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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.

Keywords: Alkaline phosphatase; Bone histomorphometry; Bone mineral density; Hyperprolactinemia; Pituitary transplantation

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

Table 1
Homo sapiens and *Rattus norvegicus* oligonucleotide sequences used in the PCR experiments (Protocol 2)

Gene	Ref. or accession no.	Primer (forward/reverse)	Product length (bps)	Cycle
Homo sapiens				
hPRLR	[17]	5′–AAATGTGGCATCTGCAACCGTTTTCAC–3′ 5′–GCACTTGCTTGATGTTGCAGTGAAGTT–3′	1790	30
hOC	[22]	5′–GGCCAGGCAGGTGCGAAGC–3′ 5′–GCCAGGCCAGCAGAGCGACAC–3′	271	30
hALP	[23]	5′–ACGTGGCTAAGAATGTCATC–3′ 5′–CTGGTAGGCGATGTCCTTA–3′	475	32
RANKL	[24]	5′–GCCAGTGGGAGATGTTAG–3′ 5′–TTAGCTGCAAGTTTTCCC–3′	486	33
hOPG	[24]	5′–GCTAACCTCACCTTCGAG–3′ 5′–TGATTGGACCTGGTTACC–3′	324	22
hGAPDH *	NM_002046	5′–CACCCACTCCTCCACCTTTG–3′ 5′–CCACCACCCTGTTGCTGTAG–3′	110	20
Rattus norvegicus				
rPRLR-S *	NM_012630	5′–TTCTACCACCATCGCAAC–3′ 5′–CTGATCTCGTTTGTCATTGAG–3′	120	–
rPRLR-L *	NM_001034111	5′–TCAAGCAACCGCAGACTC–3′ 5′–CAGTTTAGCCAATCGTTCCA–3′	107	–
rOC *	J04500	5′–CACAGGGAGGTGTGTGAG–3′ 5′–TGTGCCGTCCATACTTTC–3′	203	–
rOPG *	NM_012870	5′–ATTGGCTGAGTGTTCTGGT–3′ 5′–CTGGTCTCTGTTTTGATGC–3′	140	–
rGAPDH *	NM_017008	5′–AGTCTACTGGCGTCTTCAC–3′ 5′–TCATATTTCTCGTGGTTCAC–3′	133	–

PRLR, prolactin receptor; rPRLR-S, short-form rPRLR; rPRLR-L, long-form rPRLR; OC, osteocalcin; ALP, alkaline phosphatase; RANKL, receptor activator of nuclear factor κB ligand; OPG, osteoprotegerin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
* Custom-design primers.

histomorphometry, the right tibia was removed, dehydrated with sequential concentrations of ethanol (70%, 95%, and 100%), and embedded in methyl methacrylate resin. The embedded tibia was cut longitudinally at the thickness of 7 and 12 μm with a RM2255 microtome (Leica, Nussloch, Germany). The 12-μm section was used for determination of dynamic histomorphometric parameters under fluorescent microscope, while the 7-μm section was stained with Goldner-trichrome for determination of the static parameters under light microscope.

Bone histomorphometry

Bone histomorphometric analysis was modified from the methods of Li et al. and Lotinun et al. [10,19] with the Osteomeasure system (Osteomeasure Inc., Atlanta, GA, USA), operated with software version 4.1. The images of specimens were observed under a real-time video-assisted BX51 TRF fluorescent/light microscope (Olympus, Tokyo, Japan), and processed using a plotter. Primary static parameters obtained from stained sections included trabecular bone volume (BV/TV, %), trabecular number (Tb.N, mm^{-1}), trabecular separation (Tb.Sp, μm), trabecular thickness (Tb.Th, μm), osteoclast surface (Oc.S/BS, %), osteoblast surface (Ob.S/BS, %), and eroded surface (ES/BS, %). Dynamic parameters obtained from unstained sections were double-labelled surface (dLS/BS, %), mineral apposition rate (MAR, μm/day), and bone formation rate (BFR/BS, 10^{-2} $\mu m^3/\mu m^2$/day). The nomenclature, symbols, and units complied with the report of the American Society for Bone and Mineral Research Nomenclature Committee [20].

Immunohistochemistry

At the end of the experiment, the hypophyseal graft was dissected from the perirenal tissues. Paraffin-embedded 4.0-μm sections were used to detect PRL production in the transplanted gland. After blocking endogenous peroxidase activity with 3.0% H_2O_2 and 3.0% horse serum (Sigma), the sections were incubated with 1:300 PRL polyclonal primary antibody (Dako, Carpinteria, CA, USA) for 60 min prior to incubation 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 and perirenal fat pad were used as positive and negative controls, respectively. Hematoxylin-eosin (H&E) staining was also performed to identify structures of the graft. Images were acquired under light microscope (model BX51 TRF; Olympus, Tokyo, Japan).

Immunofluorescent analysis

MG-63 cells or primary rat osteoblasts cultured on a glass cover slip were fixed with a fixative containing 3% paraformaldehyde and 2% sucrose for 10 min at 25 °C. Cells were permeabilized with 0.5% Triton-X 100 in PBS for 5 min, and blocked with 10% FBS for 20 min at room temperature. Samples were then incubated with 1:300 PRLR polyclonal primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C, and later with FITC-conjugated goat anti-rabbit secondary antibody (Stratech, Cambridge, UK). Images were captured by an inverted fluorescent microscope (model Eclipse TE2000-U; Nikon, Tokyo, Japan) or a confocal laser-scanning microscope (model FV1000; Olympus, Tokyo, Japan).

Cell proliferation assay

MG-63 cells were inoculated in a 96-well culture plate (5000 cells/well). After 48-h PRL incubation, the culture medium was replaced by medium containing 10% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma). After a 3-h incubation with MTT at 37 °C, the absorbance of each well was determined at 540 nm by a microplate reader (model Multiscan EX; Thermo Labsystems, Cergy-Pontoise, France), as described previously [21]. Experiments were performed in 6 replications, and the relative proliferation of each *n* was averaged from three independent experiments.

