Ovariectomy worsens secondary hyperparathyroidism in mature rats during low-Ca diet

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Submitted 24 August 2006; accepted in final form 24 October 2006

Ovariectomy worsens secondary hyperparathyroidism in mature rats during low-Ca diet. Am J Physiol Endocrinol Metab 292: E723–E731, 2007. First published October 31, 2006; doi:10.1152/ajpendo.00445.2006.—Estrogen deficiency impairs intestinal Ca absorption and induces bone loss, but its effects on the vitamin D-endocrine system are unclear. In the present study, calciotropic hormones levels, renal vitamin D metabolism, 1,25-dihydroxyvitamin D3 [1,25(OH)2D3]-dependent intestinal calcium absorption, and bone properties in 3-mo-old sham-operated (sham) or ovariectomized (OVX) rats fed either a normal-Ca (NCD; 0.6% Ca; 0.65% P) or a low-Ca (LCD; 0.1% Ca; 0.65% P) diet for 2 wk were determined. LCD increased serum 1,25(OH)2D3 levels in both sham and OVX rats. Serum parathyroid hormone [PTH(1–84)] levels were highest in OVX rats fed LCD. Renal 25-hydroxyvitamin D1α-hydroxylase (1-OHase) mRNA expression was highest in OVX rats fed LCD. Renal vitamin D receptor (VDR) and mRNA expressions in rats were induced by ovariectomy in rats fed NCD but suppressed by ovariectomy in rats fed LCD. The induction of intestinal calcium transporter-1 and calbindin-D9k mRNA expressions by LCD were not altered by ovariectomy. As expected, bone Ca content, cancellous bone mineral density, and bone strength index in proximal metaphysis of rat tibia were reduced by both ovariectomy and LCD (P < 0.05) as analyzed by two-way ANOVA. Taken together, the data demonstrate that ovariectomy alters the responses of circulating PTH levels, renal 1-OHase mRNA expression, and renal VDR expression to LCD. These results suggest that estrogen is necessary for the full adaptive response to LCD mediated by both PTH and 1,25(OH)2D3.

parathyroid hormone; renal vitamin D receptor; renal 25-hydroxyvitamin D1α-hydroxylase; dietary Ca restriction

The impairment of intestinal Ca absorption in humans (19, 23, 35, 39) and in rats (2, 9, 26, 29) and induction of bone turnover (1, 15, 30) by estrogen deficiency are believed to contribute to negative Ca balance and bone loss during menopause. The cause of the impairment of intestinal Ca absorption by estrogen deficiency has been attributed to either a decrease in serum 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] levels (9, 16, 39) or a direct effect of estrogen on intestinal Ca transport (3, 9, 14, 26). During menopause, the increased Ca requirements due to poor intestinal absorption and increased urinary loss are complicated by the age-related abnormalities of the vitamin D-endocrine system, including secondary hyperparathyroidism, intestinal resistance to 1,25(OH)2D3, and decreased 1,25(OH)2D3 production due to impaired 25-hydroxyvitamin D1α-hydroxylase (1-OHase) activity (37, 38). Although the effects of menopause and estrogen on circulating levels of 1,25(OH)2D3 in postmenopausal women have been reported (13, 36), it is still unclear whether estrogen deficiency interferes with the vitamin D-endocrine system during adaptation to dietary Ca restriction.

Indeed, the effects of estrogen on vitamin D metabolism (35, 38) remain unclear. In vitro studies (24, 40, 44) using cultures of chick kidney cells showed that estrogen did not exert a direct effect on 1,25(OH)2D3 synthesis; in vivo stimulatory effects of estrogen on vitamin D metabolism in birds (8, 34, 43) and in rats (7) have been reported. The renal 1-OHase enzyme catalyzes the biosynthesis of 1,25(OH)2D3 (25). Inadequate dietary supply of Ca increases the secretion of parathyroid hormone (PTH), which in turn stimulates renal 1-OHase gene expression and the production of 1,25(OH)2D3 (11, 25, 45). Through a negative-feedback system, 1,25(OH)2D3 inhibits 1-OHase gene expression via the renal vitamin D receptor (VDR) (33). As estrogen is an important modulator in the actions of the vitamin D system, PTH, and extracellular Ca, it is not clear whether estrogen is a direct regulator of vitamin D metabolism in vivo. Most importantly, the progressive decreases in renal responsiveness of 1-OHase expression to low-Ca and low-P diets with age (5, 6, 12, 18, 28) raise the possibility that estrogen deficiency per se may be the common denominator in the altered function of the renal 1-OHase to its stimuli.

