High-calcium diet modulates effects of long-term prolactin exposure on the cortical bone calcium content in ovariectomized rats

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Charoenphandhu N, Tudpor K, Thongchote K, Saengamnart W, Puntheeranurak S, Krishnamra N. High-calcium diet modulates effects of long-term prolactin exposure on the cortical bone calcium content in ovariectomized rats. *Am J Physiol Endocrinol Metab* 292: E443–E452, 2007. First published September 26, 2006; doi:10.1152/ajpendo.00333.2006.—High physiological prolactin induced positive calcium balance by stimulating intestinal calcium absorption, reducing renal calcium excretion, and increasing bone calcium deposition in female rats. Although prolactin-induced increase in trabecular bone calcium deposition was absent after ovariectomy, its effects on cortical bones were still controversial. The present investigation, therefore, aimed to study the effect of in vivo long-term high physiological prolactin induced by either anterior pituitary (AP) transplantation or 2.5 mg/kg prolactin injection on cortical bones in ovariectomized rats. Since the presence of prolactin receptors (PRLR) in different bones of normal adult rats has not been reported, we first determined mRNA expression of both short- and long-form PRLRs at the cortical sites (tibia and femur) and trabecular sites (calvaria and vertebrae) by using the RT-PCR. Our results showed the mRNA expression of both PRLR isoforms with predominant long form at all sites. However, high prolactin levels induced by AP transplantation in normal rats did not have any effect on the femoral bone mineral density or bone mineral content. By using ^{45}Ca kinetic study, 2.5 mg/kg prolactin did not alter bone formation, bone resorption, calcium deposition, and total calcium content in tibia and femur of adult ovariectomized rats. AP transplantation also had no effect on the cortical total calcium content in adult ovariectomized rats. Because previous work showed that the effects of prolactin were age dependent and could be modulated by high-calcium diet, interactions between prolactin and these two parameters were investigated. The results demonstrated that 2.0% wt/wt high-calcium diet significantly increased the tibial total calcium content in 9-wk-old young AP-grafted ovariectomized rats but decreased the tibial total calcium content in 22-wk-old adult rats. As for the vertebrae, the total calcium contents in both young and adult rats were not changed by high-calcium diet. The present results thus indicated that the adult cortical bones were potentially direct targets of prolactin. Moreover, the effects of high physiological prolactin on cortical bones were age dependent and were observed only under the modulation of high-calcium diet condition.

bone mineral density; 45-calcium kinetic study; immunohistochemistry; pituitary transplantation; prolactin receptor

IN ADDITION TO THE NOVEL ROLES in the regulation of calcium metabolism during pregnancy and lactation (10, 33), prolactin has been shown to induce positive calcium balance in nonpregnant and nonlactating female rats (3, 39, 44). Long-term exposure to high physiological 70–100 ng/ml plasma levels of prolactin, which were comparable to the level during pregnancy (10), was found to stimulate the intestinal calcium absorption in a dose-dependent manner and reduce urinary calcium loss, both in vivo and in vitro (11, 39). Recently, we reported a stimulatory action of a high physiological level of prolactin on the duodenal calcium transport in ovariectomized rats (45), suggesting that prolactin had the capability to alleviate negative calcium balance induced by long-term estrogen depletion.

The findings that prolactin receptor (PRLR) mRNAs have been identified in dexamethasone-stimulated MG-63 and Saos-2 human osteosarcoma cell lines (7) and cultured calvarial osteoblasts (13) suggested bones as possible direct targets of prolactin. Previous investigations indicated that high physiological level of prolactin increased bone calcium deposition in normal female animals, whereas prolonged pathological levels, such as in prolactinomas or long-term antipsychotic drug uses, could induce bone resorption (18, 20, 27). A number of investigations also showed that effects of prolactin on bones were mostly confined to the primarily trabecular sites, such as sternum and vertebrae, whereas the cortical sites were not much affected (13, 40, 42). Since expression of PRLR was identified only in stimulated osteoblastic cell lines or neonatal osteoblasts derived from trabecular sites (7, 13, 15), lack of cortical bone response could be due to an absence of PRLR in cortical bones. Hence, we used the reverse transcriptase (RT)-PCR technique to identify expression of two isoforms of PRLRs, i.e., short and long form, in different cortical and trabecular bones of normal adult rats. Both isoforms shared the same extracellular and transmembrane domains but differed in the sequences and in the lengths of their cytoplasmic domains (25).

After ovariectomy (Ovx), which was a representative model of bilateral oophorectomy and menopause in the young and in adults, respectively, an increase in trabecular bone calcium deposition by 15-day administration of 2.5 mg/kg prolactin, which produced an increase in plasma prolactin level ≤ 75 ng/ml (39), appeared to be diminished (40). It was, therefore, suggested that some ovarian hormones may be required for high physiological prolactin actions on bones. Nothing was known at present regarding the effects of prolactin on the cortical bones of ovariectomized rats, although it was apparent that both prolactin and Ovx had less negative effects on cortical bones than trabecular bones (6, 40).

Besides Ovx, a number of factors, such as high-calcium diet and age of animals, could modulate actions of prolactin (4, 28, 45). High-calcium diet has been a standard regimen to alleviate negative calcium balance after Ovx (16). Tudpor and colleagues (45) recently demonstrated that high-calcium diet (2.0% wt/wt Ca) significantly abolished the stimulatory effect of prolactin on the duodenal calcium transport in young ovariectomized rats, whereas, in adult rats, combination of prolactin and high-calcium diet further reduced the duodenal calcium transport below the control level. Their results indicated an age-dependent integrative response of the small intestine to prolactin and high-calcium diet. As for bones, the stimulatory effects of prolactin on bone calcium deposition were greater in young rats than in adult rats (28), but the interaction of high-calcium diet and prolactin had not been reported. It was possible that high-calcium diet might differentially affect the actions of prolactin on the bone calcium content in young and adult ovariectomized rats.

In the present study, high physiological prolactin was obtained by two different methods, i.e., anterior pituitary (AP) transplantation and 2.5 mg/kg prolactin injection. The two techniques elevated plasma prolactin to 91 and 75 ng/ml, respectively (39). After AP transplantation, sustained hyperprolactinemia was observed within 15 days after an absence of dopaminergic inhibition of pituitary prolactin secretion (24, 39). Other pituitary hormones were not secreted due to the absence of the respective stimulatory hypothalamic hormones. In the ^{45}Ca kinetic study, which was a sensitive method to detect ^{45}Ca deposit in bone and exchangeable bone calcium pool (19, 29), high plasma prolactin levels were obtained by subcutaneous injection, because an abrupt increase in prolactin level was required at the start of the experiment (day 14 in Fig. 1). The protocol also allowed a shorter experimental period (28, 40), thus minimizing radioactive use and contamination.

The objectives of the present study were, therefore, 1) to demonstrate whether the cortical bones expressed PRLRs under normal conditions; 2) to show the effect of long-term exposure to a high physiological prolactin on the cortical bone mineral density (BMD) and content (BMC) in normal rats; 3) to elucidate the effects of prolactin on cortical bone formation, bone resorption, and calcium deposition in ovariectomized rats by using ^{45}Ca kinetic study; and 4) to investigate whether the effects of prolactin on cortical bones in ovariectomized rats were age dependent and were modulated by high-calcium diet.