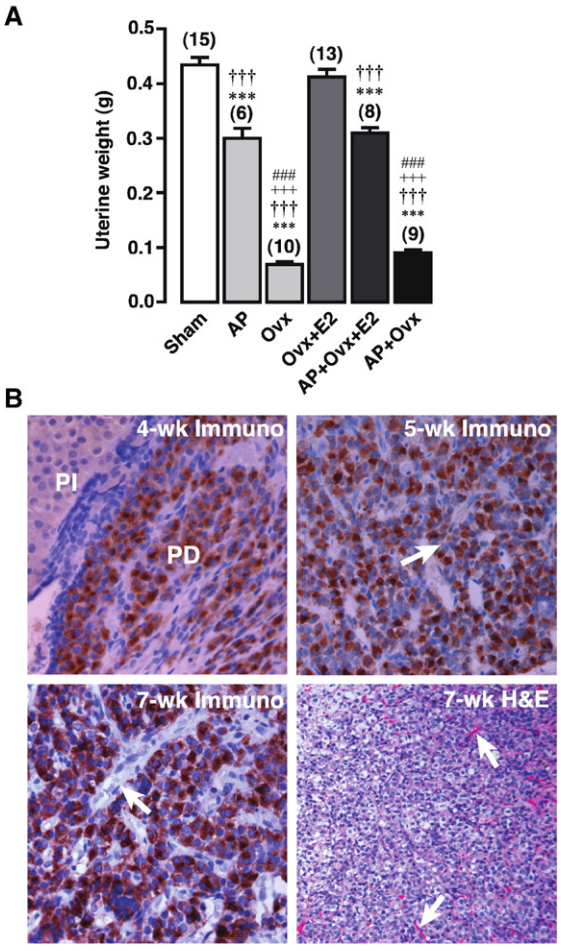


Fig. 1. (A) Uterine weight of experimental rats at 7 weeks post-surgery. ***$P<0.001$ compared with Sham. $^{\dagger\dagger\dagger}P<0.001$ compared with Ovx+E2. $^{+++}P<0.001$ compared with AP. $^{\#\#\#}P<0.001$ compared with AP+Ovx+E2. Numbers in parentheses are numbers of experimental animals. (B) Representative data of the immunohistochemical analyses (Immuno) of PRL production in the hypophyseal allografts at 4, 5 and 7 weeks post-surgery ($n=15$), magnification ×400. Corresponding 7-week graft section stained with H&E is also shown, magnification ×100. The arrows indicate sinusoids filled with numerous red blood cells. The pars distalis (PD) is strongly positive with the brownish products of peroxidase, indicating the PRL synthesis, whereas pars intermedia (PI) is negative.

Alkaline phosphatase activity assay

MG-63 cells were cultured in a 6-well culture plate (100,000 cells/well). Alkaline phosphatase activity was determined by the conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol, as previously described [13]. In brief, cells were washed twice with PBS, and incubated for 1 h with 2 mL solution containing (in mM) 100 Na_2CO_3, 10 $MgCl_2$, 20 *p*-nitrophenyl phosphate (Sigma), pH 10.3. Thereafter, 1 mL of 5 M NaOH was added. Absorbance was read at 410 nm.

Osteoblast mRNA isolation, PCR, and sequencing

The total RNA of MG-63 cells or primary rat osteoblasts were prepared by using the RNeasy mini kit (Qiagen, Valencia, CA, USA). 2.0 μg of the total RNA was reverse-transcribed with the SuperScript III (Invitrogen, Carlsbad, CA, USA) to cDNA by a thermal cycler (model Minicycler; MJ Research, Watertown, MA, USA). Sense and antisense primers for PRLR, osteocalcin, alkaline phosphatase, RANKL, OPG, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were shown in Table 1. GAPDH served as a control gene to check the consistency of the reverse transcription and to normalize values

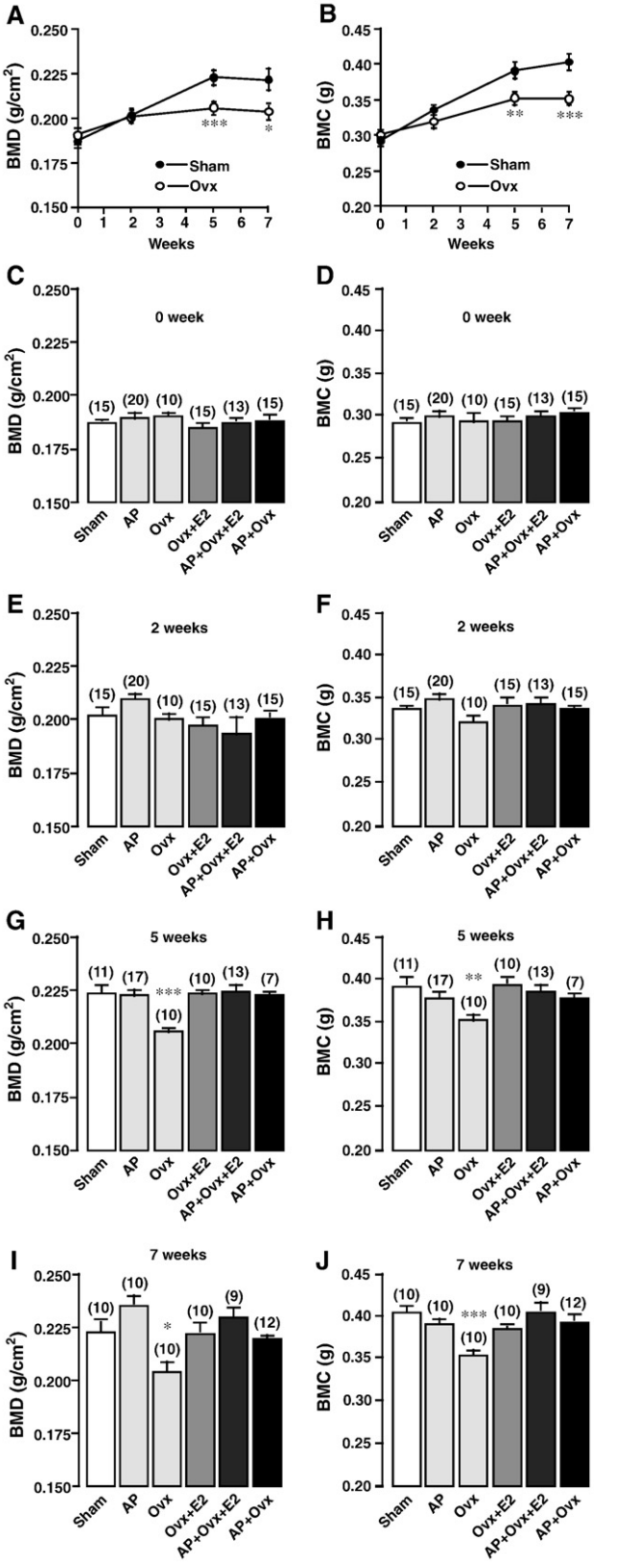
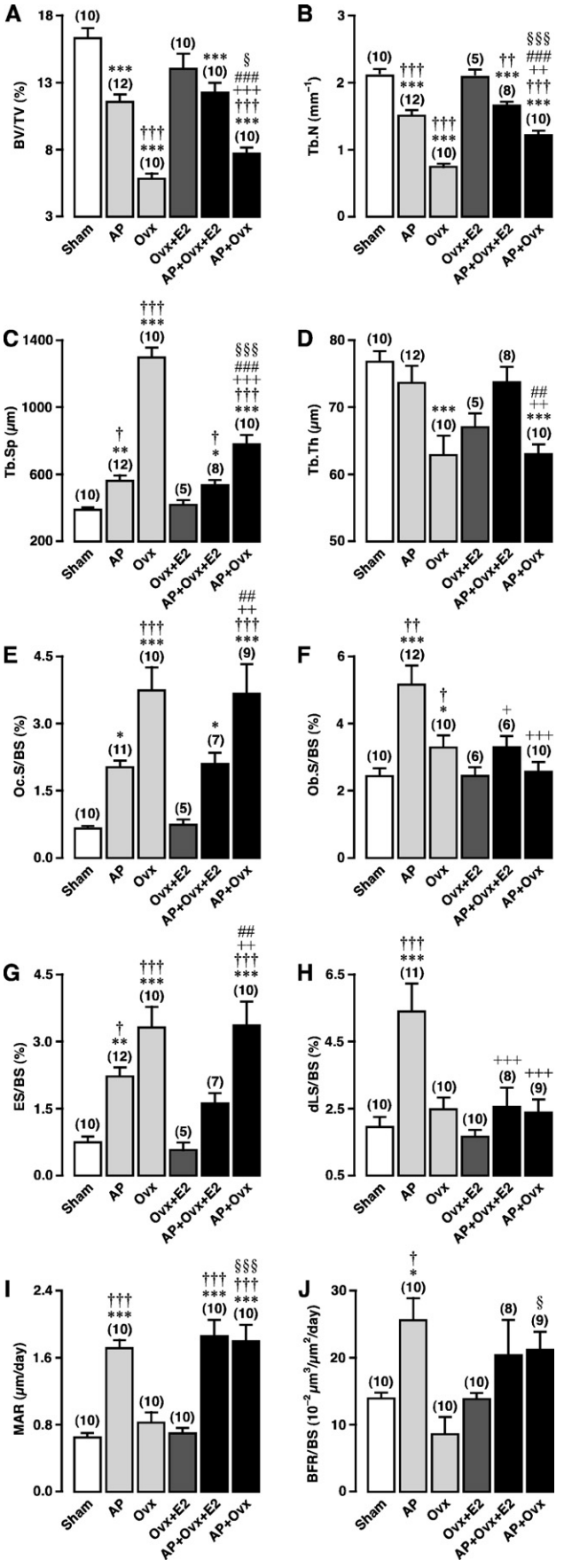