Calcium transport protein-1 (CaT1), the protein product of the TRPV6 gene, and calcium binding protein-9k (CaBP-9k) are involved in duodenal Ca2+ transcellular transport (4, 10, 42). Low-Ca diet (LCD) increases apical uptake and transcellular transport of Ca via the induction of 1,25(OH)2D3-dependent CaT1 (42) and CaBP-9k (4) expression in intestinal epithelia, respectively. However, the study by Song and Fleet (41) indicated that intestinal Ca absorption and duodenum CaT1 mRNA expression in female mice were more sensitive to changes in serum 1,25(OH)2D3 levels than in male animals, suggesting that regulation of intestinal Ca transport was gender
specific. Thus the role of estrogen in regulating the responses of intestinal Ca transport to LCD remains to be determined.

The objective of this study was to determine the role of estrogen in the responses of the vitamin D-endocrine system to dietary Ca restriction. With the use of mature female rats, the effects of ovarectomy and LCD on circulating PTH and 1,25(OH)2D3 levels, renal 1-OHase and VDR expression, intestinal CaBP-9k and CaT1 mRNA expression, and bone properties were systematically evaluated.

MATERIALS AND METHODS

Animals study design. Thirty 3-mo-old virgin female Sprague-Dawley rats (309 ± 4 g) were purchased from the GuangZhou University of Traditional Chinese Medicine (GuangZhou, China). Rats were housed in a room with alternating 12-h periods of light and dark, a constant temperature of 23 ± 1°C, and humidity of 55 ± 5%.

Fourteen rats were sham operated, and sixteen rats were ovariectomized (OVX) to make them estrogen deficient. Following ovariec-
tomy, rats were pair fed with regular chow for 5 wk before feeding for 2 days on normal-calcium diet (NCD; TD98005, 0.6% Ca, 0.65% P) for equilibrium. The animals were then randomized to either NCD or LCD (TD98006, 0.1% Ca, 0.65% P) for 2 wk. The rats were divided into four groups: sham rats fed NCD (SN, n = 7), sham rats fed LCD (SL, n = 7), OVX rats fed NCD (ON, n = 8), and OVX rats fed LCD (OL, n = 8). All diets were purchased from Harlan Teklad (Madison, WI). The compositions of the diets are shown in Table 1. All animals were allowed free access to distilled water. Body weights were measured weekly during the experimental period. Before death, each rat was individually housed in a metabolic cage, and 24-h urine samples were collected using separators. Urine samples were acidified with 5% powdered milk. The objective of this study was to determine the role of estrogen in regulating the responses of the vitamin D-endocrine system to dietary Ca restriction. With the use of mature female rats, the effects of ovarectomy and LCD on circulating PTH and 1,25(OH)2D3 levels, renal 1-OHase and VDR expression, intestinal CaBP-9k and CaT1 mRNA expression, and bone properties were systematically evaluated.

Table 1. Diet composition

<table>
<thead>
<tr>
<th></th>
<th>Normal-Ca Diet* (6 g Ca/kg diet)</th>
<th>Low-Ca Diet† (1 g Ca/kg diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casin</td>
<td>110.0</td>
<td>110.0</td>
</tr>
<tr>
<td>Egg white solids, spray dried</td>
<td>97.9</td>
<td>97.9</td>
</tr>
<tr>
<td>Bovine Methylene</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>551.0903</td>
<td>563.5803</td>
</tr>
<tr>
<td>Corn starch</td>
<td>100.0</td>
<td>100.0</td>
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<td>Corn oil</td>
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</tr>
<tr>
<td>Cellulose</td>
<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Vitamin mixture‡</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Mineral mixture§</td>
<td>0.042</td>
<td>0.042</td>
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</table>

*Harlan Teklad, Madison, WI. Normal-Ca diet (NCD; TD no. 98005). This formula is a modification of TD no. 86464. †Harlan Teklad, Low-calcium diet (LCD; TD no. 98006). This formula is a modification of TD no. 98005 to reduce calcium to 0.1%. ‡Vitamin mixture from Harlan Teklad (no. 40060) provided (mg/kg diet): p-aminobenzoic acid, 110.1; ascorbic acid, coated, 1,016.6; biotin, 0.44; vitamin B12, 29.7; calcium pantothenate, 66.1; choline chloride, 3,496.9; folic acid, 1.98; inositol, 110.1; menadione, 49.5; niacin, 99.1; pyridoxine-HCl, 22.0; riboflavin, 22.0; thiamin-HCl, 22.0; vitamin A retinyl palmitate (50,000 U/g), 39.65; dry cholecalciferol (500,000 U/g), 4.4; dry vitamin E dl-α-tocopherol acetate (500 U/g), 242.3; cornstarch (diluent), 4,666.9. §Mineral mixture from Harlan Teklad provided (mg/kg diet): potassium phosphate, monobasic, 24.6; calcium carbonate, 14.74 for NCD and 2.25 for LCD; potassium chloride, 5.6; sodium bicarbonate, 4.62; magnesium oxide, 3.83; sodium chloride, 3.7; sodium selenite, 0.5; ferric citrate, 0.21; manganese carbonate, 0.123; zinc carbonate, 0.056; chromium potassium sulfate (12H2O), 0.0193; cupric carbonate, 0.011; and potassium iodate, 0.0004.

