Effects of age and estrogen on renal vitamin D metabolism in the female rat

Sarah L. Ash, PhD, and Barry R. Goldin, PhD

ABSTRACT

Young and adult, intact and ovariectomized female rats were used to study the effects of increasing age and estrogen loss on the conversion of 25-hydroxycholecalciferol [25(OH)D] to 1,25-dihydroxycholecalciferol [1,25(OH)2D] and 24,25-dihydroxycholecalciferol [24,25(OH)2D]. The recovery level of radioactivity in plasma as 1,25(OH)2D after [3H]25(OH)D administration was lower in older animals; ovariectomy reduced [3H]1,25(OH)2D recovery in both young and adult animals. 1,25(OH)2D synthesis by kidney slices was reduced in all adult animals and in ovariectomized young animals. Calcium absorption was also lower in adult animals and in young ovariectomized animals than in controls. β-estradiol administration increased recovery of [3H]1,25(OH)2D but not [3H]24,25(OH)2D, which is parallel to the general lack of effect of ovariectomy on 24,25(OH)2D production. Parathyroidectomy eliminated the estrogen effect on 1,25(OH)2D production. Thus, estrogen loss and advancing age reduced 1,25(OH)2D production and Ca absorption independent of the estrogen effect being mediated via the parathyroid gland.

KEY WORDS

Rats, age, ovariectomized, estrogen, calcium absorption, 1,25-dihydroxycholecalciferol synthesis, estradiol, parathyroid

Introduction

The acceleration of skeletal demineralization known to occur at around age 50 in women, which can lead to the debilitating bone disease osteoporosis, was shown to be more closely associated with the onset of menopause than with the chronological age at which bone loss begins (1–3). Women who have undergone ovariectomy in young adulthood experience degrees of bone loss similar to that of postmenopausal women (4). These observations indicate that estrogen is important in maintaining calcium homeostasis. There are several ways in which the hormone could exert its effect.

For example, it has been suggested that the presence of estrogen protects against the bone resorbing properties of parathyroid hormone (PTH), a theory confirmed by some (5) and disputed by others (6). In addition, estrogen appears to improve renal Ca conservation as evidenced by Ca balance studies in postmenopausal women (7). Finally, there may also be an effect of estrogen on intestinal Ca absorption as it has been shown in both experimental animals (8, 9) and humans (10, 11) that the hormone when exogenously administered increases serum levels of 1,25-dihydroxycholecalciferol [1,25(OH)2D], the active form of vitamin D that is responsible for mediating the active transport of Ca across the intestinal brush border. Russell et al (12) recently reported that the administration of β-estradiol to ovariectomized rats increased Ca absorption to twice the rate observed for control animals. Ca absorption in the untreated surgically altered animals, however, remained unchanged. Russell et al also found that fasting and nonfasting plasma Ca levels were significantly lower in ovariectomized rats than in control rats and could be raised slightly above control values with the administration of β-estradiol.

It is possible then that an estrogen deficiency, such as that after menopause, could lower serum 1,25(OH)2D levels and decrease Ca absorption. A number of studies found that Ca absorption is substantially impaired in postmenopausal osteoporotic subjects (13–15) although there is conflicting evidence about whether serum 1,25(OH)2D levels are lower in women with the disorder than in age-matched control subjects (16–18). Only one animal study looked at the effects of estrogen loss on 1,25(OH)2D production (19). In that experiment there was a twofold but not statistically significant difference.

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in the recovery of radioactivity in plasma as 1,25(OH)2D after intraperitoneal [3H]25-hydroxycholecalciferol [25(OH)D] administration between a small number of intact and ovariectomized rats.

In this study sexually mature young and adult female rats, both intact and ovariectomized, are used to study the relative effects of increasing age and estrogen deficiency on renal 1,25(OH)2D and 24,25-dihydroxycholecalciferol [24,25(OH)2D] production and intestinal Ca absorption. The role of the parathyroid gland in mediating an estrogen effect on vitamin D metabolism is also assessed.