MATERIALS AND METHODS

Animals

Young (5-wk-old) and adult (18-wk-old) female Sprague-Dawley rats weighing 135–150 and 200–220 g, respectively, were obtained from the Animal Centre of Thailand. They were placed in hanging stainless steel cages and fed either made-to-order normal calcium (1.0% wt/wt Ca), or high-calcium (2.0% wt/wt Ca) laboratory pellets (Perfect Companion, Bangkok, Thailand) and distilled water ad libitum under 12:12-h light-dark cycle. Room temperature was controlled at 23–25°C, and relative humidity was ~50–60%. This study was approved by the Laboratory Animal Ethics Committee of Mahidol University, Bangkok, Thailand. All animals were cared for in accordance with the guidelines of the American Physiological Society's *Guiding Principles in the Care and Use of Animals*. Radioactive uses in animals were closely supervised by the Office of Atoms for Peace, Bangkok, Thailand.

AP Transplantation

The procedure was modified from the methods of Adler et al. (1) and Tudpor et al. (45). During diethylether anesthesia, a 1.0-cm paracostal incision was made to expose the left kidney, which was then covered with a warm sterile 0.9% NaCl-soaked gauze ready for transplantation. Two anesthetized 18-wk-old donors were then decapitated to remove the pituitary glands. The gland from the first donor was inserted into the prepared renal capsule of the recipient. Immediately, the second gland was implanted next to the first one and finally covered with the renal fascia. Muscle and skin were sutured and cleaned with 70% ethanol and povidone-iodine. Sham operation consisted of exposure of the left kidney and gentle touch of the renal fascia with forceps. On the experimental day, young and adult rats were 9 and 22 wk old, respectively. Visual examination of well-vascularized hypophyseal graft and immunohistochemical staining for prolactin production were performed at the end of the experiments to ensure successful 4-wk AP transplantation.

Bilateral Ovx

Bilateral Ovx has been a widely accepted surgical procedure to abolish estrogen (14). In brief, the rat was anesthetized with diethylether before two 1.5-cm paralumbar incisions were made. The distal part of the fallopian tubes was ligated before the removal of both ovaries. The skin was finally sutured and cleaned with 70% ethanol and povidone-iodine. Vital signs were carefully observed until the rat recovered from anesthesia. Sham operation was similar to bilateral Ovx, except that both ovaries were gently touched with forceps and left in place. Atrophy of the uterus and vaginal smear confirmed the success of the surgery.

BMD and BMC Measurement

BMD and BMC were determined by the modified method of Binkley et al. (9). Under 50 mg/kg ip pentobarbitone sodium (Abbott Laboratories, North Chicago, IL) anesthesia, BMD and BMC of the whole femur were assessed by using dual-energy X-ray absorptiometry (DEXA, model Lunar PIXImus2; GE Medical Systems, Madison, WI), operated with software version 2.10. The dual-energy supply was 80/35 kVp at 500 μA. Animals were laid prone on a supporting board
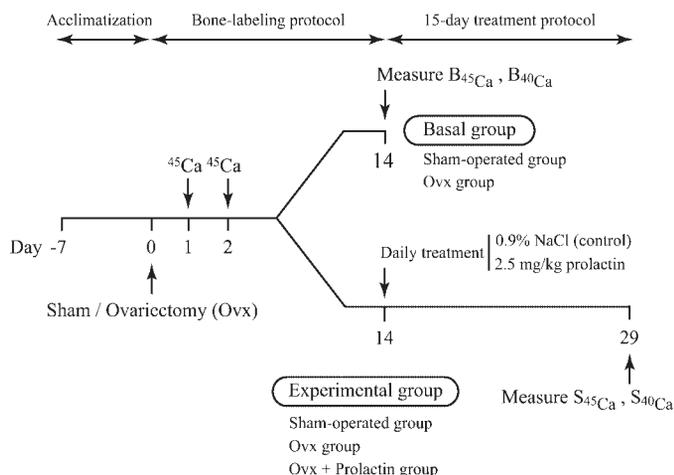

Fig. 1. Schematic diagram of the ^{45}Ca kinetic study for *protocol 2*. All rats were prelabeled with ^{45}Ca on *days 1* and *2* after 7-day acclimatization. Then they were divided into 2 groups: basal and experimental. Rats in the basal group were killed on *day 14* to determine the basal total calcium content ($B_{40_{Ca}}$) and ^{45}Ca content ($B_{45_{Ca}}$) in tibia and femur. Rats in the experimental group were injected daily with 0.9% NaCl (control) or 2.5 mg/kg sc prolactin for 15 days until *day 29*, when they were killed for determination of the total calcium content ($S_{40_{Ca}}$) and ^{45}Ca content ($S_{45_{Ca}}$) in bones. Ovx, ovariectomy.

with reproducible positioning. After intact measurement, the femur was dissected and dried for ex vivo BMD and BMC determination.

Bone Preparation

As previously described (33, 40), after the rats were killed, trabecular bones (L_{5-6} vertebrae) and cortical bones (tibia and femur) were collected and cleaned of adhering tissues. Fats and marrow tissues were eluted by 1:1 mixture of 100% ethanol and diethylether. Thereafter, bones were dried at 80°C for 48 h to obtain a constant dry weight. Finally, they were ashed in a muffle furnace (model F48020; Barnstead-Thermolyne, Dubuque, IA) at 800°C for 16 h. Ash was later dissolved in 3.0 N HCl. After centrifugation, the total calcium content in the supernatant was determined by atomic absorption spectrophotometry (model SpectrAA-300; Varian Techtron, Springvale, Australia), while ^{45}Ca content was measured by liquid scintillation spectrophotometry (model 1219; LKB-Wallac, Turku, Finland).

^{45}Ca Kinetic Study

^{45}Ca kinetic study was performed as previously described by Li and Klein (32) and Puntheeranurak et al. (40). Briefly, the adult rats were injected with 1.25 mM $CaCl_2$ solution ip (Fluka, Buchs, Switzerland) containing 6 μCi ^{45}Ca (initial specific activity of 5 mCi/ml; Radiochemical Centre, Amersham International) on *day 1* and *day 2* after acclimatization to prelabel bones (Fig. 1). The rats were then housed until *day 14*, when the 15-day treatment protocol was started. Calcium deposition was a result of bone turnover, which was a coupled process of bone formation and bone resorption. Calculations of bone formation, bone resorption, and net calcium deposition were as follows:

$$\%R_{Ca} = \frac{(B_{45_{Ca}} - S_{45_{Ca}})}{B_{45_{Ca}}} \times 100 \quad (1)$$

$$R_{Ca} = \%R_{Ca} \times B_{40_{Ca}} \quad (2)$$

$$F_{Ca} = (S_{40_{Ca}} - B_{40_{Ca}}) + R_{Ca} \quad (3)$$

$$D_{Ca} = F_{Ca} - R_{Ca} \quad (4)$$

where $B_{40_{Ca}}$ and $B_{45_{Ca}}$ were the basal total calcium (^{40}Ca) content and ^{45}Ca content, respectively, on *day 14*; $S_{40_{Ca}}$ and $S_{45_{Ca}}$ were the sample total calcium (^{40}Ca) content and ^{45}Ca content, respectively, measured at the end of each 15-day treatment protocol; $\%R_{Ca}$ was the percent calcium resorbed during the 15-day treatment; R_{Ca} was bone resorption during the 15-day treatment; F_{Ca} was bone formation; and D_{Ca} was the amount of calcium deposition over the 15-day treatment period.