Real-time quantitative PCR. Total RNA was isolated from the rat duodenal mucosa and kidney using Trizol reagent (Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. Reverse transcription of mRNA was carried out using 3 µg of total RNA in a 20-µl reaction volume containing 0.5 µg of oligo(dT)-15 primer (Promega, Madison, WI), 200 U SuperScript II RNase H-Reverse Transcriptase (Life Technologies), 0.5 mM dNTP, and 10 mM dithiothreitol in a reaction buffer of 50 mM Tris-HCl (pH = 8.3), 75 mM KCl, and 3 mM MgCl2. Total RNA was initially denatured at 70°C for 10 min and immediately chilled on ice. First-strand cDNA was synthesized after 50 min at 42°C, followed by 70°C for 15 min to inactivate the RT. Two units of RNase H (Life Technologies) were then added, followed by incubation at 37°C for another 20 min. PCR amplification and analysis for mRNA expression of duodenal CaT1, CaBP-9k, renal 1-OHase, and renal VDR were achieved using a LightCycler instrument and software version 3.5, respectively (Roche Applied Science). The sequences of the primers used in the reactions are shown in Table 2. Thermocyclings were performed in a final volume of 25 µl containing 1 µl of cDNA, 300 nM each primer, and 12.5 µl of SYBR Green Supermix (Bio-Rad Laboratory). After denaturation of cDNA at 95°C for 3 min, the cycling conditions were as follows: 45 cycles consisting of denaturation at 95°C for 15 s, annealing at 56°C for 20 s, and extension at 72°C for 20 s. Following the completion of the PCR amplification reaction, a melting curve analysis was performed by heating the sample at 0.1°C/s from 50 to 95°C while the fluorescence was measured continuously. GAPDH was used as a reference gene for internal control. The fluorescence signal was plotted in real time against the temperature to produce melting curves of each sample. Thus each specific PCR product generates a specific signal and therefore a product-specific melting peak. A relative standard curve (concentration vs. threshold cycle) of target and reference gene for quantification of PCR product was generated by dilution of cDNA from the calibrator. The untreated control sample in the SN group was used as a calibrator. The result was expressed as the concentration of the target/reference of each sample.

Western blot analysis. The proteins in the phenol phase from the initial homogenate were prepared according to the protein isolation protocol provided by the manufacturer. Protein concentrations were analyzed by the method of Bradford (Bio-Rad Laboratory). Equal amounts (30 µg) of proteins were separated by SDS-PAGE on 10% reducing gels and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, MA). Immunoblotting was performed after blocking nonspecific binding on the membrane with 5% powdered milk. The membrane was treated with polyclonal sheep anti-mouse 1-OHase antibody raised against an antigenic region of...
of the reported mouse amino acid sequence (peptide 266 to 289) at a 1:500 dilution in Tris-TBS (TTBS) containing 1% BSA as the primary antibody, followed by incubation with donkey anti-sheep antibody conjugated with horseradish peroxidase (1:20,000) in TTBS containing 1% BSA as the secondary antibody. Both antibodies were obtained from the Binding Site (Birmingham, UK). For VDR protein detection, the primary antibody was a rabbit polyclonal antibody used at a 1:1,000 dilution, and goat anti-rabbit IgG/horseradish peroxidase (1:20,000) in TTBS as the secondary antibody. Both antibodies were obtained from Santa Cruz Biotechnology. Signals of the protein bands were detected by the enhanced chemiluminescent assay (ECL, Pierce, IL) and visualized by the Lumi-Imager with the software Lumi Analyst software.

Peripheral quantitative computerized tomography analysis of rat tibia. Right tibias were thawed at room temperature before testing, and the bone mineral density (BMD) of intact tibias was measured by peripheral quantitative computerized tomography (pQCT) with an XCT2000 machine (Norland Stratec Medizintechnik, Birkenfeld, Germany) aided by specially designed software for small animals. Cone Phantom (Slice 1, 787 mm²; Slice 2, 0.539 1/cm; Slice 3, 0.333 1/cm; Slice 4, 0.436 1/cm) and Standard Phantom (0.436 1/cm) were located at 2.5 mm distal to the proximal end. Humidity was maintained at 50–60% during the measurements. Cancellous BMD (Cn-BMD), total BMD, cancellous bone mineral content (CnBMC), cancellous bone area (CnBAr), total bone area (total BAr), and strain stress index-polar (SSI-p) were recorded as given by the pQCT software.