Methods

Animals and diet

Female Holtzman rats (Charles River Breeding Laboratories, Wilmington, MA) aged 6–7 wk (young, sexually mature) and 9–10 mo (adult) ovariectomized, parathyroidectomized, and intact were used. Mean animal weights for young intact, young ovariectomized, adult intact, and adult ovariectomized were 242 ± 7, 278 ± 26, 356 ± 10, and 380 ± 9 g, respectively. Ovariectomy was confirmed with vaginal smears using Wright’s stain. Irregular appearance of cells and lack of regular cycling indicated loss of ovarian function. Animals were housed in groups of 3 or 4 in lucite cages with wood-shaving bedding changed twice weekly in a temperature-controlled room with a 12-h light-dark cycle. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Tufts University.

All animals were fed ad libidem a semipurified diet containing the following per 100 g: 40 g corn starch, 20 g casein, 20 g sucrose, 10 g corn oil, 5 g Rogers-Harper Mineral Mix (20), 3.5 g nonnutritive fiber, 1 g vitamin mix (AIN760A) (21), 0.3 g DL-methionine, and 0.2 g choline chloride, for at least 3 wk prior to experimentation. The diet provided adequate amounts of Ca and phosphorus (0.59 and 0.39 g/100 g diet respectively) and cholecalciferol (1000 IU/kg [65 nmol]) diet. All diet ingredients were purchased from Teklad Test Diets, Madison, WI.

Experiments were carried out within 7 wk after the arrival of each group of animals.

In vivo measurement of 25(OH)D metabolism

Radiolabeled 25(OH)D (25(OH)[26,27-methyl3H]D3; and [1,25(OH)2D]1,25(OH)2[26,27-methyl3H]D) were purchased from Amersham Corp, Arlington Hts, IL. Unlabeled 25(OH)D, 1,25(OH)2D, and 24,25(OH)2D were kindly provided by Dr Milan Uskokovic, Hoffman-LaRoche Inc, Nutley, NJ, and Dr Michael Holick, USDA Human Nutrition Research Center, at Tufts University, Boston, MA.

Animals were given an intraperitoneal injection of 2 µCi (or 7.4 × 1010 Bq) [3H]25(OH)D (20 Ci/mmole or 7.4 × 1011 Bq) and sacrificed 24 h later via heart puncture performed under ether anesthesia. Time course experiments indicated that recovery of [3H]25(OH)D was maximal at this time. The purity of [3H]25(OH)D was measured by high-performance liquid chromatography (HPLC) and found to be > 99%.

Two milliliters of plasma were extracted in 1 mL portions with 3.75 mL methanol:chloroform (2:1) followed by 2 mL chloroform to produce a phase separation. The organic phase was removed and dried under nitrogen. The extract was then prepared for HPLC using a modified procedure of Adams et al (22). The extract was redissolved in 0.3 mL ethanol and applied to a Sep-Pak silica cartridge (Waters Associates, Milford, MA), presaturated with 5 mL of starting solvent. The cartridge was then eluted with 10 mL portions of 25, 60, and 100% ethylacetate in hexanes. The mean percentage ± SEM of recovery of radioactivity for the extraction procedure for 25(OH)D and 1,25(OH)2D was determined to be 90.6 ± 77.4 ± 0.7, respectively.

The fractions were pooled, dried down under N and redissolved in 0.25 mL of HPLC running solvent. Vitamin D metabolites were separated with HPLC on a Microsorb Short One silica column (5 μm, 100 × 4.6 mm (Rainin Instrument Co, Woburn, MA) with a running solvent of hexanes:isopropanol (93:7) and a flow rate of 1.5 mL/min. One-minute fractions were collected in Betalfluor liquid scintillation cocktail (National Diagnostics, Somerville, NJ) and counted in a Beckman LS-313P liquid scintillation counter (Beckman Instruments, Inc, Fullerton, CA). Results were expressed as the amount of radioactivity recovered in the 1,25(OH)2D or 24,25(OH)2D zones (as determined by the elution of concurrently run unlabeled standards) as a percentage of the total amount of radioactivity eluted from the column. Column elution continued until no further radioactivity could be detected.

In those experiments measuring the effects of estrogen administration on in vivo 25(OH)D metabolism, 0.015 mg of β-estradiol (Sigma Chemical Co, St Louis, MO) in 0.1 mL corn oil was injected subcutaneously for 4 d before [3H]25(OH)D dosing.