Fig. 2. (A) BMD and (B) BMC measurements of the right femur of anesthetized Sham ($n=10$–15) and Ovx ($n=10$) rats. (C–J) Femoral BMD and BMC of anesthetized Sham, AP, Ovx, Ovx+E2, AP+Ovx+E2, and AP+Ovx rats at 0, 2, 5, and 7 weeks post-surgery. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with their respective values of Sham rats. Numbers in parentheses are numbers of experimental animals.



between samples. After amplification with Taq polymerase (Qiagen), the PCR products were visualized on a 2% agarose gel stained with 1.0 μg/mL ethidium bromide under a trans-UV system (model Quantity One 2000; Bio-Rad, Hercules, CA, USA). The cycle–band intensity curve was plotted for each gene to obtain an optimal PCR cycle which fell in the exponential phase. For a semi-quantitative analysis (semi-qRT-PCR), the expression of a studied gene in the control group was considered to be 100%, while that in the experimental group was calculated as a percent change relative to the value of the control group. Expressions of rat PRLR (rPRLR), osteocalcin and OPG mRNAs were quantitatively determined by a real-time PCR (model MiniOpticon; Bio-Rad) with iQ SYBR Green Supermix kit (Bio-Rad). Amplicon sequencing was performed by the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), as described previously [25].

Western blot analysis

MG-63 cells or primary rat osteoblasts were lysed in the lysis buffer (150 mM Tris at pH 7.4, 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 1 mM DTT, 1 mM NaF, 0.5 mM Na_3VO_4, 1 mM β-glycerophosphate, 0.1% SDS, 1% DOC, 1% NP-40, and protease inhibitor cocktail) (Sigma). After a 30-min incubation at 4 °C, lysates were sonicated and centrifuged at 12,000 × g for 10 min at 4 °C, and then heated for 5 min at 95 °C before being loaded on a gel. Proteins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently transferred to a nitrocellulose membrane (Amersham, Buckinghamshire, UK) by electroblotting. Membranes were probed overnight at 4 °C with 1:500 rabbit anti-rPRLR (for primary rat osteoblasts), 1:1000 rabbit anti-human RANKL or OPG antibodies (Santa Cruz), and re-probed with 1:5000 mouse anti-β-actin antibody (Santa Cruz). After 1-h incubation at 25 °C with 1:2000 goat anti-rabbit or anti-mouse secondary antibodies (Santa Cruz), blots were visualized using the enhanced chemiluminescence kit (Amersham).

Experimental protocols

Protocol 1

The objectives of this protocol were to demonstrate changes in bone remodeling during chronic hyperprolactinemia, and to elucidate whether hyperprolactinemia had an estrogen-independent effect on bones of adult female rats. Hyperprolactinemia and estrogen deficiency were induced by AP transplantation and Ovx, respectively. Rats were randomly divided into 5 groups, i.e., sham-operated (Sham), AP-grafted (AP), Ovx supplemented with 2.5 μg/kg 17β-estradiol s.c. (Sigma) 3 times a week (Ovx+E2), AP+Ovx+E2, and AP+Ovx. Sham operation was performed in all rats which were not subject to AP transplantation or Ovx. An equivalent volume of purified corn oil was administered s.c. to all non-Ovx rats. Intact BMD and BMC of all rats were measured prior to surgery, and at 2, 5 and 7 weeks post-surgery. BMD and BMC of Ovx rats served as a positive control to demonstrate the efficiency of the densitometric technique. Then, 25 mg/kg tetracycline i.p. (Sigma) was administered twice to label bone for the histomorphometric analysis of dynamic parameters 6 days and 1 day before the animals were killed. After the rats were sacrificed (7 weeks), uterine weight measurement, PRL immunohistochemistry, ex vivo BMD and BMC measurements, and bone histomorphometry were

Fig. 3. Bone histomorphometry performed on the right tibia of Sham, AP, Ovx, Ovx+E2, AP+Ovx+E2, and AP+Ovx rats at 7 weeks post-surgery. Primary static parameters were obtained from the Goldner-trichrome-stained sections under a light microscope, including trabecular bone volume (BV/TV, A), trabecular number (Tb.N, B), trabecular separation (Tb.Sp, C), trabecular thickness (Tb.Th, D), osteoclast surface (Oc.S/BS, E), osteoblast surface (Ob.S/BS, F), and eroded surface (ES/BS, G). Dynamic parameters obtained from unstained tetracycline-labelled bone sections under a fluorescent microscope included double-labelled surface (dLS/BS, H), mineral apposition rate (MAR, I), and bone formation rate (BFR/BS, J). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with Sham. $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ compared with Ovx+E2. $^{+}P<0.05$, $^{++}P<0.01$, $^{+++}P<0.001$ compared with AP. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ compared with AP+Ovx+E2. $^{\S}P<0.05$, $^{\S\S\S}P<0.001$ compared with Ovx. Numbers in parentheses are numbers of experimental animals.

