal steroid hormones *(24)*, or ovarian sex hormones *(24–26)*. We proposed that other hormones such as PRL may be involved in bone adaptation associated with lactation. In our previous reports *(21,27)*, we proposed that PRL played a significant role in the regulation of calcium absorption and bone metabolism in young and sexually mature rats. We were able to demonstrate the physiologic roles of endogenous PRL in the retention of calcium in pregnant rats *(19)*, regulation of the concentration of calcium in milk *(19)*, and stimulation of calcium absorption and maintenance of bone calcium content in young growing rats *(20)*. Thus, it appears that PRL may also have some roles in the regulation of calcium metabolism during the lactating period.

With more than 20 times higher serum concentrations of PRL than the levels in nonpregnant rats *(28)*, the lactating period in some ways resembles hyperprolactinemia, a commonly encountered clinical disorder often present with hypogonadism. However, hyperprolactinemia may be associated with serum levels of PRL as high as 100 times that of a normal range *(29)*. Decreased bone mineral content in hyperprolactinemic women with amenorrhea is suggested to result

not directly from PRL but from PRL-induced ovarian hormone deficiency *(30)*. However, a direct action of PRL on bone metabolism was also possible, as suggested by a report of decreased bone formation rate in PRL receptor knockout mice *(31)*. Furthermore, PRL receptors have been demonstrated in human osteoblastic cell lines *(32)*, and bone tissues of humans, mice, and rats *(30,33)*.

In the present study, two cortical bones of the weight-bearing type (i.e., femur and tibia) and two cancellous bones (i.e., lumbar vertebrae and sternum) in the lactating rats were investigated. Regarding the two types of bone, besides a clear structural distinction, cortical and cancellous bones vary in the degree of mineralization and pattern of metabolism. Inequality between the rates of bone formation and resorption was shown in both types of bone, i.e., in cephalic bone, appendicular bone (femur), axial bones (lumbar vertebrae and sternum), and the pelvis *(34)*. Inequality apparently diminished toward equality with increase in age *(34)*. Consistent with previous reports *(34–36)*, we found bones of lactating rats to have different rates of bone turnover, with femur, tibia, and lumbar vertebrae showing net loss in calcium at the end of 1 wk of study. The different individual rates of bone turnover may also be owing to several other factors, such as blood supply, type of bone marrow, number of marrow cells, mechanical load, muscle distribution, and physical activities *(34,37,38)*. It was noted that the sternum, in contrast to the other bones, exhibited a net gain in calcium at the end of the 1-wk study. It is possible that the continuous and frequent cyclic physical activities of the sternum owing to breathing may have a positive effect on the calcium turnover, which resulted in a net calcium gain.

To evaluate the role of PRL in the regulation of bone turnover, we used three approaches: histomorphometric analyses of tibia, ^{45}Ca kinetic studies of four bones, and biochemical markers of bone turnover. All of these methods are useful and provide information on bone metabolism at different levels of organization (i.e., whole body, organ, and cellular). In the present study, a drastic reduction in the histomorphometric indexes of bone formation and resorption in the tibia of the bromocriptine-treated rats agreed with changes in the rates of bone calcium deposit and resorption in the kinetic study, whereas the biochemical markers of bone turnover showed no change. A limitation to the use of biochemical markers is that they provide only a qualitative assessment of bone formation and resorption and their levels in serum and urine cannot be directly translated into rates of bone formation and resorption. Although they have proved useful for estimation of the overall effects of lactation-associated changes in the whole skeleton, the present finding confirms a previous report *(39)* that the biochemical markers only weakly correlate with the histomorphometric indexes. Calcium kinetics, on the other hand, provide more direct measures of bone turnover at the organ level and allow evaluation of the total calcium content turnover at the organ level, which includes different pools of calcium *(39,40)*. As for the histomorphometric technique, it may represent an accurate and dynamic assessment of a bone, but the results from a single bone such as tibia may not apply to other sites in the skeleton. In the present study, there was a clear correlation between the ^{45}Ca kinetics and histomorphometric measurements in the bromocriptine-treated group, showing decreases in the rates of bone formation and bone resorption. However, the histomorphometric analyses of tibia of the PRL-supplemented group showed only a tendency for bone resorption to increase, while the kinetic study showed a significant increase in the net loss of calcium.