Statistical analysis. The data from these experiments were reported as means ± SE for each group. Statistical differences between groups were evaluated by both one-way and two-way ANOVA analysis (Prism4 for Windows, GraphPad product). Analysis of the effects of operation and diet and interaction of both factors as grouping variables was performed, and the significance between individual groups was determined using the Bonferroni method. P values < 0.05 were considered statistically significant. Pearson correlation between serum 1,25(OH)₂D₃ and duodenal mRNA expression was performed, and the statistical significances of the relationship were assessed by Prism statistical analysis software.

RESULTS

Body weight, uterine weight, and serum and urine chemistries. As early as 2 wk after ovariectomy and sham operation, differences in weight gain between OVX and sham rats were observed (P < 0.05). Thus, at the baseline of the feeding study, OVX rats weighed much more than the sham-operated rats despite the fact that they were pair fed similar amounts of food as the sham-operated group (P < 0.0005, 5 wk after operation). At the end of the feeding study, two-way ANOVA analysis showed that neither diet nor operation affected the weight gain of rats during the 2 wk of treatment (Table 3).

Table 3. Weight gain in 2 wk of dietary treatment, uterine weight, and serum and urine chemistries in sham and OVX rats fed normal- or low-Ca diet for 2 wk

<table>
<thead>
<tr>
<th>Operation</th>
<th>Diet</th>
<th>Weight Gain, g</th>
<th>Uterine Weight, mg/g body wt</th>
<th>Serum Ca, mmol/l</th>
<th>Serum P, mmol/l</th>
<th>Urine Ca/ Cr, mg/mg</th>
<th>Urine P/Cr, mg/mg</th>
</tr>
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<tbody>
<tr>
<td>Sham</td>
<td>Normal</td>
<td>25.0±4.9</td>
<td>2.33±0.22*</td>
<td>2.18±0.07</td>
<td>1.57±0.11</td>
<td>0.33±0.04</td>
<td>18.7±1.5*</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>20.0±1.2</td>
<td>2.38±0.23*</td>
<td>2.04±0.08</td>
<td>1.65±0.11</td>
<td>0.27±0.04</td>
<td>43.7±4.3*</td>
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<tr>
<td>OVX</td>
<td>Normal</td>
<td>28.0±4.2</td>
<td>0.53±0.02*</td>
<td>2.13±0.04</td>
<td>1.75±0.07</td>
<td>0.37±0.06</td>
<td>15.6±1.8*</td>
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<tr>
<td></td>
<td>Low</td>
<td>24.5±5.0</td>
<td>0.50±0.02*</td>
<td>2.02±0.05</td>
<td>1.71±0.09</td>
<td>0.32±0.04</td>
<td>41.3±3.6*</td>
</tr>
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Two-way ANOVA P value

<table>
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<tr>
<th>Operation</th>
<th>Diet</th>
<th>P value</th>
<th>P value</th>
<th>P value</th>
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<td></td>
<td></td>
<td>0.4016</td>
<td>&lt;0.0001</td>
<td>0.5212</td>
</tr>
<tr>
<td>Diet</td>
<td></td>
<td>0.3428</td>
<td>0.9630</td>
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<tr>
<td>Interaction</td>
<td></td>
<td>0.8659</td>
<td>0.8165</td>
<td>0.7952</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 7–8 in each group. OVX, ovariectomized. Means in columns with unlike superscript letters are significantly different: *P < 0.001.

AJP-Endocrinol Metab • VOL 292 • MARCH 2007 • www.aipendo.org
Uterine weight of OVX rats was significantly lower than that of sham rats at 7 wk after operation ($P < 0.001$, Table 3), suggesting that the bilateral oophorectomy operations in these animals were successful. Because uterine weight is a sensitive indicator for in vivo estrogenic activities, the results indicated that OVX rats had minimal estrogen in the circulation.

Two-way ANOVA analysis indicated that 2 wk of LCD feeding reduced serum Ca levels in both sham and OVX rats ($P < 0.001$, Table 3). Neither serum P level nor urine Ca excretion was altered by either ovariectomy or LCD. In contrast, LCD, but not ovariectomy, significantly increased urinary P excretion in both OVX and sham rats ($P < 0.0001$, Table 3).

Serum PTH and 1,25(OH)$_2$D$_3$ levels. As shown in Fig. 1A, ovariectomy significantly increased serum bioactive PTH(1–84) levels in rats fed LCD by fourfold ($P < 0.01$ vs. SN group). Two-way ANOVA analysis indicated that ovariectomy, but not LCD, significantly altered serum PTH in these rats ($P < 0.02$). Serum 1,25(OH)$_2$D$_3$ levels increased in response to LCD in both sham and OVX rats ($P < 0.001$, Fig. 1B). Basal serum 1,25(OH)$_2$D$_3$ levels and responses to LCD were not altered by ovariectomy. These results indicate that the increases in serum PTH levels were not paralleled by similar changes in serum 1,25(OH)$_2$D$_3$ levels in response to ovariectomy or LCD.