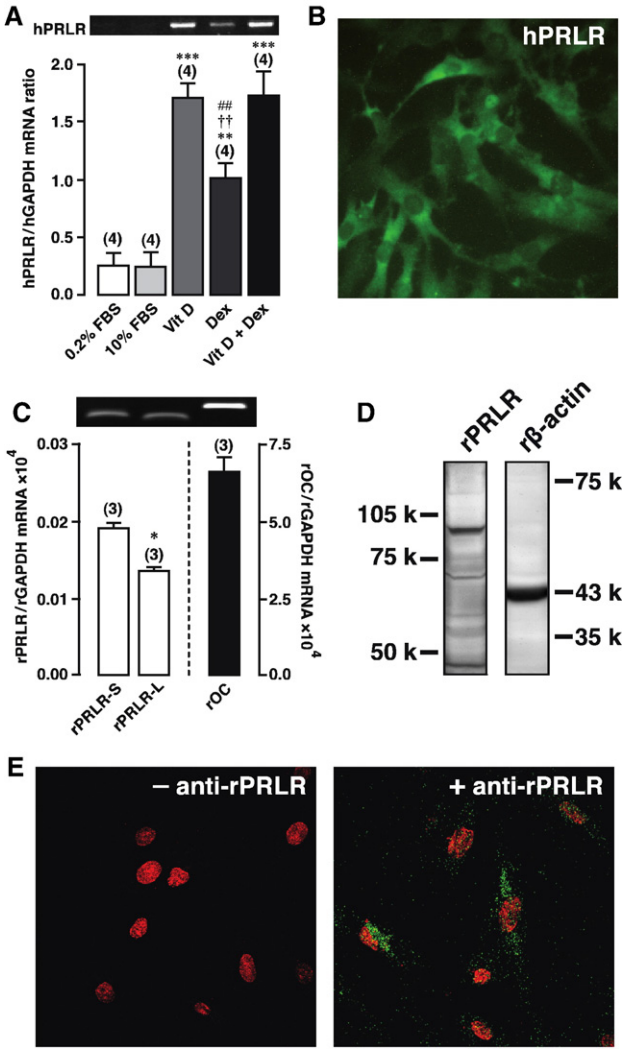


Fig. 4. (A) Expression of hPRLR mRNA (semi-qRT-PCR) in the osteoblast-like MG-63 cells exposed for 48 h to 0.2% and 10% fetal bovine serum (FBS, control), 0.1 μM 1,25-$(OH)_2D_3$ (Vit D), 1 μM dexamethasone (DEX), or a combination of Vit D and DEX (Vit D+DEX). $^{**}P<0.01$, $^{***}P<0.001$ compared with 0.2% FBS group. $^{\dagger\dagger}P<0.01$ compared with Vit D group. $^{\#\#}P<0.01$ compared with DEX group. Numbers in parentheses are numbers of independent flasks. (B) Immunofluorescent analysis of hPRLR protein expression in MG-63 cells exposed for 48 h to Vit D+DEX ($n=5$), magnification ×400. (C) Quantitative real-time PCR study of short (rPRLR-S) and long (rPRLR-L) isoforms of rPRLR and osteocalcin (rOC) in cultured osteoblasts derived from rat tibiae. $^{*}P<0.05$ compared with rPRLR-S. Numbers in parentheses are numbers of experimental animals. All experiments were performed in triplicate. (D) Western blot analysis of rPRLR protein expressions in primary rat osteoblasts ($n=5$). rβ-actin was a housekeeping protein. (E) Representative confocal immunofluorescent images of primary rat osteoblasts ($n=5$), magnification ×60. Cells were incubated in the presence (+anti-rPRLR) and absence (−anti-rPRLR) of primary antibody against rPRLR proteins. rPRLRs and nuclei were labelled in green and red, respectively.

performed. Immunohistochemical analysis of PRL production was also performed separately in 4-, 5-and 6-week AP-grafted rats. Serum OPG levels of Sham and AP-grafted rats were measured by a sandwich ELISA kit (Biomedica, Vienna, Austria), according to the manufacturer's instruction.

Protocol 2

The objective of this protocol was to elucidate the direct effects of hyperprolactinemia on osteoblast functions. To show that PRL could exert a direct action on bone, expression of rPRLR in rat cultured osteoblasts was demonstrated. Expression of human PRLR (hPRLR) in MG-63 cells was determined in the presence of 0.2% and 10% fetal bovine serum (FBS; controls), 0.1 μM 1,25-$(OH)_2D_3$ (Vit D) (Sigma), 1 μM dexamethasone (DEX) (Sigma), and a combination of Vit D and DEX by using RT-PCR and immunofluorescent analysis. Since both Vit D and DEX enhanced expression of hPRLR without effect on the studied parameters (Fig. 4), both chemicals were added to the culture medium. Thereafter, MG-63 or hFOB cells were incubated with normal medium (Control) or medium containing various concentrations of recombinant human PRL (rhPRL) (purity>97% as determined by SDS-PAGE; R&D Systems, Minneapolis, MN, USA) of 1, 10, 100, 1000 ng/mL at 37 °C for 0.5, 3, 6, 12, 24 and 48 h prior to determination of osteoblast functions. Parameters of bone formation included osteoblast proliferation, osteocalcin and alkaline phosphatase mRNA expressions, and alkaline phosphatase activity, while those of bone resorption were the ratios of RANKL/OPG mRNA and protein expressions. In some experiments, OPG mRNA expression was determined in primary rat osteoblasts incubated for 48 h with 1000 ng/mL PRL (Sigma). Expressions of mRNAs and proteins were analyzed in triplicate by RT-PCR and Western blot analysis, respectively.

Statistical analyses

Results were expressed as mean±SE. Two sets of data were compared using the unpaired Student's *t*-test. Multiple comparisons were performed by one-way analysis of variance (ANOVA) with Newman–Keuls post-test. The difference between expressions of short and long isoforms of rPRLRs was compared by Mann–Whitney test. 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 Inc., San Diego, CA, USA).

Results

Uterine weight and PRL immunohistochemistry confirmed successful Ovx and AP transplantation

In the present study, estrogen deficiency and hyperprolactinemia were induced by Ovx and AP transplantation, respectively. After a 7-week period of Ovx, the uterine weight was markedly decreased as expected, and 2.5 μg/kg 17β-estradiol supplementation (E2) completely reversed the effect of Ovx (Fig. 1A). Both AP and AP+Ovx+E2 rats showed similar decreases in the uterine weight, which was greater than those of Ovx rats, suggesting an anti-estrogen-like effect of hyperprolactinemia on the uteri. Uterine weights of AP+Ovx rats were not different from that of Ovx rats.