In vitro measurement of 25(OH)D metabolism

1-Hydroxylase activity was measured in isolated renal slices by a modification of the method of Armbruch et al (23). Starring animals were sacrificed by decapitation, and kidneys were placed on ice in NaCl (0.09 g/L). After removal of the renal capsule, cortical slices were prepared with a Stadie-Riggs microtome. Kidney slices weighing a total of 150–250 mg were placed in one-dram (3.7 mL) glass vials with 1 mL of Ringerbicarbonate buffer, pH 7.4, containing 1 mg glucose, 1 mg 25(OH)D and 0.5 μCi (or 1.85 × 109 Bq) [3H]25(OH)D. Average thickness of slices was 0.5 mm and surface area ranged from 1.5 ± 0.2 cm2 for young animals to 1.9 ± 0.5 cm2 for adult animals. Vials were filled with oxygen, stoppered, and incubated in a shaking water bath for 1 h at 37 °C.

After incubation, kidney slices were homogenized in the incubation medium and the homogenate was extracted with 3 mL methanol:chloroform (1:1) and 1 mL chloroform. The organic layer was removed, dried under N2 and redissolved in 0.2 mL ethylacetate. This fraction was prepared for thin-layer chromatography, by applying it to a Sep-Pak silica cartridge presaturated with 5 mL ethylacetate, and eluted with 3 mL ethylacetate. The eluate was dried under N2 and redissolved in 0.06 mL acetone.

Vitamin D metabolites were separated by applying the eluate to thin-layer chromatography plates (Silica Gel 60, E Merck, Darmstadt, Germany) and developed for 1 h in benzene:ethylacetate (1:1). Zones corresponding to 25(OH)D, 1,25(OH)2D, and 24,25(OH)2D, as determined by concurrently run unlabeled standards visualized with an ultraviolet light source, were scraped from the plates into 2 mL methanol: chloroform (1:1), mixed in a vortex mixer, and centrifuged. The supernatant was dried in scintillation bottles and counted in Hydrofluor liquid scintillation cocktail (National Diagnostics, Somerville, NJ). The kidney-slice assay was done in duplicate for each animal and the average of the two results was...
expressed as picograms (femts) of 1,25(OH)₂D or femtomoles of 24,25(OH)₂D produced per milligram tissue per hour.

_in vitro determination of intestinal Ca absorption_

⁴⁴Ca was purchased from Amersham Corp, Arlington Hts, IL. Intestinal Ca absorption was determined by the method of Martin and DeLuca (24). Results are expressed as the ratio of inside (serosal) to outside (mucosal) ⁴⁴Ca counts, or S:M ratio.

_Estrone and estradiol determinations_

The measurements of plasma estrone and estradiol levels were kindly performed by Dr Christopher Longcope at the University of Massachusetts Medical Center, Worcester, MA using the methods of Longcope et al (25). The coefficient of variation, calculated from differences between duplicate analyses of control samples, was 6% for both estrone and estradiol. The coefficient of variation between assays was 12% for estrone and 11% for estradiol.

Statistical analysis

Statistical comparisons of the multiple groups were done using Duncan’s multiple range test (26). Student’s t test was used for comparisons of two groups.

Results

The percent recovery of radioactivity as 1,25(OH)₂D and 24,25(OH)₂D 24-h after an intraperitoneal dose of [³H]25(OH)D was measured in the plasma. The results, as presented in Table 1, were expressed on a per kilogram body weight basis for two reasons. First, body weight was found to be proportional to kidney size (r = 0.776, p < 0.001). In addition, the increase in body weight known to occur after ovariectomy, as it did in these animals, was shown to be the result of an increase in the size of all body components, not just fat as previously believed (27).

After adjustments for body weight were made, the level of radioactivity in the 1,25(OH)₂D fraction was significantly lower in the intact adult rats than in the young intact animals (p < 0.01). There was no difference between young and adult ovariectomized animals. Removal of the ovaries did result in a significant reduction in [³H]1,25(OH)₂D recovery in both young and adult animals (p < 0.01 and p < 0.05, respectively).

The recovery of tritium in fractions corresponding to 24,25(OH)₂D after adjusting for body weight, as indicated in Table 1, was not affected by ovariectomy in young animals or by age in intact animals. However, ovariectomy of adult rats did significantly reduce recovery of radioactivity as 24,25(OH)₂D as compared with young ovariectomized or adult intact animals (p < 0.05).

To determine if the decrease in recovery of radioactivity in the 1,25(OH)₂D fraction found to occur with increasing age and estrogen loss was due to decreased activity of renal 1-hydroxylase, the enzyme was measured directly in an in vitro experiment with isolated renal slices.

