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Prolactin directly enhances bone turnover by raising osteoblast-expressed receptor activator of nuclear factor κB ligand/osteoprotegerin ratio

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Abstract

Hyperprolactinemia leads to high bone turnover as a result of enhanced bone formation and resorption. Although its osteopenic effect has long been explained as hyperprolactinemia-induced hypogonadism, identified prolactin (PRL) receptors in osteoblasts suggested a possible direct action of PRL on bone. In the present study, we found that hyperprolactinemia induced by anterior pituitary transplantation (AP), with or without ovariectomy (Ovx), had no detectable effect on bone mineral density and content measured by dual-energy X-ray absorptiometry (DXA). However, histomorphometric studies revealed increases in the osteoblast and osteoclast surfaces in the AP rats, but a decrease in the osteoblast surface in the AP+Ovx rats. The resorptive activity was predominant since bone volume and trabecular number were decreased, and the trabecular separation was increased in both groups. Estrogen supplement (E2) fully reversed the effect of estrogen depletion in the Ovx but not in the AP+Ovx rats. In contrast to the typical Ovx rats, bone formation and resorption became uncoupled in the AP+Ovx rats. Therefore, hyperprolactinemia was likely to have some estrogen-independent and/or direct actions on bone turnover. Osteoblast-expressed PRL receptor transcripts and proteins shown in the present study confirmed our hypothesis. Furthermore, we demonstrated that the osteoblast-like cells, MG-63, directly exposed to PRL exhibited lower expression of alkaline phosphatase and osteocalcin mRNA, and a decrease in alkaline phosphatase activity. The ratios of receptor activator of nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG) proteins were increased, indicating an increase in the osteoclastic bone resorption. The present data thus demonstrated that hyperprolactinemia could act directly on bone to stimulate bone turnover, with more influence on bone resorption than formation. PRL enhanced bone resorption in part by increasing RANKL and decreasing OPG expressions by osteoblasts.
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Introduction

Hyperprolactinemia is associated with a number of physiological and pathological conditions, such as pregnancy, lactation, prolactinoma and chronic uses of dopamine-related antipsychotic drugs [1,2]. High physiological levels of prolactin (PRL) of ∼200–350 ng/mL in prolonged lactation led to transient osteopenia and reversible negative calcium balance [3]. On the other hand, sustained pathological PRL exposure (up to ∼1000 ng/mL) not only stimulated bone turnover but also produced a massive calcium loss, overt osteopenia, and osteoporosis [2,4,5]. Further studies using the ^{45}Ca kinetic technique showed the impact of hyperprolactinemia on trabecular sites, but not on cortical sites [6,7]; however, it was not known

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whether the microstructures of cortical and trabecular bones were altered in chronic hyperprolactinemia.

Accelerated bone turnover is a common feature of physiological and pathological hyperprolactinemia [8]. During pregnancy and lactation, high bone turnover was a mechanism to rapidly supply calcium for fetal growth and milk production [9]. Our histomorphometric study in lactating rats showed that suppression of endogenous PRL by bromocriptine induced a decrease in maternal bone turnover [10]. From the studies of pathological hyperprolactinemia, several investigators suggested that PRL-accelerated bone turnover was likely due to estrogen deficiency, since hyperprolactinemia suppressed estrogen production [4,5,11]. However, we recently demonstrated the expressions of both short and long isoforms of PRL receptors (PRLR) in tibiae, femora and vertebrae of adult female rats [6]. Therefore, we hypothesized that PRL could directly exert its effects on bone to stimulate bone turnover.

It is known that osteoblasts expressed the receptor activator of nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG) [12]. Binding of RANKL to its receptors on the osteoclast progenitor cells induces bone resorption, whereas binding of RANKL to its decoy receptor OPG inhibits bone resorption. Thus, the ratio of RANKL/OPG expression by osteoblasts was an important determinant of osteoclastogenesis and osteoclast activity as well as bone turnover [12]. Osteoblasts also expressed alkaline phosphatase and osteocalcin, both of which were used as indicators of the osteoblast activity [13,14]. Since high bone turnover resulted from enhanced bone formation and resorption, we hypothesized that the osteoblast activity as well as the expression ratio of RANKL and OPG would be increased by hyperprolactinemia.

Studies of chronic hyperprolactinemia in rats require an induction of highly sustained plasma PRL without the stress-induced PRL release. To achieve this, we used the anterior pituitary (AP) transplantation instead of the more stressful daily PRL injections [6,15]. Within 15 days, after transplanting two extra AP glands from donors under the renal capsule of the recipient, continuous PRL secretion from the AP allografts in the absence of hypothalamic dopaminergic inhibition resulted in a physiological hyperprolactinemia of 90–100 ng/mL, comparable to the levels reported during pregnancy [6,15,16]. Other pituitary hormones were not secreted from the grafts due to the absence of the stimulatory hypothalamic hormones.