To examine the viability and function of the AP allografts, the grafts were dissected from the perirenal tissues of 4-, 5-, 6- and 7-week AP rats, and analyzed histologically and immunohistochemically. They were highly vascularized reddish tissue with a diameter of ~2 mm. H&E staining showed typical cells of varying sizes, arranged in irregular cords and clusters, consistent with normal pituitary glands (Fig. 1B). Numerous sinusoidal networks were seen between the clusters of cells (arrows in Fig. 1B). Immunohistochemistry revealed active PRL production in the pars distalis, but not in the pars intermedia or surrounding connective tissues (Fig. 1B). As microvascular endothelial damage and lymphoid infiltration were not seen, there was neither graft rejection nor allograft vasculopathy. The AP rats thus possessed two healthy ectopic pituitary glands which, unlike the normal pituitary gland under the hypothalamic dopaminergic control, actively and continuously produced PRL. Sustained plasma PRL levels induced by this procedure

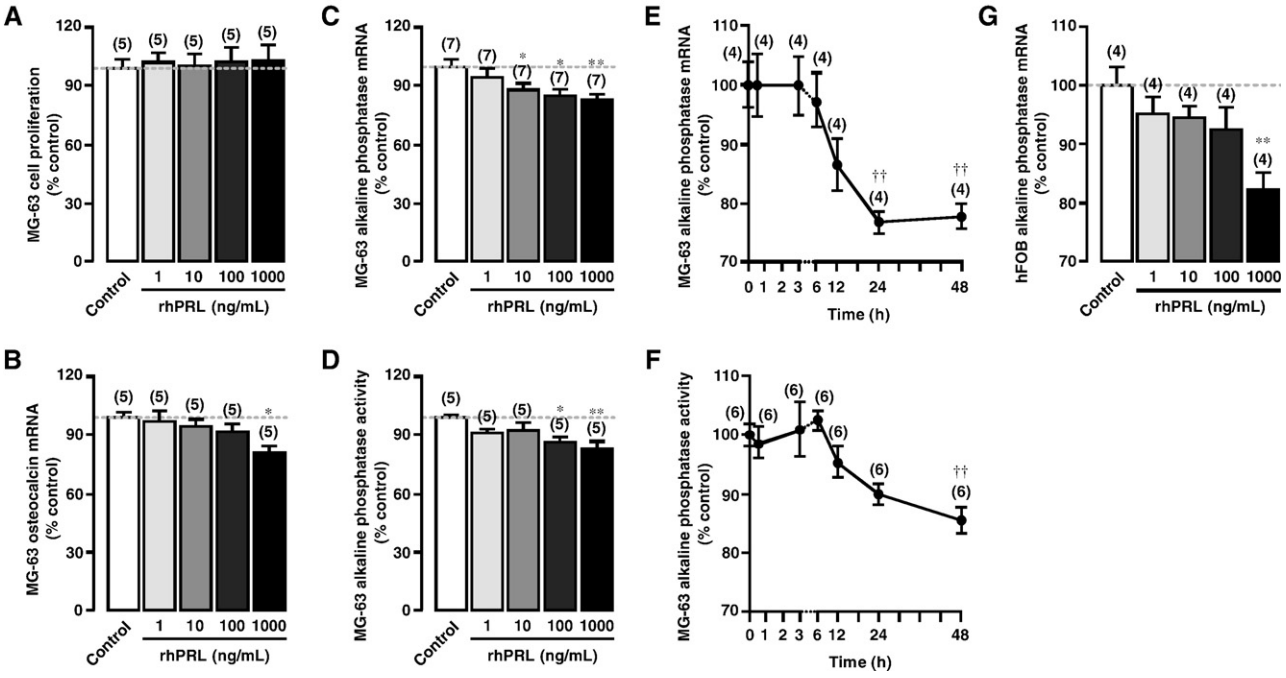


Fig. 5. Dose-dependent changes in bone formation parameters which are osteoblastic cell proliferation (A), osteocalcin mRNA expression (B), alkaline phosphatase mRNA expression (C), and alkaline phosphatase activity (D) in MG-63 cells incubated for 48 h with 1, 10, 100 or 1000 ng/mL rhPRL. (E) and (F) present time-dependent changes in alkaline phosphatase mRNA expression and activity, respectively, in MG-63 cells directly exposed to 1000 ng/mL rhPRL. (G) dose-dependent changes in alkaline phosphatase mRNA expression in hFOB cells incubated for 48 h with 1, 10, 100 or 1000 ng/mL rhPRL. mRNA expressions were demonstrated by semi-qRT-PCR. $^{*}P<0.05$, $^{**}P<0.01$ compared with the control group. $^{\dagger\dagger}P<0.01$ compared with the values at 0 h. Numbers in parentheses are numbers of independent flasks. Experiments were performed in triplicate.

averaged about 91 ng/mL [16] which was comparable to the levels in pregnant rats [26], and much higher than ~7–10 ng/mL in normal non-pregnant adult female rats [16,26].

Hyperprolactinemia did not change femoral bone mineral density (BMD) or content (BMC)

In situ femoral BMD and BMC were determined at 0-, 2-, 5- and 7-week post-surgery to evaluate the effect of Ovx and hyperprolactinemia. In all groups, BMD and BMC gradually increased with age. Ovx rats characteristically showed BMD and BMC that were lower at weeks 5 and 7 (Figs. 2A and B), but were fully restored to the Sham values by estrogen supplementation. BMD and BMC of ex vivo femurs also exhibited consistent results (data not shown). Hyperprolactinemia did not affect BMD or BMC in AP and AP+Ovx+E2 groups (Figs. 2C–J). Interestingly, BMD and BMC of AP+Ovx rats were comparable to those of the age-matched Sham rats (Figs. 2C–J). Our results suggested that hyperprolactinemia may have an estrogen-independent action on bone since it maintained, instead of reduced, bone mass in AP+Ovx rats.

Histomorphometric analyses revealed complex patterns of microscopic changes in AP rats, and suggested a direct action of PRL on bone

Although densitometric analyses did not show any change in BMD or BMC of AP rats, histomorphometric analyses revealed high bone turnover in AP rats, as shown in Fig. 3. Our data indicated enhanced bone resorption with decreases in bone volume (BV/TV) and trabecular number (Tb.N) in AP rats, whereas trabecular separation (Tb.Sp), osteoclast surface (Oc.S/BS), and eroded surface (ES/BS) were increased with no change in the trabecular thickness (Tb.Th). Meanwhile, increases in the osteoblast surface (Ob.S/BS), double-labelled surface (dLS/BS), mineral apposition rate (MAR), and bone formation rate (BFR/BS) indicated enhanced bone formation. However, it was likely that changes in the resorptive parameters were predominant as BV/TV was decreased.