It is clear that calcium turnover in bone does not strictly correspond to the cell-mediated bone formation and resorption. Since bone calcium exists as rapidly exchangeable calcium and bound calcium of various forms including the crystallized hydroxyapatite, under certain conditions calcium can be released from bone into the extracellular compartment without the breakdown of hydroxyapatite by bone resorption. Calcium can also enter the bone compartment without bone formation *(41)*.

Bromocriptine given in the dosage used in our previous and present studies completely inhibited the endogenous secretion of PRL *(20)* so that the bromocriptine-treated group represented an absence of endogenous PRL. From the results of the bromocriptine-treated lactating rats, we found that the high circulating levels of endogenous PRL in lactating rats had the same stimulatory effect on bone turnover as supraphysiologic doses of PRL in nonpregnant rats *(22,32)*. However, the skeletal response of nonpregnant rats to PRL was a net gain of calcium content, whereas the response of lactating rats was a net loss of calcium content, owing to milk secretion. Further evidence of the stimulatory effect of PRL on bone calcium turnover came from changes observed in an absence of endogenous PRL: i.e., higher bone volume, lower mineral apposition rates, and reduction in bone resorption biochemical indexes. It was found that endogenous PRL-induced bone loss resulted primarily from perforation and loss of whole trabeculae rather than the thinning of existing trabeculae. These changes represent a picture similar to the model of estrogen deficiency *(42,43)*.

Estrogen levels change dramatically during pregnancy and lactation. Pregnancy is a hyperestrogenic period and lactation is a PRL-induced hypoestrogenic state. There has been a report that bone turnover in pregnancy is similar to control but is increased during lactation *(44)*. Other studies, on the other hand, provided evidence of a decrease in bone density and an increase in bone turnover during pregnancy *(45,46)* and lactation with different patterns of BMD changes at different measuring sites *(45)*. Although there is no conclusive evidence regarding the association between estrogenic status and BMD, changes in bone turnover in lactation or hyperprolactinemia owing to estrogen deficiency could not be excluded. Since bone volume is lower despite higher rates of bone formation and bone resorption in control lactating rats when compared to the bromocriptine-treated

group, it is possible that on activation with endogenous PRL, osteoblasts and osteoclasts are activated simultaneously but osteoblast function lags behind. In the cortical bone, an increase in mineral apposition rate and bone formation rate without any change in the double-labeled surface indicates that osteoblast activity but not recruitment is increased by the endogenous PRL. This finding is consistent with a previous report in knockout mice *(31)*. Furthermore, PRL supplementation returned bone turnover rate to control conditions. However, the significantly higher longitudinal growth rate seen in this group could be owing to the fact that these 13-wk-old lactating rats had not ceased growing. This increased bone growth in response to exogenous PRL was consistent with data from previous study in growing animals *(20)*.

Another contender that may be part of the alternative mechanism associated with bone loss of lactation is PTH-related peptide (PTHrP). PTHrP is produced by lactating mammary gland, possibly under the influence of PRL, and is secreted in significant amounts into breast milk *(47)*. Elevated plasma levels of PTHrP released from the mammary gland are negatively associated with change in BMD at a number of skeletal sites *(6)*. Thus, PRL and PTHrP may have some independent associations with the increased bone turnover or bone loss during lactation *(6)*. However, there was discordance in the cross-sectional studies that examined the potential association of PTHrP and lactation in women *(48–50)*. In addition, sc administration of PTHrP to nonpregnant nonlactating women resulted in increases in $1,25(OH)_2D_3$, urinary phosphate and calcium excretion with decreases in serum phosphate *(51)*, a pattern of changes that did not resemble the metabolic response to lactation. Thus, there is no conclusion regarding the role of PTHrP in bone metabolism in lactation.

In sum, it is likely that bone loss during lactation has multiple hormonal mechanisms. The present data support previous findings from the kinetic studies that the high circulating level of PRL is likely to have a physiologic role in the regulation of bone metabolism in lactating rats. PRL probably acts directly on bone cells as well as stimulates production of PTHrP, which, in turn, stimulates bone resorption. In addition, PRL-induced hypoestrogenic conditions may also increase bone resorption. Whatever the mechanisms are, it is clear that in lactation the increased rate of bone turnover with net loss of calcium serves to provide calcium for milk production. The underlying mechanisms of action of PRL on bone cells and possible integrative actions of PRL and other hormones in the control of calcium mobilization in lactation are being investigated.