Bone mRNA Isolation and RT-PCR

Fresh tibia, femur, calvaria, and L_{5-6} vertebrae were excised from 10 normal adult rats and cleaned with ice-cold 0.1 M phosphate-buffered saline, pH 7.4. Adhesive connective tissues, muscles, and bone marrow were removed at 4°C before all bones were immediately cryopreserved in liquid nitrogen to make them breakable. Gastrocnemius and soleus dissected from the same rat were used as negative controls for PRLR mRNA expression, whereas liver was used as a positive control. Sense and antisense primers for the rat long-form PRLR (rPRLR), short-form rPRLR, osteocalcin (an osteoblast-specific gene), and glyceraldehyde-3-phosphate dehydrogenase (a housekeeping gene, GAPDH) were designed by OLIGO 6 (Molecular Biology Insights, Cascade, CO) and Primer Validator 1.3 (Naratt Software, Bangkok, Thailand), as shown in Table 1. The antisense primer of the long-form rPRLR matched the unique sequence in its cytoplasmic domain, which was absent in the short isoform. To collect the mRNA sample, each bone was homogenized in liquid nitrogen with a ceramic grinder. The total RNA was prepared from the homogenate by using the TRIzol reagent (Invitrogen, Carlsbad, CA), as previously described (12). One microgram of the total RNA was reverse transcribed with the ImProm-II kit (Promega, Madison, WI) to cDNA by a thermal cycler (model MyCycler; Bio-Rad, Hercules, CA). GAPDH served as a control gene to check the consistency of reverse transcription (percent coefficient of variation <1%, $n = 10$). The amplification reaction was performed with the GoTaq Green Master Mix (Promega) according to the manufacturer's instruction. The cycle band intensity curve was plotted for each calvarial gene to obtain an optimal PCR cycle, which fell in the exponential phase (Table 1). The PCR products were visualized on a 2% agarose gel stained with 1.0 μg/ml ethidium bromide under a trans-UV system (model FluorChem SP; Alpha Innotech, San Leandro, CA).

Immunohistochemistry

After 4 wk of AP transplantation, the hypophyseal graft was dissected from the perirenal tissues for immunohistochemical analysis. Formalin-fixed, paraffin-embedded 4.0-μm sections were used to detect prolactin production in the implanted gland. Endogenous peroxidase activity and nonspecific background were blocked by 3.0% H_2O_2 and 3.0% horse serum (Sigma, St. Louis, MO), respectively. The sections were later incubated with 1:300 prolactin polyclonal primary antibody (Dako, Carpinteria, CA) for 60 min. After being washed with phosphate-buffered saline, pH 7.6, the sections were then incubated for 10 min with biotin-conjugated anti-rabbit secondary antibody and peroxidase-conjugated streptavidin (Dako). The chromogenic reaction was carried out with 3′,3′-diaminobenzidine (Dako) to produce a brownish product. The slides were finally counterstained with hematoxylin (Sigma) for 5 min. The normal pituitary gland was used as a positive control, whereas the perirenal fat pad was used as a negative control. Hematoxylin-eosin staining was also routinely performed to histologically identify structures of hypophyseal graft. Digital images were acquired from a light microscope (model BX51 TRF; Olympus, Tokyo, Japan) operated with Image-Pro Plus 5.1 (Media Cybernetics, Silver Spring, MD).

Table 1. *Rattus norvegicus oligonucleotide sequences used in the RT-PCR experiment (protocol 1)*

Gene	Accession No.	Primer (Forward/Reverse)	Product Length, bp	Cycles
rPRLR (long)	NM_001034111	5′-CCTCGGAACCTGACATTAGA-3′ 5′-GGGGAAAGTCTTGGCACC-3′	520	30
rPRLR (short)	NM_012630	5′-CCTCGGAACCTGACATTAGA-3′ 5′-CTTGTATTTGCTTGGAGAACC-3′	492	30
rOC	X04141	5′-GAACAGACAAGTCCCACACAG-3′ 5′-GTCTATTCACCACCTTACTGC-3′	187	28
GAPDH	NM_017008	5′-GGTGATGCTGGTGCTGAGT-3′ 5′-CAGTCTTCTGAGTGGCATTG-3′	301	21

rPRLR (long), long-form rat prolactin receptor; rPRLR (short), short-form rat prolactin receptor; rOC, rat osteocalcin.
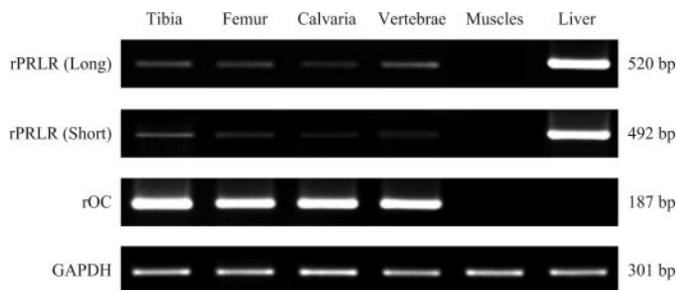
Fig. 2. mRNA expression of long and short form of rat prolactin receptors (rPRLR), osteocalcin (rOC), and GAPDH in tibia, femur, calvaria, and L_{5-6} vertebrae. Muscles were used as negative controls, whereas liver was used as a positive control. RT-PCR was carried out with 1.0 μg total RNA. The figure shows representative data from 10 normal 18-wk-old adult rats. Each sample was determined in triplicate.

Experimental Protocols

Protocol 1. This study was to verify the previous report (40) that high physiological levels of prolactin did not have any effect on cortical bones. Before studying the effects of prolactin on bones, mRNA expression of rPRLR was determined in triplicate on tibia, femur, calvaria, and L_{5-6} vertebrae of 10 normal adult rats. High plasma prolactin was induced by AP transplantation. After 4-wk AP transplantation, the femoral BMD and BMC were assessed with DEXA. Precision measurements were conducted with six intact rats and excised rat femurs. All data were obtained in triplicate. Prolactin production from implanted AP glands was confirmed by anti-prolactin immunohistochemistry.

Protocol 2. The objective of this protocol was to investigate the effects of 2.5 mg/kg prolactin (Sigma), a high physiological dose, on bone calcium metabolism in tibia and femur of ovariectomized adult rats by using the ^{45}Ca kinetic study. Prolactin or 0.9% NaCl (control) were administered subcutaneously once daily at 9:00 AM. Our prolactin radioimmunoassay revealed that, 5 h after prolactin injection (2:00 PM), plasma prolactin level was 75 ng/ml (39). After the 15-day treatment, total calcium content and ^{45}Ca content were measured (Fig. 1). Obtained parameters were bone formation, bone resorption, and calcium deposition in two cortical bones.

Protocol 3. This study was to investigate possible integrative effects of high-calcium diet and age of animals on the actions of prolactin in ovariectomized rats. The rats were fed either with 1.0% wt/wt Ca (normal calcium) or 2.0% wt/wt Ca (high-calcium) diet throughout the entire experimental period. Total bone calcium contents of 4-wk AP-grafted young (9-wk-old) and adult (22-wk-old) ovariectomized rats were determined by atomic absorption spectrophotometry. The L_{5-6} vertebrae, as primarily trabecular sites, and tibia, as a primarily cortical site, were examined.