In the Ovx rats, most histomorphometric parameters including BV/TV, Tb.N, Tb.Sp, Tb.Th, Oc.S/BS and ES/BS showed explicit bone resorption, whereas a bone formation parameter, Ob.S/BS, was also increased. Effects of Ovx on bone were completely prevented by estrogen supplementation as indicated by all histomorphometric parameters in the Ovx+E2 group being comparable with those of the Sham group. AP+Ovx+E2 rats, similar to the AP rats, manifested high bone turnover although some parameters were not significantly different from those in the Sham or Ovx+E2 rats, i.e., Ob.S/BS, ES/BS, dLS/BS, and BFR/BS. In the AP+Ovx group, BV/TV, Tb.N and Tb.Th were much lower than those in the Sham or AP groups, whereas Tb.Sp, Oc.S/BS and ES/BS were greater, consistent with those in estrogen-deficient animals. However, BV/TV, Tb.N and BFR/BS in AP+Ovx rats were greater, while Tb.Sp was less than those in Ovx rats. Of interest, in contrast to the Ovx rats, Ob.S/BS in the AP+Ovx rats were not increased, and MAR was significantly higher than that in Sham and Ovx rats. We therefore postulated that the effects of hyperprolactinemia on bone were

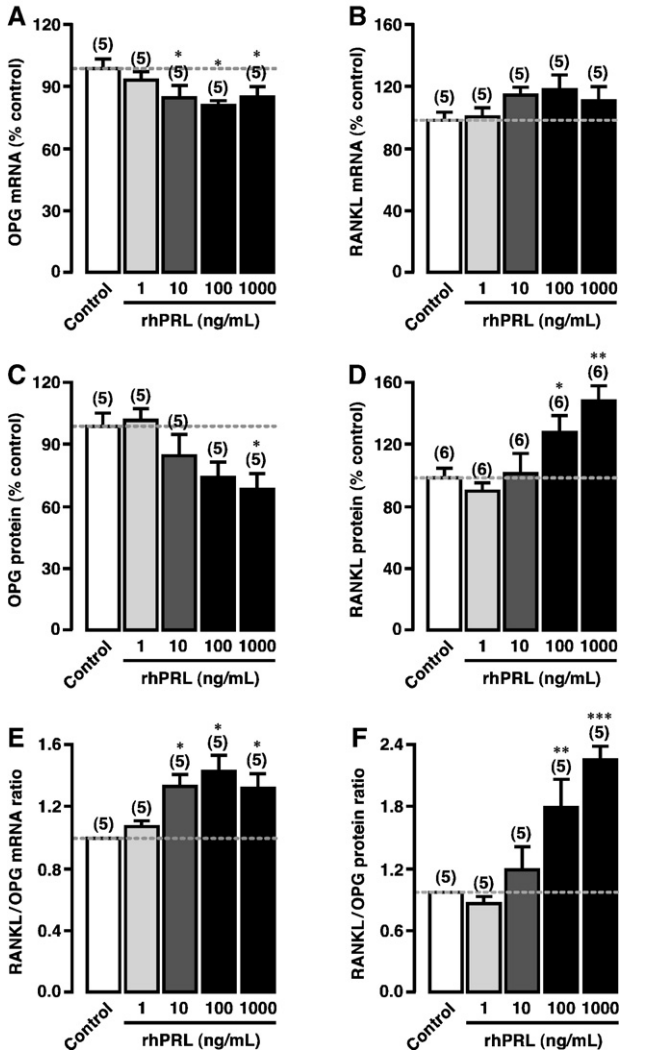


Fig. 6. Dose-dependent changes in the bone resorption-associated parameters which are mRNA expressions of OPG (A) and RANKL (B), protein expressions of OPG (C) and RANKL (D), and the ratios of RANKL/OPG mRNA (E) and protein (F) expressions in MG-63 cells incubated for 48 h with 1, 10, 100 or 1000 ng/mL rhPRL. mRNA expressions were demonstrated by semi-qRT-PCR, while protein expressions were studied by Western blot analysis. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with the control group. Numbers in parentheses are numbers of independent flasks. Experiments were performed in triplicate.

different from those of estrogen depletion, and PRL possibly exerted a direct action on bone partly in an estrogen-independent manner.

The osteoblast-like cells MG-63 and rat cultured osteoblasts expressed PRLR

To investigate if PRL had a direct action on bone, we studied bone formation and resorption parameters in human osteoblast-like cells (MG-63). As shown in Fig. 4A, the expression of human PRLR (hPRLR) mRNA, which was considerably weak in 0.2% and 10% fetal bovine serum (FBS)-treated groups (controls), was strongly enhanced by 0.1 μM 1,25-$(OH)_2D_3$ (Vit D), 1 μM dexamethasone (DEX), and the combination of both. The effect of DEX on hPRLR mRNA expression was lower than that of Vit D. Since Vit D and DEX affected neither studied parameters nor cell proliferation (data not shown), both agents were added in the culture media in the following experiments. Vit D + DEX not only upregulated hPRLR mRNA but also stimulated hPRLR protein expression in MG-63 cells (Fig. 4B).

Moreover, by using the quantitative real-time PCR, cultured osteoblasts derived from the rat tibiae were shown to express mRNAs of short and long isoforms of rat PRLR (rPRLR) with a predominant short isoform, as well as bone-specific marker, osteocalcin (Fig. 4C). Several isoforms of rPRLR proteins migrated as multiple bands in the molecular weight range of 40 to 100 kDa (Fig. 4D). Confocal fluorescent imaging also demonstrated expressions of rPRLR proteins in primary rat osteoblasts (Fig. 4E). These results confirmed that osteoblasts were targets of PRL.

Expressions of bone formation markers in MG-63 were decreased by recombinant human PRL (rhPRL)

Fig. 5 shows the effects of 48-h rhPRL (1, 10, 100 and 1000 ng/mL) on the osteoblastic markers of bone formation. While none of the concentrations used changed the rate of MG-63 proliferation (Fig. 5A), rhPRL of 1000 ng/mL significantly downregulated osteocalcin mRNA expression to 81.65±3.40% of control ($P<0.05$) (Fig. 5B). Alkaline phosphatase mRNA expression was decreased by 10, 100 and 1000 ng/mL rhPRL to 88.62±3.02 ($P<0.05$), 85.43±2.57 ($P<0.05$), 83.11±2.57 ($P<0.01$) % of control (Fig. 5C), respectively, while 100 and 1000 ng/mL rhPRL reduced the activities of alkaline phosphatase to 87.46±2.89 and 84.19±2.77% of control (Fig. 5D). In the time–response studies, 1000 ng/mL rhPRL significantly decreased mRNA expression (Fig. 5E) and activity of alkaline phosphatase (Fig. 5F) at 24 h and 48 h after exposure, respectively. A decrease in the mRNA expression of alkaline phosphatase was also observed in hFOB cells (Fig. 5G). Therefore, our findings indicated that the direct effects of PRL on osteoblasts were on the osteoblastic functions rather than on cell proliferation.