Therefore, the objectives of the present investigation were (*i*) to demonstrate that bone was a direct target of PRL by PRLR expression studies; (*ii*) to evaluate changes in the microscopic osseous structures after chronic hyperprolactinemia; (*iii*) to demonstrate the estrogen-independent effects of PRL on bone; and (*iv*) to confirm the direct effect of hyperprolactinemia on bone by showing changes in the osteoblast activity at the molecular level.

Materials and methods

Animals

Female Sprague–Dawley rats (10-week-old, weighing 200–220 g), were obtained from the Animal Centre of Thailand, Salaya, Thailand. They were placed in hanging stainless steel cages, fed standard pellets containing 1% w/w calcium (Perfect Companion Co., Ltd., Bangkok, Thailand) and distilled water ad libitum under 12 h:12 h light:dark cycle. Room temperature was controlled at 23–25 °C, and the relative humidity was about 50–60%. This study was approved in accordance with the principles and guidelines of the Laboratory Animal Ethics Committee of Mahidol University, Bangkok, Thailand. After acclimatization, bone mineral density (BMD) and content (BMC) were measured before surgery (0 week).

Cell culture

Osteoblast-like MG-63 cell line (ATCC No. CRL-1427; a kind gift from Dr. Suttatip Kamolmatyakul, Prince of Songkla University, Thailand) or human fetal osteoblast 1.19 (hFOB) cells (ATCC No. CRL-11372) were cultured in 75-cm^2 T flasks with α-MEM (for MG-63) or DMEM/F-12 (for hFOB) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin/streptomycin, and 0.25 μL/mL amphotericin B (Sigma, St. Louis, MO, USA). 1 μM dexamethasone and 0.1 μM 1,25-$(OH)_2D_3$ (Sigma) were also added to the medium to induce maximal expression of PRLR as previously described [17]. Cells were incubated at 37 °C with 5% CO_2, and subcultured according to the ATCC's protocol.

As for the primary osteoblast culture, two tibiae were removed from a 10-week-old rat by sterile surgical technique. After removing the connective tissues and marrow cells, bones were cut into small dice, and cultured in DMEM supplemented with 15% FBS, 100 U/mL penicillin/streptomycin and 100 μg/mL ascorbate-2-phosphate (Sigma). Cells were incubated at 37 °C with 5% CO_2, and subcultured every 3 days.

Anterior pituitary (AP) transplantation

The procedure was modified from the methods of Adler et al. [15] and Charoenphandhu et al. [6]. In brief, during diethylether anesthesia, a 1.0-cm paracostal incision was made to expose the left kidney of the recipient rat. Two anesthetized donors were then decapitated to remove the pituitary glands which were immediately inserted into the prepared left renal capsule of the recipient. 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 a gentle touch of the renal fascia with forceps. Visual examination of the well-vascularized hypophyseal graft and immunohistochemical staining for PRL production were performed at the end of the experiments to confirm successful AP transplantation. Animals with unsuccessful transplantation were excluded from data analyses.

Bilateral ovariectomy

Bilateral ovariectomy (Ovx) has been an accepted surgical procedure for inducing estrogen deficiency [18]. In brief, the rat was anesthetized with diethylether before two 1.5-cm paralumbar incisions were made. The fallopian tubes were then ligated prior to the removal of both ovaries. The skin was finally sutured and cleaned with 70% ethanol and povidone–iodine. Sham operation was similar to the bilateral ovariectomy except that both ovaries were gently touched with forceps and left in place. Uterine weight and vaginal cytology confirmed the success of the surgery.

BMD and BMC measurements

Both parameters were determined as previously described [6]. Under 50 mg/kg sodium pentobarbitone i.p. (Abbott, North Chicago, IL, USA) anesthesia, BMD and BMC of the right femur were assessed by dual-energy X-ray absorptiometry (DXA) (model Lunar PIXImus2; GE Medical Systems, Madison, WI, USA), operated with a software version 2.10. The dual-energy supply was 80/35 kVp at 500 μA. Animals were laid prone on a supporting board with reproducible positioning. To confirm that the surrounding connective tissue and fat did not interfere with the in situ BMD and BMC measurement, femur was dissected for ex vivo BMD and BMC determination at the end of the 7-week experiment.

Bone preparation

After sacrifice, the right femur was dissected 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. BMD and BMC of the ex vivo femur were determined. For bone

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