Statistical Analyses

Results were expressed as means $\pm$ SE. Two sets of data were compared using the unpaired Student's *t*-test. Multiple comparisons were performed by ANOVA with Newman-Keuls posttest. The level of significance for all statistical tests was $P < 0.05$. Data were analyzed by GraphPad Prism 4.0 for Mac OS X (GraphPad Software, San Diego, CA).

RESULTS

RT-PCR Revealed mRNA Expression of rPRLRs in Various Bones

RT-PCR demonstrated expression of both short- and long-form rPRLR mRNA in tibia, femur, calvaria, and vertebrae of normal adult female rats (Fig. 2). Both rPRLR isoforms were also detected in liver (positive control), but not in muscles (negative control). All four bones expressed the long-form rPRLR mRNA more than the short-form rPRLR. The sample bones also strongly expressed mRNA of osteocalcin, indicating that the extracted total RNA contained mRNA synthesized by osteoblasts. Expression levels of GAPDH, a housekeeping gene, were not statistically different in the six sampled tissues, thus confirming equal total RNA template used. The present result suggested that both cortical or trabecular sites of bones were potential target tissues of prolactin.

Immunohistochemical Analyses Showed Prolactin Production in the Hypophyseal Allograft

After 4-wk AP transplantation, two implanted pituitary glands were removed from the perirenal connective tissues and were analyzed for their viability and function histologically and immunohistochemically. The excised grafts were highly vascularized reddish-gray tissue with an average diameter of 2–3 mm. Hematoxylin-eosin staining (Fig. 3A) revealed a group of epithelial cells of varying size, arranged in irregular cords and clusters, agreeing with histological morphology of normal pituitary gland. Numerous sinusoidal networks were seen between the clusters of cells (Fig. 3, A and D). Immunohistochemical analyses demonstrated active prolactin production inside the pars distalis of the implanted glands (Fig. 3, B, C, and D). Surrounding fat tissues, fascia (arrows in Fig. 3C), and the pars intermedia (arrow in Fig. 3B) did not contain prolactin immunoreactive signal. Active signs of microvascular endothelial damage and lymphoid proliferation were not seen, suggesting the absence of graft rejection and allograft vasculopathy. The results corroborated that the AP-grafted rats possessed two healthy ectopic pituitary glands, which actively produced extra prolactin. According to the previous report of Piyabhan et al. (39), sustained plasma prolactin level induced by this procedure was ~91 ng/ml, while the level in normal adult female rats was 7 ng/ml.

Long-Term Prolactin Exposure Had No Effect on the BMD and BMC in Normal Rats

To demonstrate the effect of long-term prolactin exposure induced by AP transplantation in normal adult rats fed normal calcium diet, femoral BMD and BMC were measured. DEXA assessment in vivo showed that the femoral BMD and BMC in AP-grafted rats were comparable to those in sham-operated rats (Fig. 4), thus suggesting the absence of prolactin effect on the cortical bones. The ex vivo femoral BMD and BMC experiments revealed similar findings (data not shown).

Long-Term Prolactin Exposure Had No Significant Effect on Cortical Bone Remodeling in Ovariectomized Rats

To determine the effect of long-term prolactin exposure by 2.5 mg/kg prolactin administration on cortical bones of adult ovariectomized rats fed a normal calcium diet, a highly sensitive ^{45}Ca kinetic study was performed. As shown in Fig. 5, although Ovx alone had no significant effect on bone formation, bone resorption, and total calcium content in cortical bones, net calcium deposition showed reduction with statistical significance in the femur. We further found that ovariectomized rats exposed to exogenous prolactin (Ovx+P) did not manifest any detectable change in bone formation (Fig. 5A),
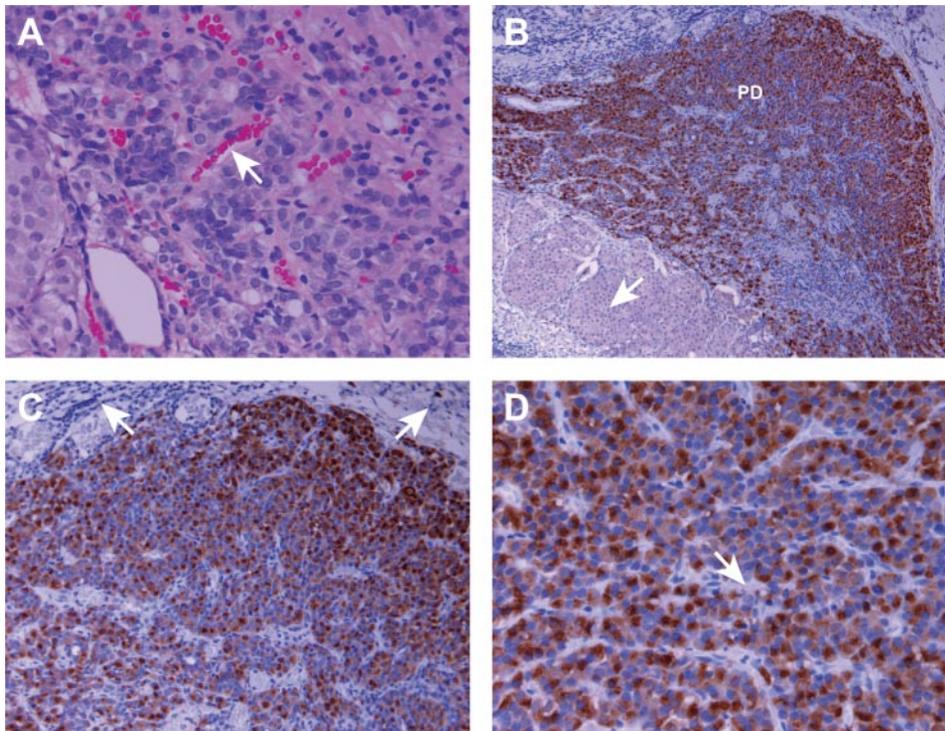
Fig. 3. Representative data of prolactin immunohistochemical analyses from 16 anterior pituitary (AP)-grafted rats. *A*: hematoxylin-eosin (H&E) staining of a hypophyseal allograft excised from a 4-wk AP-grafted rat; magnification ×400. The arrow indicates a sinusoid filled with numerous red blood cells. *B*: immunohistochemical staining with anti-prolactin antibody of implanted pituitary glands; magnification ×100. The pars distalis (PD) is strongly labeled with brownish products of peroxidase, whereas the pars intermedia (arrow) is not stained. *C*: the PD of implanted pituitary gland stained with anti-prolactin antibody; magnification ×200. Surrounding perirenal connective tissues (arrows) are negative for anti-prolactin staining. *D*: anti-prolactin staining of the PD; magnification ×400. A complex hypophyseal sinusoidal network stained with hematoxylin is indicated by the arrow.

bone resorption (Fig. 5*B*), or calcium deposition (Fig. 5*C*). With no change in bone remodeling, total calcium content measured from the ashed tibia and femur was also unaltered in Ovx+P (Fig. 5*D*). Our results indicated that long-term high physiological prolactin exposure had no significant effect on cortical bones in adult ovariectomized rats.