Renal 1-OHase expression. The renal mitochondrial 1-OHase is an important enzyme that catalyzes the biosynthesis of 1,25(OH)$_2$D$_3$ and is upregulated in response to LCD in young rats. To determine whether ovariectomy alters the responses of renal 1-OHase to LCD feeding, the levels of its mRNA (Fig. 2A) and protein (Fig. 2B) expression in kidney were determined. As shown in Fig. 2A, renal 1-OHase mRNA was significantly increased by fourfold in OVX rats fed LCD ($P < 0.001$ vs. SN group). Both ovariectomy and LCD could significantly upregulate the 1-OHase mRNA level ($P < 0.0001$). LCD increased renal 1-OHase protein expression by twofold in both sham and OVX rats ($P < 0.01$ vs. NCD-fed rats, Fig. 2B). LCD, but not ovariectomy, significantly altered renal 1-OHase protein expression ($P < 0.001$). Ovariectomy altered the responses of renal 1-OHase mRNA but not protein expression to LCD in these rats. The changes in renal 1-OHase protein expression (Fig. 2B) paralleled the changes in serum 1,25(OH)$_2$D$_3$ levels (Fig. 1B) in response to LCD in both sham and OVX rats.

VDR expression. VDR is a nuclear transcription factor that mediates the biological activities of 1,25(OH)$_2$D$_3$ on Ca homeostasis and vitamin D metabolism. To determine whether ovariectomy alters the renal responses to LCD, the levels of renal VDR mRNA and protein expression in both sham and OVX rats were compared. Renal VDR mRNA levels were significantly increased by ovariectomy alone ($P < 0.05$ vs. SN group, Fig. 3A). The increase in VDR by LCD alone did not reach statistical significance. In contrast, renal VDR mRNA levels were significantly reduced in OVX rats fed LCD diet ($P < 0.05$ vs. SN group, Fig. 3A). The changes in renal VDR

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**Fig. 1.** Intact parathyroid hormone [PTH(1–84)] and active vitamin D [1,25(OH)$_2$D$_3$] in serum from 4-mo-old sham-operated and ovariectomized (OVX) rats fed either normal-calcium diet (NCD; 6 g Ca/kg diet) or low-calcium diet (LCD; 1 g Ca/kg diet) for 14 days were measured by ELISA kits. Values are means ± SE of individual measurements in each rat (n = 6–8 in each group). SN, sham rats fed NCD; SL, sham rats fed LCD; ON, OVX rats fed NCD; OL, OVX rats fed LCD. *$P < 0.05$, **$P < 0.01$ vs. SN group; ##$P < 0.01$ vs. ON group.

**Fig. 2.** Renal expression of 25-hydroxyvitamin D$_3$-1α-hydroxylase (1-OHase) mRNA and protein in sham-operated and OVX rats fed NCD (6 g Ca/kg diet) or LCD (1 g Ca/kg diet) for 14 days. Expression level was shown as ratio of target gene/GAPDH (n = 6 in each group). Values are means ± SE. **$P < 0.01$, ***$P < 0.001$ vs. SN group. ##$P < 0.01$ vs. ON group.
protein expression (Fig. 3B) in response to LCD and ovariectomy treatment were in parallel with the changes in renal VDR mRNA expression (Fig. 3A). Renal VDR protein levels were significantly induced in OVX rats fed NCD (\(P < 0.01\) ON vs. SN, Fig. 3B) and suppressed in OVX rats fed LCD (\(P < 0.05\) OL vs. SN, Fig. 3B). The interactions between LCD and ovariectomy were highly significant for regulation of both renal VDR mRNA (\(P < 0.0025\)) and protein (\(P < 0.001\)) expressions. These results suggest that ovariectomy indeed altered the responses of renal VDR protein and mRNA expression to LCD.

Intestinal CaT1 and CaBP-9k mRNA expression. CaT1 and CaBP-9k are 1,25(OH)\(_2\)D\(_3\)-dependent proteins involved in apical Ca transport and transcellular transport of Ca, respectively. To determine whether the intestinal adaptation to LCD is affected by ovariectomy, CaT1 and CaBP-9k mRNA expressions were determined (Fig. 4). Ovariectomy did not alter basal level of CaT1 (Fig. 4B) or CaBP-9k expression (Fig. 4C). LCD significantly increased intestinal CaBP-9k mRNA expression in sham rats (\(P < 0.05\) vs. SN group, Fig. 4C). As shown by the analysis of two-way ANOVA, LCD indeed increased intestinal CaT1 and CaBP-9k mRNA expression in both sham and OVX rats (\(P < 0.05\)), indicating that ovariectomy did not alter the intestinal responses of 1,25(OH)\(_2\)D\(_3\)-dependent mRNA expression during LCD.