Materials and Methods

Animals

Thirteen-week-old lactating rats on d 5 of lactation were used. Wistar rats were obtained from the Animal Center of Thailand, Salaya Campus, Mahidol University. They were housed in hanging wire cages under a 12-h light:12-h dark cycle at 22ºC and provided unlimited access to tap water and a commercial chow that contained 1.0% calcium, 0.9% phosphorus, and 4000 IU/kg of vitamin D. Experiments were approved by the Animal Ethics Committee of the Faculty of Science, Mahidol University. Calcium kinetic studies were commenced on d 5 of lactation, while the histomorphometric studies were conducted on d 9 of lactation. Both studies ended on d 19 of lactation. Animals were divided into three groups subjected to two separate experiments.

Ca^{45} Kinetic Studies

Bone turnover is a coupled process of bone resorption and bone formation. Similarly, bone calcium turnover involves calcium release and calcium deposit. We estimated bone calcium turnover by a modified method of Li and Klein *(52)*. Briefly, the total amount of calcium resorbed from each bone over 7 d was computed by multiplying basal mean calcium by the percentage of basal bone calcium release (determined from rate of loss of ^{45}Ca from prelabeled bone). The amount of calcium deposit in each bone was quantified by adding the amount of calcium resorbed over 7 d to the net increase in calcium content of each bone. Thus, total calcium deposit was the sum of newly added calcium and resorbed calcium.

The 13-wk-old lactating rats on d 5 of lactation were divided into two groups: basal and sample. The sample group was subdivided into the saline control, bromocriptine-treated, and bromocriptine plus PRL–treated groups. All animals (basal and sample groups) received ip injection of 1.25 m*M* $CaCl_2$ solution containing approx 6 μCi of ^{45}Ca on d 5 and 6 to prelabel bone (Fig. 1). Six days later, on d 12 of lactation, the basal group was sacrificed and femur, tibia, lumbar vertebrae 5 and 6, and sternum were harvested to analyze total calcium and ^{45}Ca contents. On d 12, the sample group received the first dose of daily ip injection of 0.9% NaCl (control), 3 mg of bromocriptine/kg of body wt twice a day (Bromo), or 3 mg of bromocriptine/kg of body wt twice a day plus sc injection of 2.5 mg of PRL/kg of body wt (PRL). Dams were housed with their pups throughout the 7-d treatment. On d 18, dams were separated from their pups and kept individually in the metabolic cage to collect 24-h fasting urine for determination of deoxypyridinoline (Pyrilinks®-D kit), a biochemical marker for bone resorption. Bones of the sample group were harvested on d 19. Blood samples were collected for serum determination of alkaline phosphatase.

Handling and Preparation of Bone

On d 19 of lactation, after the animal was anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg of body wt, intraperitoneally), femur, tibia, sternum, and lumbar vertebrae 5 and 6 were harvested and cleaned of adhering tissues. Each bone was rinsed in warm saline, blotted dry, cut

in half, and had its bone cavity flushed. Bones were extracted in a 1:1 mixture of absolute alcohol and diethyl ether for 48 h and with diethyl ether for 24 h to remove fat. Then they were dried at 80ºC for 48 h to obtain a constant dry weight. Dry defatted and dehydrated bones were then ashed in a muffle furnace at 600ºC for 16 h. The ash was dissolved in 3 *M* HCl, and the supernatant was determined for total calcium and ^{45}Ca contents.

Calculations

Calculations of bone calcium deposit and bone calcium resorption were as follows:

$$\%R = \frac{B^{45}_{Ca} - S^{45}_{Ca} \times 100}{B^{45}_{Ca}}$$

$$R_{Ca} = \%R \times B_{Ca}$$

$$D_{Ca} = (S_{Ca} - B_{Ca}) + R_{Ca}$$

in which B_{Ca} is the basal mean total calcium content in bone on d 12; B^{45}_{Ca} is the basal mean ^{45}Ca in bone on d 12; S_{Ca} is the sample total calcium content in bone on d 19; S^{45}_{Ca} is the sample ^{45}Ca in bone on d 19, $B^{45}_{Ca} - S^{45}_{Ca}$ is the difference between bone calcium ^{45}Ca content on d 12 of the basal group and d 19 of the sample group, which represents the apparent resorption of ^{45}Ca over 7 d; %R is the percentage of basal calcium resorption (or percentage of loss of ^{45}Ca from d 12); R_{Ca} is the total amount of calcium resorption from bone between d 12 and 19; and D_{Ca} is the total amount of calcium deposit in bone between d 12 and d 19.