High PRL exposure increased the ratios of MG-63-expressed RANKL/OPG mRNAs and proteins

Effects of rhPRL exposure on the markers of bone resorption are presented in Fig. 6. The ratio of RANKL and OPG, both of

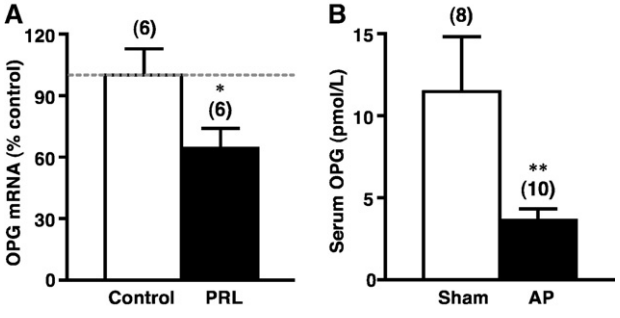


Fig. 7. (A) Expression of OPG mRNA (real-time PCR) in primary rat osteoblasts incubated for 48 h with 1000 ng/mL PRL. (B) Serum OPG levels in Sham and AP rats. $^{*}P<0.05$ compared with control. $^{**}P<0.01$ compared with Sham rats. Numbers in parentheses are numbers of experimental animals.

which were synthesized by osteoblasts, represented bone resorption [12]. In the present investigation, 48-h exposure to 10, 100 or 1000 ng/mL rhPRL decreased OPG mRNA expression in MG-63 cells to 85.83±4.49 ($P<0.05$), 82.68±1.05 ($P<0.05$), and 85.37±4.37 ($P<0.05$) % of control (Fig. 6A), respectively, while the same concentrations had no effect on RANKL mRNA expression (Fig. 6B). At the translational level, 48-h 1000 ng/mL rhPRL exposure downregulated OPG protein expression to 69.23±7.58% of control ($P<0.05$) (Fig. 6C), whereas 100 and 1000 ng/mL rhPRL increased RANKL protein expression to 127.73±10.07 ($P<0.05$) and 147.30±9.22 ($P<0.01$) % of control (Fig. 6D), respectively. The RANKL/OPG mRNA ratios were therefore increased by 10, 100 and 1000 ng/mL rhPRL to 1.35±0.08 ($P<0.05$), 1.43±0.11 ($P<0.05$) and 1.32±0.12 ($P<0.05$), respectively (Fig. 6E). Similarly, the ratios of RANKL/OPG protein were increased by 100 and 1000 ng/mL rhPRL to 1.81±0.25 ($P<0.01$) and 2.27±0.19 ($P<0.001$) (Fig. 6F). rhPRL of 10 ng/mL tended to increase the ratio of RANKL/OPG protein; however, the change was statistically insignificant (Fig. 6F).

In primary rat osteoblasts, 1000 ng/mL PRL decreased OPG mRNA expression to 64.21±10.12% of control ($P<0.05$) (Fig. 7A). Moreover, high PRL in AP-grafted rats showed a decrease in serum OPG level (Fig. 7B), i.e., from 11.47±3.35 to 3.63±0.71 pmol/L ($P<0.01$). Our results suggested that PRL enhanced bone resorption by increasing RANKL expression and decreasing OPG expression, thereby raising the RANKL/OPG ratio.

Discussion

Physiological hyperprolactinemia occurs during pregnancy and lactation, while pathological hyperprolactinemia is a common presentation in clinical practice. Effects of hyperprolactinemia on bone have been documented in patients and experimental animals [2,6,9], but it was not known whether PRL exerted its actions on bone directly or via PRL-induced hypogonadism. Therein, we provided supportive evidence that high physiological levels of PRL (~90–100 ng/mL) could directly regulate the osteoblast functions with a consequent increase in bone turnover, that involved a complex microscopic change leading to bone loss in an estrogen-independent manner.

In the present investigation, we used Ovx and AP transplantation to induce estrogen deficiency and hyperprolactinemia, respectively, and the differential bone responses in these conditions were evaluated. AP transplantation has two advantages over PRL injection in that, (*i*) it does not induce chronic stress in animals, and (*ii*) it produces sustained PRL levels of ~90–100 ng/mL, comparable to the levels during pregnancy in humans and rats [16,26]. Consequently, our results represent changes in bone calcium metabolism under the physiological hyperprolactinemia. The present data show that, during the 7-week AP transplantation, there was no sign of graft rejection or atrophy, and the AP allografts continued to synthesize PRL. Regarding the uterine weight which is normally used to indicate estrogenic status in rats, no change in the uterine weights of Ovx+E2 rats from those of Sham indicated that 2.5 μg/kg 17β-estradiol supplementation was sufficient to replace the circulating estrogen in Ovx rats. Since the serum estrogen concentrations in both AP and Ovx rats were reported to be comparable and less than 50 pg/mL [27], the estrogen levels in the AP group after estrogen supplementation (AP+Ovx+E2) should be close to normal. However, the uterine weights in AP+Ovx+E2 rats were still significantly lower than those in Ovx+E2 rats. Hence, PRL may have some direct antiestrogenic actions on the uterus independently of circulating estrogen. In other words, if the effect of PRL on the uterus is solely due to the PRL-suppressed serum estrogen level, this dose of estrogen supplement should be sufficient to restore the uterine weight. Such antiestrogenic action of PRL has been reported before in the mouse uterus, in which PRL prevented the formation of atypical hyperplasia [28].

Although several investigators suggested that the effects of hyperprolactinemia on bone were produced by prolactin-induced estrogen deficiency [5,11], the presence of PRLRs in osteoblasts, but not in osteoclasts, implicated osteoblasts as a direct target of PRL [6,13,17]. PRLRs were identified in the dexamethasone-stimulated osteoblast-like cells MG-63 and Saos-2 human osteosarcoma cell lines [17], as well as in the primary osteoblasts obtained from neonatal calvaria [29]. MG-63 cells normally express a very low amount of hPRLR mRNA. However, consistent with the previous report [17], hPRLR expression could be upregulated by dexamethasone and the physiological concentration of 1,25-$(OH)_2D_3$. Moreover, we recently demonstrated that tibia, femur, calvaria and L5-6 vertebrae of adult female rats expressed both short and long isoforms of rPRLR, suggesting that both cortical and trabecular sites were targets of PRL [6]. Expressions of rPRLR transcripts and proteins in rat cultured osteoblasts, as seen in the present study, further confirmed this hypothesis. In addition, decreases in BMD, MAR and BFR in $PRLR^{-/-}$ mice strongly suggested a direct action of PRL on bone [29]. We therefore performed a series of in vivo experiments to demonstrate mechanisms underlying changes in bone remodeling induced by hyperprolactinemia and estrogen deficiency.

An absence of change in the femoral BMD, BMC and bone calcium content during hyperprolactinemia has been reported before [6]. In the present study, unaltered BMD and BMC in the 2-, 5-and 7-week AP rats could be interpreted as PRL having no effect on bone, bone formation and resorption being equally affected, or changes being too small to be detected by DXA. However, the fact that high PRL could restore BMD and BMC in the AP+Ovx rats to Sham level suggested differential responses of bone to Ovx-induced estrogen deficiency and hyperprolactinemia. If hyperprolactinemia reduced bone mass solely through hypogonadism, AP+Ovx rats should have exhibited osteopenia as in the Ovx rats. Therefore, it was likely that PRL had an estrogen-independent action on bone.