The renal conversion of [³H]25(OH)D to [³H]1, 25(OH)₂D, and 24,25(OH)₂D was measured in young and adult, intact and ovariectomized animals. Results are presented in Table 2. There was a significant decline in 1,25(OH)₂D production after estrogen loss in young (p < 0.01) but not adult animals (p < 0.10). Increasing age was associated with a significant decrease in the conversion of 25(OH)D to 1,25(OH)₂D in both intact (p < 0.01) and ovariectomized animals (p < 0.01).

The in vitro conversion of 25(OH)D to 24,25(OH)₂D appeared to decrease with age in both intact (p < 0.01) and ovariectomized animals (p < 0.01). Estrogen loss did not significantly affect 24-hydroxylase activity.

The functional significance of these findings was measured by the in vitro determination of ⁴⁴Ca absorption using everted intestinal sacs. As shown in Table 3, there

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Percentage of radioactivity recovered in plasma as vitamin D metabolites 24 h after [³H]25(OH)D injection*</th>
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<tbody>
<tr>
<td></td>
<td>1,25(OH)₂D/</td>
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<tr>
<td></td>
<td>kg body wt</td>
</tr>
<tr>
<td>Young intact (n = 9)</td>
<td>2.10 ± 0.26*</td>
</tr>
<tr>
<td>Young ovex (n = 7)</td>
<td>1.01 ± 0.08bc</td>
</tr>
<tr>
<td>Adult intact (n = 11)</td>
<td>1.31 ± 0.10b</td>
</tr>
<tr>
<td>Adult ovex (n = 8)</td>
<td>0.91 ± 0.10bc</td>
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</tbody>
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* Values with different superscript notation within a column are significantly different from each other (p < 0.05). Values are mean ± SEM. Ovex = ovariectomized.

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<th>TABLE 3</th>
<th>⁴⁴Ca absorption in everted intestinal sacs—ratio of serosal to mucosal radioactivity*</th>
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<tbody>
<tr>
<td></td>
<td>Serosal/mucosal</td>
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<tr>
<td>Young intact (n = 9)</td>
<td>1.516 ± 0.143*</td>
</tr>
<tr>
<td>Young ovex (n = 11)</td>
<td>1.196 ± 0.163b</td>
</tr>
<tr>
<td>Adult intact (n = 6)</td>
<td>0.889 ± 0.036c</td>
</tr>
<tr>
<td>Adult ovex (n = 7)</td>
<td>0.862 ± 0.014c</td>
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was a marked decline in Ca absorption in ovariectomized young animals as compared with intact controls ($p < 0.05$). Increasing age also was associated with decreased Ca absorption in both intact ($p < 0.01$) and ovariectomized animals ($p < 0.05$).

Results of the serum estrogen determinations indicated that there was a significant positive correlation between estrone levels and in vitro 1,25(OH)$_2$D production in all groups of animals as shown in Figure 1 ($r = 0.500$, $p < 0.01$). The correlation between estradiol and 1-hydroxylase activity was not as strong ($r = 0.332$, $p < 0.1$). There was only one significant difference in mean estrogen values for the four groups: a decrease in estradiol after ovariectomy in young animals ($p < 0.05$).

To determine if the decrease in recovery of radiolabeled as 1,25(OH)$_2$D observed in the in vivo experiment in ovariectomized animals could be reversed with estrogen treatment, a physiological dose of \( \beta \)-estradiol was injected subcutaneously into one of two groups of ovariectomized young animals for 4 d before intraperitoneal \([\text{3H}]\)25(OH)D administration.

As indicated in Table 4, percent recovery of tritium as 1,25(OH)$_2$D increased significantly after treatment with estrogen ($p < 0.005$). The percent recovery of radioactivity in the 24,25(OH)$_2$D fraction, however, remained unchanged, which is in agreement with previous results indicating no significant effect of estrogen on 25(OH)D-24-hydroxylase (24-hydroxylase) activity.

\( \beta \)-estradiol was also administered to intact adult animals to ascertain whether the decrease in enzyme activity found to occur with increasing age was refractory to stimulation by estrogen. Again, as shown in Table 4, there was a significant increase in recovery of \([\text{3H}]\)25(OH)$_2$D ($p < 0.005$), and no effect on \([\text{3H}]\)24,25(OH)$_2$D recovery. No corrections for body weight were made in these two experiments because the animals being compared were of similar size.