Bone Ca content, BMD, and biomechanical strength of rat tibia. Either dietary Ca restriction or ovariectomy could independently reduce Ca content in rat tibia. Ca content of rat tibia was significantly decreased by LCD (\(P < 0.0015\)) and by ovariectomy (\(P < 0.0001\)). In addition, OVX rats fed LCD had the lowest tibial Ca content, suggesting that the negative effects of ovariectomy and dietary Ca restriction on bone Ca content were cumulative.

The effects of ovariectomy and dietary Ca restriction on BMD and bone mineral content (BMC) in the proximal metaphysis of rat tibia are summarized in Table 4. In general, the CnBMD, the CnBMC, and the total BMD of the tibial proximal metaphysis were lower in rats fed LCD than in those fed NCD, and these parameters were also lower in the OVX than in the sham-operated rats. Two-way ANOVA analysis showed that both diet (\(P = 0.0278\)) and operation (\(P < 0.0001\)) had significant effects on the CnBMD of tibial proximal metaphysis in mature rats (Table 4). Ovariectomy, but not LCD, had significant effects on total BMD (\(P < 0.0001\)) and BMC (\(P = 0.01\)) of tibial proximal metaphysis. The effects of ovariectomy and LCD on CnBMD, total BMD, and CnBMC of tibial proximal metaphysis were also cumulative, as these parameters in the OL group were significantly lower than those of the SN group (\(P < 0.001\)) and SL group (\(P < 0.05\)). Neither ovariectomy nor LCD altered Ca content of rat femur.
ectomy nor LCD had a significant effect on CnBAr and total BAr of the tibial proximal metaphysis. Significant decreases of CnBAr and total BAr were only observed in the OL group (P < 0.05 vs. SN group, Table 4).

SSI-p, an index that estimates torsion bone strength, was significantly reduced by ovariectomy (P = 0.0127) in the proximal end of rat tibia (Table 4). SSI-p was significantly reduced in the proximal end of rat tibia in the OL group (P < 0.01 vs. SN; P < 0.05 vs. SL, ON). The predicted bending bone strength (BSICSA), as estimated by the product of volumetric BMD and cross-sectional area, was significantly reduced by both ovariectomy (P < 0.005) and LCD (P < 0.02) (Table 4).

Correlation analysis of serum 1,25(OH)₂D₃ and intestinal vitamin D-dependent mRNA expression. As shown in Fig. 5A, duodenal CaT1 mRNA was highly correlated with serum 1,25(OH)₂D₃ in sham [duodenal CaT1 mRNA = 0.0058 × serum 1,25(OH)₂D₃ + 0.2951; P < 0.05, Pearson r = 0.9022] and OVX rats [duodenal CaT1 mRNA = 0.0034 × serum 1,25(OH)₂D₃ + 0.3927; P < 0.05, Pearson r = 0.8226]. Moreover, a strong positive relationship between serum 1,25(OH)₂D₃ and duodenal CaBP-9k mRNA was found in both the sham [duodenal CaBP-9k mRNA = 0.0120 × serum 1,25(OH)₂D₃ + 0.7604; P < 0.05, Pearson r = 0.8830] and OVX rats [duodenal CaBP-9k mRNA = 0.0109 × serum 1,25(OH)₂D₃ + 0.5984; P < 0.05, Pearson r = 0.9027] as shown in Fig. 5B. The results suggest that the intestinal Ca transport responses to increased serum 1,25(OH)₂D₃ levels were not altered by ovariectomy.

DISCUSSION

Estrogen deficiency causes postmenopausal osteoporosis through an increase in bone turnover that results in bone loss (20, 37). In addition, recent reports that estrogen plays an important role in regulating intestinal Ca absorption suggest that estrogen deficiency also contributes to the pathogenesis of age-related osteoporosis (38, 39). The present study reveals that ovariectomy further increases circulating PTH levels in mature rats during dietary Ca restriction. The increased PTH levels do not result in a proportional increase in serum 1,25(OH)₂D₃ or in the expression of 1,25(OH)₂D₃-dependent Ca transport proteins. However, renal VDR expression is reduced, and CnBMD and calculated bending strength of rat tibia are dramatically reduced.