The relative amount (percentage) of calcium deposited into and resorbed from each bone was calculated by dividing each absolute value by the corresponding baseline value (basal group) of that group *(52)*.

Histomorphometric Studies

Each animal received sequential sc injections of calcein (30 mg/kg of body wt) and tetracycline-HCl (25 mg/kg of body wt) 10 d and 1 d, respectively, prior to sacrifice (i.e., on d 9 and 18 of lactation). During this period, the animals received daily ip injection of 0.9% NaCl (control), ip injection of 3 mg of bromocriptine/kg of body wt twice daily, or ip injection of 3 mg of bromocriptine/kg of body wt twice daily plus sc injection of 2.5 mg of PRL/kg of body wt (PRL). After sacrifice, the left tibia was removed from each animal and trimmed to remove the muscle, and the proximal metaphysis of tibia was separated from the diaphysis at the tibiofibular junction. The distal end of the proximal tibia was further cut at about 2 mm. Both metaphysis and diaphysis were then fixed in 70% ethanol for 48 h and dehydrated through a graded series of alcohols (80% ethanol, 90% ethanol), then three changes of 100% ethanol for 24 h each. Bones were then cleared in chloroform for 24 h, placed for a further 24 h in 100% ethanol, and embedded in LR White Hard Grade (London Resin, Reading, UK). Longitudinal sections of the proximal tibial metaphyses were cut on a Jung K microtome (Carl Zeiss, Heidelberg, Germany). The 7-μm sections were stained with 1% toluidine blue for morphologic assessment, and 12-μm sections were assessed unstained by fluorescent microscopy. After embedding in resin, the tibiofibular section was cut and the 12-μm section was also assessed unstained by fluorescent microscopy. Cancellous bone measurement modified from the method of Chow et al. *(53)* was performed in an area approx 4.2 mm^2 on the proximal tibial metaphyses located 1.2 mm distal from the growth plate to exclude the primary spongiosa *(35)*. Bone histomorphometric parameters were measured as described in the report of the ASBMR Histomorphometry Nomenclature Committee *(54)* using an image analyzer (Osteomeasure; Osteometrics, Atlanda, GA).

Histomorphometric measurements of cancellous and cortical bone were made on two sections per animals. Cancellous bone volume was expressed as a percentage of tissue volume (BV/TV). Trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), osteoblast surface per bone surface (Ob.S/BS), osteoclast surface per bone surface (Oc.S/BS), and eroded surface per bone surface (ES/BS) were determined. Bone surface covered by double fluorochrome label was expressed with reference to total bone surface (dL.S/BS). Mineral apposition rate (MAR) was the mean distance between the calcein and tetracycline label divided by labeling interval. Bone formation rate per bone surface (BFR/BS) was determined from the product of dL.S/BS and MAR. Longitudinal growth rate (LGR) was the distance between the calcein and tetracycline bands located distal to the growth plate. Cortical bone area and marrow area were calculated as a percentage of cross-sectional area. Bone formation rate was calculated at the periosteal surface.

Biochemical Analyses

Total calcium concentration was determined by atomic absorption spectrophotometry (Spectra AA-300; Varian Techtron Pty., Springvale, Australia). The ^{45}Ca radioactivity was measured by the standard liquid scintillation technique (LKB Rackbeta 1219; LKB Wallac, Turku, Finland) with quench correlated by the external standard ratio method. The total ^{45}Ca contents were presented as a percentage of the administered dose and expressed per gram of dry weight of tissue. Serum and bone alkaline phosphatases were measured by an alkaline phosphatase kit (Bio-Medical, Bangkok, Thailand) containing *p*-nitrophenyl phosphate as a substrate. Absorption was measured at 410 nm, and conversion to enzyme activity was made using a *p*-nitrophenol standard curve. Urinary deoxypyridinoline was measured by an enzyme-linked immunoassay kit (Pyrilinks-D; Metra Biosystem, Mountain View, CA). Calcein and tetracycline were purchased from Sigma (St Louis, MO). Creatinine was determined by kit from Bio-Medical. Ovine PRL (Sigma) was dissolved in isotonic saline adjusted to pH 9.0 with 2 *M* NaOH.

Statistical Analyses

All data are presented as mean ± SEM. Differences between treatment groups were analyzed by one-way analysis of variance and Student's Neuman-Keuls test. Statistical significance was taken as $p < 0.05$.

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