High-Calcium Diet Differentially Affected the Cortical Bone Calcium Content in Young and Adult AP-Grafted Ovariectomized Rats

According to our previous investigation (45), the intestinal calcium transport in young and adult rats differentially responded to prolactin. We, therefore, studied the effects of long-term prolactin exposure induced by 4-wk AP transplantation on a cortical bone in both young (9-wk-old) and adult (22-wk-old) ovariectomized rats. In young rats (Fig. 6*A*), tibial total calcium content in ovariectomized rats was similar to that in sham-operated rats. AP transplantation, like 2.5 mg/kg prolactin injection (Fig. 5*D*), had no effect on tibial total calcium content in ovariectomized rats fed a normal calcium diet (1.0% wt/wt; N). Interestingly, although high-calcium diet (2.0% w/w; H) had no significant effect on ovariectomized young rats, it increased tibial total calcium content in AP-grafted young ovariectomized rats from 13.84 ± 2.17 (Ovx+AP+N, $n = 6$) to 19.01 ± 0.29 mmol/g dry wt (Ovx+AP+H, $n = 6$, $P < 0.05$).

In contrast, Ovx significantly reduced the tibial total calcium content in adult rats fed a normal calcium diet compared with sham-operated rats, i.e., 23.77 ± 2.05 (sham, $n = 12$) vs. 19.54 ± 1.62 mmol/g dry wt (Ovx+N, $n = 13$, $P < 0.05$), suggesting that long-term estrogen deficiency distinctly affected the cortical calcium in adult rats, but not in young rats. High-calcium diet alone or AP transplantation alone did not alter the tibial total calcium content in adult ovariectomized

rats. However, AP-grafted ovariectomized rats fed a high-calcium diet manifested a further decrease in the tibial total calcium content from 17.00 ± 0.55 (Ovx+AP+N, $n = 10$) to 13.27 ± 0.49 mmol/g dry wt (Ovx+AP+H, $n = 10$, $P < 0.05$). The results indicated that high-calcium diet differentially modulated the effects of long-term prolactin exposure on tibial total calcium content in young and adult ovariectomized rats.

High-Calcium Diet Did Not Modulate the Action of Prolactin in the Trabecular Bone of Ovariectomized Rats

Despite the presence of an integrative effect between age and high-calcium diet on the cortical bone in AP-grafted ovariectomized rats, it was not known whether those factors were able to modulate the action of prolactin on trabecular bones. Therefore, we studied the effects of high-calcium diet on the L_{5-6} vertebrae in young and adult AP-grafted ovariectomized rats. We found that, similar to the results from tibia, young ovariectomized rats fed a normal-calcium diet (Ovx+N) did not show any change in the vertebral total calcium content (Fig. 7*A*). Neither AP transplantation alone, high-calcium diet alone, nor a combination of both affected the vertebral total calcium content in young ovariectomized rats.

Similar to the observation in cortical bone, total calcium content from ashed vertebrae of adult ovariectomized rats fed a normal-calcium diet (Fig. 7*B*) was significantly lower than that in sham-operated rats, i.e., 18.41 ± 0.97 (sham, $n = 17$) vs. 14.96 ± 0.91 mmol/g dry wt (Ovx+N, $n = 16$, $P < 0.01$). However, long-term prolactin exposure, high-calcium diet, and a combination of both had no significant effect on the vertebral total calcium content. Our results suggested that high-calcium diet did not modulate the actions of prolactin on the trabecular bone in ovariectomized rats. In addition, the effects of long-term estrogen deficiency were also age dependent, since its
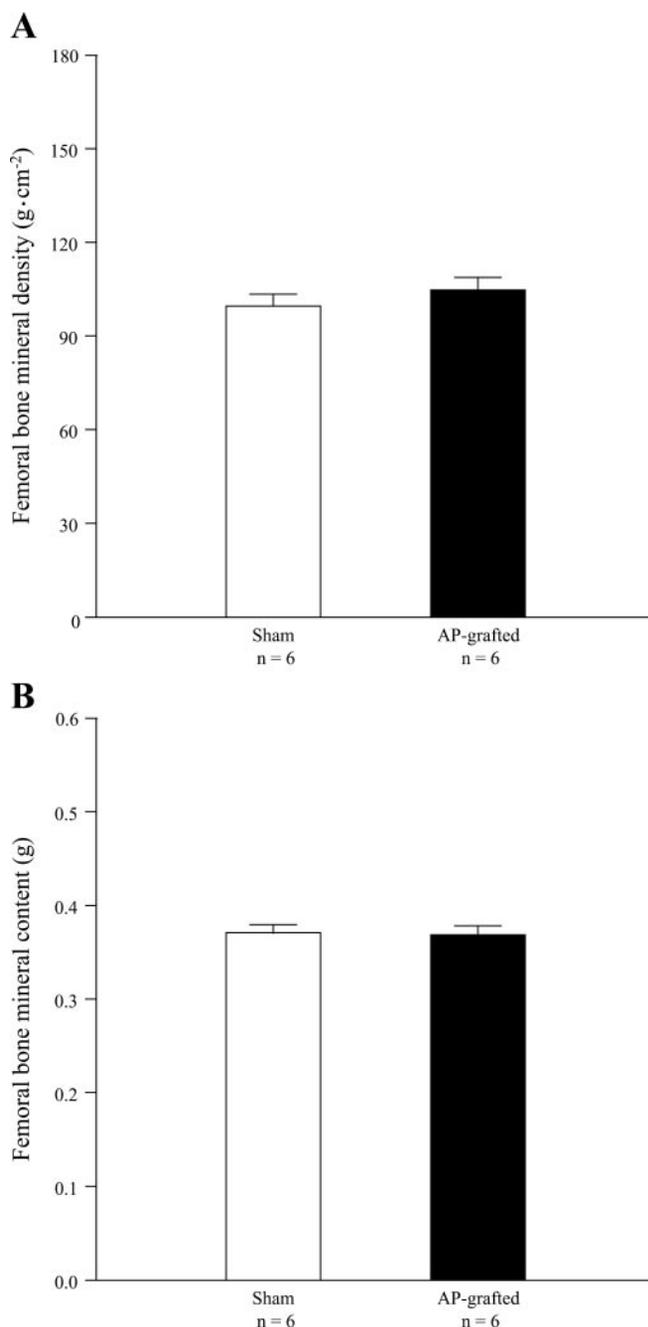
Fig. 4. Femoral bone mineral density (BMD; *A*) and bone mineral content (BMC; *B*) measured by dual-energy X-ray absorptiometry in sham-operated and 4-wk AP-grafted nonovariectomized adult rats. Results were obtained in triplicate. *n*, Number of experimental animals.

effects on cortical and trabecular bones were observed only in adult ovariectomized rats (Figs. 6*B* and 7*B*).