By using bone histomorphometry, it was found that hyperprolactinemia in AP and AP+Ovx rats differentially stimulated tibial bone turnover, possibly in an estrogen-independent fashion. In the Ovx rats, most parameters, i.e., BV/TV, Tb.N, Tb.Sp, Tb.Th, Oc.S/BS and ES/BS, implicated severe bone resorption, whereas increased Ob.S/BS indicated enhanced bone formation. These data confirmed accelerated bone turnover with

bone formation coupled to bone resorption, as previously reported in Ovx rats [30,31]. This osteopenic state could be detectable by DXA as early as 5 weeks post-Ovx. However, despite having changes in parameters such as Tb.N, Tb.Sp, Oc.S/BS and ES/BS indicative of enhanced bone resorption as in the Ovx rats, the small decrease in BV/TV in the AP rats which was not large enough to be detected by DXA was probably a result of the concurrent increase in bone formation as indicated by two-fold increase in Ob.S/BS, MAR, and BFR/BS. Increases in Ob.S/BS and Oc.S/BS further suggested the coupled activation of osteoblastic and osteoclastic activities, and/or proliferation of both cell types in the AP rats. Thus, unlike in Ovx, bones exposed to high physiological PRL were in a high bone turnover state, and not in an overt osteopenic state.

Estrogen-independent action of PRL was further demonstrated in the AP+Ovx rats. Unchanged Ob.S/BS, dLS/BS, and BFR/BS in AP+Ovx rats not only indicated bone formation being uncoupled from bone resorption, but also demonstrated the differential responses of in vivo bone to hyperprolactinemia and estrogen deficiency. Increases in MAR and BFR/BS in AP rats were also in agreement with the report of Clément-Lacroix et al., which showed decreases in both parameters in $PRLR^{-/-}$ mice [29]. Interestingly, the AP+Ovx group, despite having a high osteoclastic activity, also exhibited a high MAR similar to the AP rats. Therefore, under the hyperprolactinemic condition, there was a PRL-mediated increase in the mineralization process which, in turn, helped prevent a further decrease in BMD. Incidentally, we have recently demonstrated a ~64% increase in the transcellular active calcium transport in the duodenum of the 4-week AP+Ovx rats [32]. In the presence of a high physiological PRL (~90–100 ng/mL), the extra calcium from the enhanced intestinal calcium absorption probably contributed to the increased mineralization, and in doing so, alleviated bone loss during estrogen deficiency. Increased bone calcium deposition also partly explained the undetectable change in BMD in the AP+Ovx rats.

Our densitometric and histomorphometric findings corroborated the estrogen-independent effects of PRL, and strengthened the hypothesis of a direct PRL action on bone. However, since the serum estrogen and PRL concentrations were not measured, the presents results provided only a suggestive evidence for the estrogen-independent effects of PRL. Indeed, direct actions of PRL could not be concluded solely from the in vivo findings, since effects of other factors, e.g., changes in the gonadotrophin levels, extraovarian estrogen and dietary calcium, could not be excluded. To show that PRL had a direct action on bone, we studied the in vitro effects of rhPRL on the synthesis of bone formation and bone resorption markers by the osteoblast-like MG-63 and hFOB cells.

Even though PRL was known to stimulate cell proliferation and tumorigenesis in mammary epithelial and Nb2 cells [33,34], a previous study by Coss et al. [13] and the present study found no effect of PRL on osteoblast proliferation. Nevertheless, the mRNA expression of osteocalcin and alkaline phosphatase, markers of osteoblast functions, were attenuated by rhPRL. The PRL-induced decrease in alkaline phosphatase activity in MG-63 cells was consistent with the findings in primary neonatal rat osteoblasts [13] and human fetal osteoblast (hFOB) 1.19 cells (Fig. 5G). These in vitro findings were contrary to the histomorphometric data of the AP rats that indicated an enhanced mineral apposition and bone formation. Increased bone formation seen in vivo could be due to the coupling of bone formation to bone resorption, which was absent in vitro. Alternatively, an increase in bone formation could have resulted from the increased calcium supply from the PRL-stimulated intestinal calcium absorption [16,32]. As bone formation is a complex process consisting of osteoid formation and mineralization, availability of calcium in the plasma is crucial for mineralization, thereby increasing the rate of bone formation even in the vitamin D-deficient rats [35]. Further investigations are required to demonstrate the effects of PRL on the intricate interactions between bone and intestine.

Regarding markers of bone resorption, we determined the change in the RANKL/OPG expression ratio in MG-63 cells. A number of studies reported that the age-related osteopenia, eroded bone surface, and high incidence of hip fracture in humans were positively correlated with an increase in RANKL/OPG ratio [12,36–38]. So far, there has been no report on the effect of PRL on the osteoblast expression of RANKL and OPG. Expression of PRLR exclusively in osteoblasts, but not in osteoclasts [13,39], evinced the osteoblast as the modulator of PRL-induced bone resorption. Herein, we demonstrated, for the first time, that rhPRL upregulated the expression of RANKL but downregulated that of OPG, thus leading to an increase in the RANKL/OPG ratio both at the transcriptional and translational levels. Downregulation of OPG mRNA expression in primary rat osteoblasts and a decrease in serum OPG levels in AP rats were consistent with the findings in MG-63 cells.

The concentration of PRL may be a salient determinant of bone loss, because PRL regulated the expression of bone markers, including osteocalcin, alkaline phosphatase, RANKL and OPG, in a dose-response manner. A pathological level of rhPRL (1000 ng/mL) markedly changed all measured parameters of bone turnover, whereas 100 ng/mL rhPRL had less effect on the osteoblastic function. It was also apparent from our previous report that decreases in bone formation and bone mass may be prevented by the concurrent stimulation of the intestinal calcium absorption with ~100 ng/mL PRL [32,40]. Our postulation corroborated findings that pregnancy did not induce significant osteopenia, whereas long-term lactation with ~200–400 ng/mL plasma PRL led to reversible bone loss [3]. Since PRLR knockout impaired ossification in neonatal mice and decreased BMD in adult mice [29], the basal circulating PRL (~7–10 ng/mL) must also be essential for the maintenance of normal bone growth and remodeling. In the present study, 10 ng/mL PRL maintained a slightly elevated expression ratio of RANKL/OPG mRNA which was apparently appropriate for normal bone functions. On the other hand, pathological levels of PRL often resulted in overt osteopenia similar to estrogen deficiency [1,2]. Our findings confirmed that 1000 ng/mL PRL could increase bone resorption, as indicated by a marked increase in the RANKL/OPG ratio. Of note was our previous finding that 1000 ng/mL PRL exerted a typical biphasic action on the small intestine, i.e., the enhanced calcium absorption was diminished

to the basal level [41,42], thereby aggravating a negative calcium balance. Thus, the massive bone loss in pathological hyperprolactinemia could result from the PRL-enhanced bone resorption without matching increase in the intestinal calcium absorption.

In conclusion, hyperprolactinemia in adult rats resulted in a high bone turnover and bone loss, but its effects on bone resorption were not as prominent as in Ovx rats. High physiological PRL and estrogen deficiency each rendered distinct patterns of bone remodeling, supporting our hypothesis that PRL had some estrogen-independent actions in bone. Further investigation in MG-63 cells confirmed the direct actions of PRL on osteoblasts in which PRL suppressed the osteocalcin expression and alkaline phosphatase activity while increasing the RANKL/OPG ratio. Our results were able to explain the clinical findings of the hyperprolactinemia-induced high bone turnover and osteopenia.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

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