The present study demonstrated that circulating PTH levels increase during LCD only in OVX rats. In contrast to the normal induction of PTH secretion by LCD in male rats, circulating PTH levels did not increase in response to LCD in sham-operated female rats. These data suggest that estrogen plays a role in preventing the upregulation of PTH secretion in response to LCD. The observed effect of estrogen on PTH

Table 4. Bone parameters in the proximal metaphysis of tibia of sham and OVX rats fed normal- or low-Ca diet for 2 wk

<table>
<thead>
<tr>
<th>Operation</th>
<th>Diet</th>
<th>CnBMD, mg/ccm</th>
<th>Total BMD, mg/ccm</th>
<th>CnBAr, mg/mm</th>
<th>Total BAr, mm²</th>
<th>SSI-p</th>
<th>BSICSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>Normal</td>
<td>583.9±46.4</td>
<td>645.2±23.2</td>
<td>15.2±1.5</td>
<td>11.1±0.9</td>
<td>24.7±1.9</td>
<td>15.2±2.3</td>
</tr>
<tr>
<td>OVX</td>
<td>Normal</td>
<td>512.9±52.1</td>
<td>592.3±45.2</td>
<td>13.7±1.7</td>
<td>10.4±1.1</td>
<td>23.3±2.4</td>
<td>12.2±2.2</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>398.2±53.4</td>
<td>513.1±27.0</td>
<td>12.3±1.7</td>
<td>10.5±0.9</td>
<td>23.5±2.0</td>
<td>10.9±1.7</td>
</tr>
</tbody>
</table>

Two-way ANOVA P value

- Operation: <0.0001
- Diet: 0.0278
- Interaction: 0.4668

Values are means ± SE; n = 6–8 in each group. Means in columns with unlike superscript letters are significantly different: *P < 0.05; **P < 0.01; ***P < 0.001. Cn, cancellous; BMD, bone mineral density; BMC, bone mineral content; BAr, bone area; SSI-p, stress strain index-polar; BSICSA, bone strength index-cylindrical section area.

Fig. 5. Correlation between serum 1,25(OH)₂D₃ content and duodenal calcium transport gene expression. Duodenal CaT1 (A) or CaBP-9k (B) mRNA expression was plotted as a function of serum 1,25(OH)₂D₃. Solid and dashed lines represent the OVX and sham groups, respectively. A: regression line in OVX group is duodenal CaT1 mRNA = 0.0034 × serum 1,25(OH)₂D₃ + 0.3927 (P < 0.05, Pearson r = 0.8226) and in sham group is duodenal CaT1 mRNA = 0.0008 × serum 1,25(OH)₂D₃ + 0.2951 (P < 0.05, Pearson r = 0.9022). B: regression line in OVX group is duodenal CaBP-9k mRNA = 0.0109 × serum 1,25(OH)₂D₃ + 0.5984 (P < 0.05, Pearson r = 0.9027) and in sham group is duodenal CaBP-9k mRNA = 0.0120 × serum 1,25(OH)₂D₃ + 0.7604 (P < 0.05, Pearson r = 0.8830).
secretion appears to be independent of serum Ca and serum 1,25(OH)2D3, as both sham and OVX rats fed LCD have similar levels of serum Ca and 1,25(OH)2D3. These results are in agreement with clinical studies reported by Riggs et al. (27, 31, 32), in which estrogen deficiency was identified as the principal cause of secondary hyperparathyroidism in postmenopausal women. Furthermore, the increased PTH levels could be reversed by sufficient Ca intake.

The results in the present study were not in total agreement with those reported in an earlier study by Dick et al. (17). In their study, the levels of serum PTH in both sham and OVX rats in response to LCD feeding were similar. In other words, the highly induced PTH levels of OVX rats in response to LCD as observed in our study were not detected in their study. Several differences in study design may reconcile the disparate observations. The age of the rat model used by Dick et al. (6 mo old) vs. ours (3 mo old) was different, and these two models indeed represented very different skeletal structures. While the rat skeleton is continually growing, the longitudinal growth rate slows dramatically by 6 mo of age. Thus the older rat model represented a primarily remodeling skeleton, while the younger model was a modeling/remodeling skeleton instead. This difference in age in the model might explain, at least partially, the differences in the response of PTH to LCD in OVX rats. Moreover, despite a similar level of dietary Ca (0.1%) used, the level of dietary P was lower in their study (0.3 vs. 0.65%). In addition, Dick et al. studied the responses of OVX and sham-operated animals to LCD at baseline and 1, 3, and 6 wk after operation, whereas OVX and sham rats in our study were subjected to 2 wk of LCD or NCD at 5 wk after operation. Thus, in our study, the animals were allowed to recover from the operation with sufficient Ca intake before being subjected to the challenges of dietary Ca restriction, whereas the animals in the study of Dick et al. were facing challenges of rapid bone Ca loss as a result of ovariectomy as well as insufficient dietary Ca intake simultaneously.