DISCUSSION

High physiological levels of prolactin in the range of 70–100 ng/ml have been known to produce positive calcium balance by stimulating intestinal calcium absorption in a dose-dependent manner (11, 44) and by reducing renal calcium loss (39). High physiological prolactin induced by AP transplantation was also reported to increase duodenal calcium absorption in ovariectomized rats, suggesting that prolactin may be used to mitigate negative calcium balance during long-term estrogen deficiency. However, since there were reports that trabecular osteopenia could develop in patients with hyperprolactinemic hypogonadism (8), the effects of prolactin on bone calcium content were investigated by our group. Herein, we were able to show that the high physiological level of prolactin, unlike the pathological level, did not induce bone loss, especially after Ovx. The present investigation demonstrated, for the first time, that the primarily cortical sites, such as tibia, in ovariectomized rats were possible target tissues of high physiological prolactin, the action of which was dependent on dietary calcium availability. The results were also consistent with a report of differential responses of the trabecular and cortical sites to prolactin (40). In the present study, we paid special attention to the total calcium contents that were known to correlate well with BMD and BMC (2, 48).

Two models have been widely used to study the effects of high physiological prolactin in vivo, i.e., daily prolactin injection and AP transplantation (28, 45). Our laboratory has reported that both techniques produced high physiological prolactin levels of 70–100 ng/ml (39), which were comparable to the levels during pregnancy in rats (10). Generally, plasma prolactin level and the presence of vascular structure within the implanted pituitary gland were used to indicate its survival and normal function (45). We further demonstrated by using a immunohistochemical technique that the graft survived and actively produced prolactin. Importantly, the absence of vascular endothelial damage and active lymphoid proliferation indicated that AP graft rejection did not occur during the 4-wk posttransplantation. Although 4-wk AP transplantation was a useful method for inducing sustained high prolactin level in vivo, it was not suitable for use in the ^{45}Ca kinetic study, which needed to coincide exactly with the elevation of the plasma prolactin level. Therefore, in those studies, high prolactin levels were attained by daily prolactin injection.

Although the role of prolactin in bone metabolism has not been elucidated, PRLR mRNA expression was shown in two human osteosarcoma cell lines, MG-63 and Saos-2, but only when stimulated by $1\alpha,25\text{-}(OH)_2D_3$ and dexamethasone (7). PRLRs were also visualized immunocytochemically on the calvarial osteoblasts from neonatal rats (15). In addition, the fact that PRLR-knockout mice manifested delayed bone formation and minor osteopenia suggested a physiological role of prolactin in neonatal bone development (13). However, besides calvaria from neonates and stimulated osteoblastic cell lines, there had been no evidence pertaining to the expression of PRLRs in bones of adult rats. Herein, we first demonstrated that primarily cortical sites, i.e., tibia and femur, and trabecular sites, i.e., calvaria and vertebrae, of normal adult rats expressed PRLR mRNA. Although both short- and long-form receptors were identified, all four bones expressed more long form than short form, similar to that observed in other well-established target tissues of prolactin, such as mammary glands and ovaries (25, 35). Relative physiological significance of the two isoforms in bones has not been addressed, but the long form was believed to be responsible for important signal transduction cascades in the mammary gland cells for lactogenesis, while the short form seemed to be important in the regulation or fine-tuning of the long-form signaling (25). Because effective separation of different types of bone cells from living
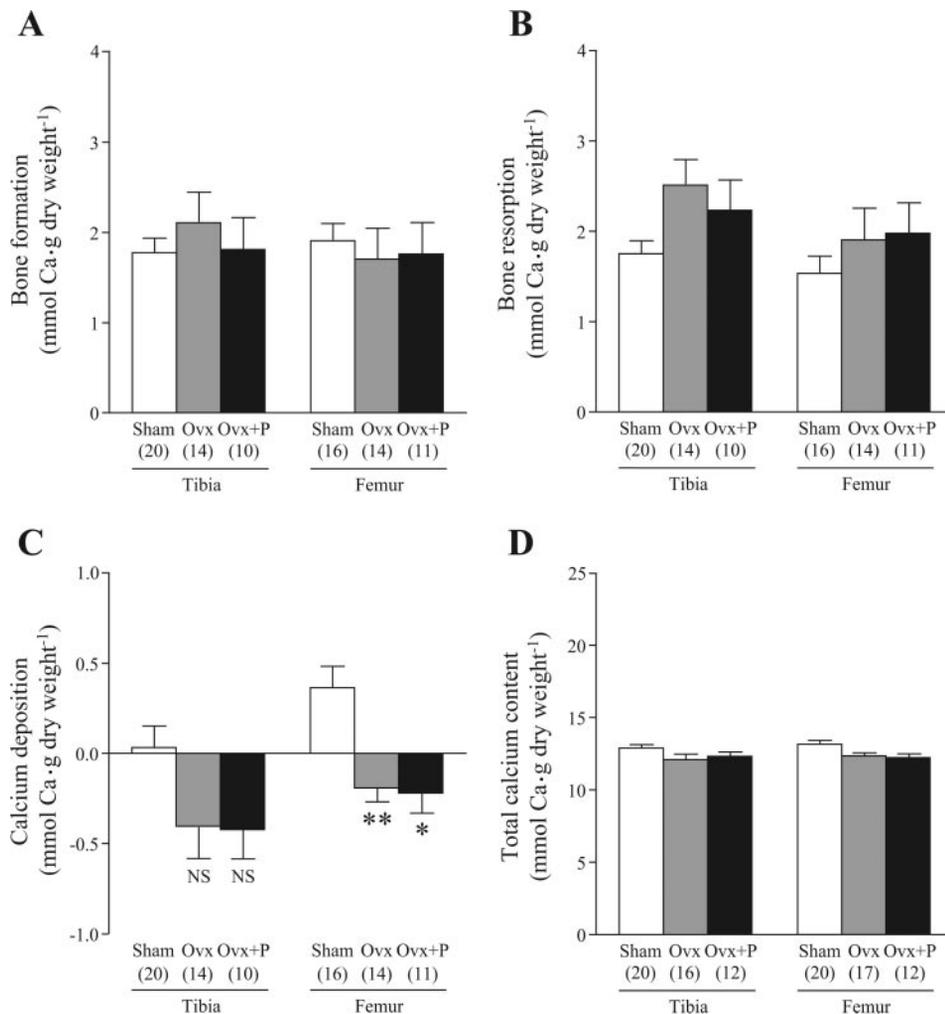
Fig. 5. Bone formation (*A*), bone resorption (*B*), calcium deposition (*C*), and total calcium content (*D*) in tibia and femur obtained from sham-operated (Sham), Ovx, and 2.5 mg/kg prolactin-injected ovariectomized (Ovx+P) adult rats. Sham and Ovx rats were injected daily with 0.9% NaCl sc. NS, $P > 0.05$ (not significant). *$P < 0.05$. **$P < 0.01$ compared with its respective sham-operated group. Numbers in parentheses represent the number of animals in each group.

bones was not technically possible at present, we did not provide direct evidence for the type of intraosseous cells that expressed PRLRs. Since osteoclasts and osteocytes have never been reported to express PRLR, it was most likely that osteoblasts were responsible for the expression of PRLRs. Therefore, the present demonstration of PRLR expression in bone tissues supported the hypothesis of a direct action of prolactin on trabecular and cortical bones in adult rats.

Since there were only a few studies of prolactin and bone, the actions of prolactin in bones were largely unknown. Human studies showed that long-term hyperprolactinemia over 1.7 yr led to low trabecular bone density and progressive trabecular osteopenia (8). Psychiatric patients given some antipsychotic drugs, which reduced hypothalamic activity of dopamine, also manifested bone loss and reduction in BMD (5, 21). Interestingly, although plasma prolactin level was markedly increased during pregnancy, reduction in total body BMD and BMC was not detected (17, 42), leading to the postulation that high physiological levels of prolactin, in contrast to sustained pathological levels, regardless of its cause, produced only a minor effect on bones. Molecular mechanisms of prolactin in bones have been related to the osteoblastic functions rather than osteoblastic proliferation (15).

Results from the ^{45}Ca kinetic study showed that, in adult rats, trabecular bones, but not cortical bones, responded to a

high physiological dose of 2.5 mg/kg prolactin by increasing the rate of bone calcium deposition (40). BMD and BMC from the DEXA confirmed the absence of prolactin action on cortical bones in adult rats. Moreover, our laboratory's previous bone histomorphometric study in lactating rats that showed inhibition of endogenous prolactin secretion resulting in decreased bone formation in tibial metaphyses (33) indicated a significant effect of prolactin on the trabecular components of cortical bones. Our present results could not exclude possible effects of prolactin on femoral trabecular microstructure; however, the absence of changes in the femoral BMD and BMC suggested only a minor effect of prolactin on the calcified structure at the cortical sites in adult rats.

Of interest in the present study was the effect of high physiological prolactin on bones in ovariectomized rats. Puntheeranurak et al. (40) in 2006 showed that, after Ovx, prolactin-enhanced bone calcium deposition in trabecular bones of adult rats was diminished, but cortical bones were not studied. Because the ^{45}Ca kinetic technique was a highly sensitive method that could detect the vertebral and sternal calcium turnover within 60 min after prolactin administration (28, 29), the present findings (Fig. 5), therefore, reliably indicated a persistent lack of prolactin action on tibia and femur after Ovx. An absence of changes in the tibial total calcium content in adult AP-grafted ovariectomized rats fed a
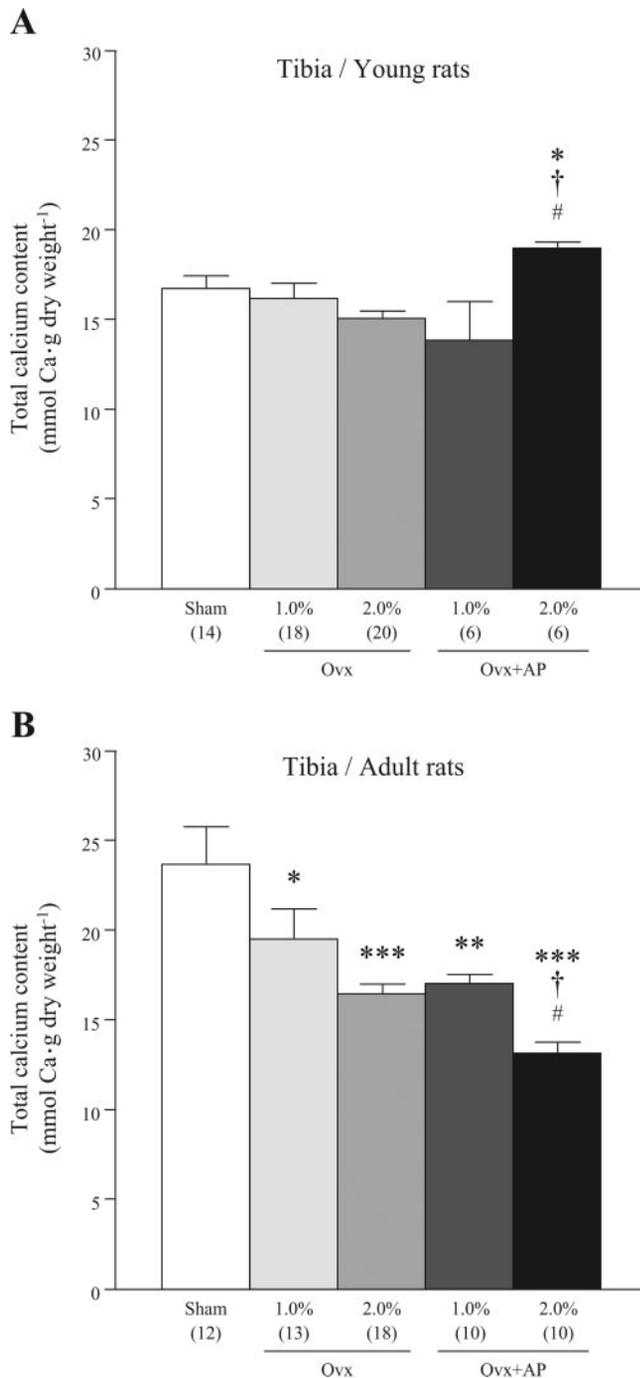
Fig. 6. Total calcium content in tibia of young (*A*) and adult (*B*) Ovx and AP-grafted ovariectomized (Ovx+AP) rats fed normal (1.0% wt/wt Ca) or high-calcium (2.0% wt/wt Ca) diet. Sham-operated rats were fed 1.0% wt/wt Ca diet. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the sham-operated rats. †$P < 0.05$ compared with Ovx rats fed high-calcium diet. #$P < 0.05$ compared with Ovx+AP rats fed normal calcium diet. Numbers in parentheses represent the number of animals in each group.

in the sternal and vertebral ^{45}Ca deposit 60 min after acute prolactin administration (28). However, this age-dependent effect of prolactin on total calcium deposition was not evident after 15-day long-term prolactin administration (28). These different results suggested that acute prolactin exposure probably affected the rapidly exchangeable bone calcium pool, but not the calcium content in the mineralized bone. Similar to the previous findings (28), bones of the 4-wk AP-grafted ovariec-
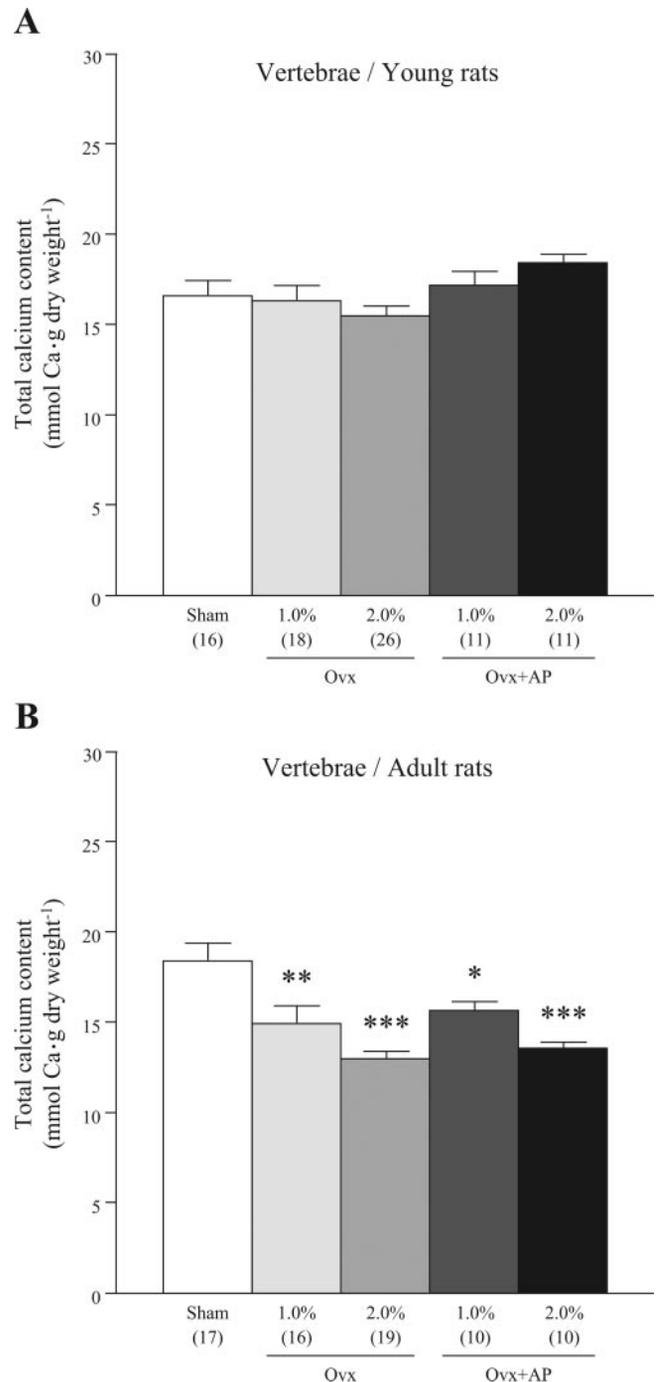