Serum 1,25(OH)2D3 levels were increased in response to LCD in both sham and OVX rats but not by ovariectomy alone. Thus it appears that the regulation of circulating levels of 1,25(OH)2D3 by dietary Ca restriction is not affected by ovariectomy. However, serum 1,25(OH)2D3 increased during LCD in sham rats in the absence of an increase in serum PTH levels, and further increases in PTH levels in OVX rats fed LCD did not result in higher serum 1,25(OH)2D3 levels. Thus the results indicate that the regulation of serum 1,25(OH)2D3 is complex and suggest that factors in addition to serum PTH are involved in regulating the circulating levels of 1,25(OH)2D3. It should also be noted that the changes in serum 1,25(OH)2D3 levels corresponded to changes in renal 1-OHase protein expression, which suggests that the increase in circulating levels of 1,25(OH)2D3 is at least in part due to increased biosynthesis by the renal 1-OHase enzyme.

The responses of renal 1-OHase mRNA to LCD and/or ovariectomy appear to be different from those of serum 1,25(OH)2D3 or renal 1-OHase protein expression. Renal 1-OHase mRNA increased in response to LCD and ovariectomy, and its expression level in OVX rats was proportional to the circulating PTH level. These results are in agreement with the well-known actions of PTH to directly induce renal 1-OHase mRNA expression (11, 33). However, the magnitude of induction of renal 1-OHase mRNA (4-fold increase) did not result in a proportional increase in renal 1-OHase protein (2-fold increase) in OVX rats fed LCD compared with sham rats fed NCD. These results suggest that the regulation of renal 1-OHase activity may involve both transcriptional and translational control of the renal 1-OHase enzyme. In a previous study (28), we demonstrated increased protein stability of renal 1-OHase as one mechanism by which mature rats increase serum 1,25(OH)2D3 levels during low-P diet. Thus, in OVX rats, the lack of a proportional increase in renal 1-OHase protein during high renal 1-OHase mRNA expression could also be due to an alteration of the protein stability of renal 1-OHase or a change in rate of protein translation by ovariectomy. Future studies will be required to delineate the mechanisms involved in regulation of renal 1-OHase during ovariectomy.

The present study clearly demonstrates that the regulation of renal VDR expression during dietary Ca restriction is altered by ovariectomy, whereas LCD alone did not alter renal VDR expression in sham rats. Moreover, it appears that the regulation of renal VDR expression by LCD in female rats is different from that in male counterparts, as renal VDR expression did not decrease during LCD in female rats (22). Both observations might be explained by the responses of circulating PTH to LCD in female rats. The reduction of renal VDR expression is associated with a significant induction of circulating PTH levels in OVX rats during LCD. In contrast, circulating PTH levels in sham female rats were not upregulated in response to LCD as usually observed in male animals. Our interpretation is consistent with the recent report by Healy et al. (21) in which PTH downregulates renal VDR expression in mice. It remains unclear how ovariectomy alone may induce renal VDR expression, as it cannot be accounted for by the level of circulating PTH or 1,25(OH)2D3 in OVX rats. Future study will be required to determine whether estrogen is involved in downregulation of renal VDR expression in female rats.

The results of the present study also clearly demonstrate that ovariectomy does not alter intestinal responses of 1,25(OH)2D3-dependent transcripts of Ca transport proteins during LCD. The basal and stimulated levels of CaT1 and CaBP-9k mRNA expression were not affected by ovariectomy. The correlation analysis (Fig. 5) provides additional evidence of the relationships between serum 1,25(OH)2D3 and intestinal vitamin D-dependent gene expressions and the lack of influence by ovariectomy. However, because we only measured mRNA expressions, it remains possible that intestinal vitamin D-dependent Ca transport might be affected by ovariectomy, as reported previously (9, 29), via the alteration of translations or the activities of these proteins directly.

The most obvious consequences of secondary hyperparathyroidism induced by LCD feeding in OVX rats are the dramatic reductions of total BMD and CnBMD, bone Ca content, SSI-p, and bone strength index (BSI) as mathematically derived from pQCT data (as shown in Table 4). In particular, the loss of cancellous BMD and BSI was increased dramatically in OVX rats by 34 and 49%, respectively, when fed with LCD. These results highlight the importance of sufficient Ca intake during menopause to minimize circulating PTH levels, as secondary hyperparathyroidism during LCD greatly increases the risk of fracture through loss of bone mass and reduced bone strength.

In summary, ovariectomy altered the responses of serum PTH, renal VDR expression, and bone properties to LCD.
These results provide insight into the pathological effects of estrogen deficiency as the principal cause of secondary hyperparathyroidism, as observed in clinical studies in postmenopausal women (27, 31, 32). The potential therapeutic role of high-Ca intake is also supported.

ACKNOWLEDGMENTS

We thank the State Key Laboratory of Chinese Medicine and Molecular Pharmacology for providing support in carrying out this study.

GRANTS

This work was supported by the Areas of Excellence Scheme Established under the University Grants Committee of the Hong Kong Special Administrative Region (HKSSR), China (AOEP-10/01), and the Central Allocation Grant from the Research Committee of the Hong Kong Polytechnic University (G-YD53, G-YD95), HKSSR.

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