Fig. 7. Total calcium content in L$_{5-6}$ vertebrae of young (*A*) and adult (*B*) Ovx and Ovx+AP rats fed normal-(1.0% wt/wt Ca) or high-calcium (2.0% wt/wt Ca) diet. Sham-operated rats were fed 1.0% wt/wt Ca diet. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the sham-operated rats. Numbers in parentheses represent the number of animals in each group.

normal calcium diet also confirmed that prolactin did not have a significant effect in cortical bones.

The effects of prolactin on calcium metabolism in normal rats were also known to be age and dietary calcium dependent (4, 45). For instance, 3-wk-old weaned and 5-wk-old young female rats, but not adult rats, manifested significant increases

tomized rats (a model of long-term prolactin exposure) fed a normal-calcium diet did not show the age-related response to high physiological prolactin. On the other hand, differential age-dependent responses to prolactin were seen in ovariectomized rats fed a high-calcium diet, i.e., tibial total calcium content was increased in young, but decreased in adult AP-grafted ovariectomized rats. The mechanism underlying these findings was not presently known, although it was possible that the age-dependent effects of prolactin on bone might be related to the differential responses of the intestinal calcium absorption (34, 38, 45) and bone turnover to prolactin in the two age groups (28). It was interesting to find that young rats with higher capacity for bone calcium acquisition (26, 37, 41), higher rate of intestinal calcium absorption (22), and greater responsiveness to prolactin (28) compared with adult rats could best benefit from a high-calcium diet (43). Furthermore, the observed prolactin actions may be indirectly dependent on the presence of $1\alpha,25\text{-}(OH)_2D_3$, since 2–3% wt/wt high-calcium diet, although it is used to remedy negative calcium balance, has been known to decrease the circulating level of $1\alpha,25\text{-}(OH)_2D_3$ as a result of 86% reduction in $1\alpha,25\text{-}(OH)_2D_3$ production (36). In addition, we also found that the effect of high-calcium diet was confined to the tibia but was not observed in the primarily trabecular sites, i.e., vertebrae. Physiological significances of the differential integrative responses of trabecular and cortical bones in ovariectomized rats to high physiological prolactin and high-calcium diet have not been known.

Regarding the effects of Ovx alone in young and adult rats, our results showed a different age-related change in tibial and vertebral total calcium contents (Figs. 6 and 7). Whereas total calcium contents in young ovariectomized rats receiving either normal or high-calcium diet were unaltered, those in adult ovariectomized rats were significantly decreased, both in tibia and vertebrae. Yamazaki and Yamaguchi (49) also provided supporting evidence that young ovariectomized rats lost calcium from bone at a slower rate than adult ovariectomized rats. Similar to the age-related differential responses to AP transplantation plus Ovx, the underlying mechanism of this finding has not been established. Possible explanations for less calcium loss in young ovariectomized rats included higher calcium deposit in bones, more rapid skeletal growth, and greater rate of intestinal calcium absorption in the young rats (22, 37). Age-dependent resistance to $1\alpha,25\text{-}(OH)_2D_3$ in adult rats could partially explain the decrease in tibial and vertebral total calcium contents (Figs. 6B and 7B), because $1\alpha,25\text{-}(OH)_2D_3$ resistance led to negative calcium balance, which rendered ovariectomized adult rats more susceptible to osteopenia (23, 30, 46). Interestingly, a high-calcium diet did not affect tibial total calcium content in ovariectomized rats without AP transplantation, indicating that the effect of high-calcium diet on tibia required the presence of a high circulating prolactin level. In other words, the observed effects were not possible in the absence of estrogen alone or high-calcium diet alone.

However, there were some inconsistent data concerning cortical calcium deposition and the total calcium contents in ovariectomized rats. After Ovx, there was no change in tibial or femoral total calcium content (Fig. 5D), whereas ^{45}Ca kinetic technique showed a decrease in calcium deposition in femur, but not in tibia (Fig. 5C). These controversial findings were likely to be due to the fact that Ovx-induced bone resorption

was generally detectable after 1 mo and was substantial after 3 mo (31, 47). It appeared that a 1-mo experimental period may be too short to congruously demonstrate the effect of estrogen depletion on all sampled bones, since some bones may be more susceptible to estrogen depletion, resulting in high variations among different animals. A detectable decrease in femoral calcium deposition might implicate the femur being more susceptible to estrogen depletion than the tibia (Fig. 5C). On the other hand, a 1-mo period was sufficient to show the effects of prolactin alone or prolactin plus estrogen depletion on bone calcium deposition (28, 39, 40). Indeed, using short-term ^{45}Ca kinetic technique, prolactin actions on bone calcium metabolism were seen in <2 h, suggesting that it affected the rapidly exchangeable calcium pool (29).

It could be concluded that, first, tibia, femur, calvaria, and vertebrae removed from adult rats expressed mRNA of short- and long-form PRLRs, suggesting that bones were possible direct targets of prolactin. Second, by using DEXA, we confirmed the previous report that long-term high physiological prolactin did not affect the cortical bone calcium metabolism (40). Third, by using the highly sensitive ^{45}Ca kinetic method to detect bone calcium turnover, we did not find any effect of a high physiological dose of prolactin on ovariectomized adult rats. Fourth, 2.0% wt/wt high-calcium diet differentially modulated tibial, but not vertebral, total calcium content in young and adult AP-grafted ovariectomized rats, indicating that dietary calcium was one of the main determinants of prolactin actions on bone calcium metabolism. In contrast to a high pathological prolactin in prolactinomas or long-term use of antipsychotic drug, which aggravated bone resorption (8, 20), high physiological prolactin did not produce further bone loss in ovariectomized rats. According to our recent investigation, which showed 4-wk AP transplantation markedly stimulating intestinal calcium absorption in ovariectomized rats (45), high physiological prolactin could be a potential agent to alleviate negative calcium balance after long-term estrogen depletion.

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