The final question asked was whether the apparent increase in 1,25(OH)$_2$D production after estrogen treatment represented direct stimulation of the 1-hydroxylase enzyme in the kidney or a PTH-mediated response because that hormone is considered to be a major regulator of 1,25(OH)$_2$D production. Parathyroidectomized adult animals were injected subcutaneously with \( \beta \)-estradiol for 4 d followed by intraperitoneal injection of \([\text{3H}]\)25(OH)D. The percent recovery of radioactivity as 1,25(OH)$_2$D was not significantly different between the \( \beta \)-estradiol–treated group (0.366 ± 0.024, $n = 7$) and control animals (0.426 ± 0.07, $n = 4$).

### Discussion

Results from both the in vitro and in vivo experiments indicate that production of the active form of vitamin D, 1,25(OH)$_2$D, decreases as a result of estrogen loss as well as with advancing age. Given that these findings were produced in vitamin D- and Ca-replete animals, they are not confounded by the question of differing responses to a nutrient deficiency among the treatment groups.

The loss of estrogen as a result of ovariectomy in young animals administered an intraperitoneal dose of \([\text{3H}]\)25(OH)$_2$D resulted in a greater than twofold decrease in the recovery of radioactivity as 1,25(OH)$_2$D than in age-matched controls after correcting for body weight. In the adult animals, the effect of an estrogen deficiency on \([\text{3H}]\)1,25(OH)$_2$D recovery, though still significant, was not as striking. It is possible that some of the adult intact animals were approaching natural menopause and therefore also had reduced circulating estrogen levels. The mean value for plasma estradiol in the adult intact rats was in fact 25% lower than in their younger counterparts; however, the difference was not significant because of the high degree of variability of the estradiol assay.

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**Table 4**

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* Values are percentage of radioactivity recovered in the plasma 24 h after intraperitoneal injection of \([\text{3H}]\)25(OH)D. Values are mean ± SEM. Ovex = ovariectomized.
† \( \beta \)-Estradiol is significantly higher than control at $p < 0.005$. 

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**FIG 1.** The relationship between in vitro renal 1,25(OH)$_2$D production and plasma estrone concentration. The production of 1,25(OH)$_2$D was significantly correlated with plasma estrone concentration at $p < 0.01$. 

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Because the in vivo technique cannot distinguish between decreased enzymatic production and increased catabolism of 25(OH)D, the effect of estrogen loss on renal 1-hydroxylase activity was assessed directly in an in vitro experiment. Conversion of tritiated 25(OH)D to 1,25(OH)2D in isolated kidney slices was significantly reduced as a result of ovariectomy in young rats, and there was a positive correlation between plasma estrogen levels and 1-hydroxylase activity for all animals. These results confirm the in vivo finding and indicate that the action of estrogen is on renal 1,25(OH)2D production rather than breakdown.

Results of the experiment using parathyroidectomized animals suggest that this estrogen effect requires the presence of PTH, because the removal of the gland prevented completely the previously demonstrated increase in vivo 1,25(OH)2D production after β-estradiol administration. Therefore estrogen’s action is indirect, presumably triggering increased 1,25(OH)2D production via increases in PTH. This conclusion regarding the indirect effect of estrogens is supported by Henry (28), who found no increase in 1,25(OH)2D production after the addition of estradiol to chick kidney cell cultures.

It appears that the mechanism whereby estrogen protects the skeleton from demineralization involves more than changes in Ca metabolism at the bone and kidney as had been suggested (5, 7). Estrogen loss, by decreasing 1,25(OH)2D production, also contributes to diminished bone density because of decreased intestinal absorption of Ca. As shown here estrogen deficiency did reduce the uptake of Ca by isolated intestinal sacs in young animals, thus supporting the functional significance of the finding of decreased 1,25(OH)2D production following ovariectomy.

Advancing age in intact rats also was associated with reduced in vitro 1,25(OH)2D production and in vivo recovery of [3H]1,25(OH)2D after adjustments for body weight, confirming earlier findings (23). The age effect in vitro was independent of estrogen, as evidenced by the significant decrease in adult ovariectomized rats as compared with young animals. However, a similar difference was not observed in vivo. In fact, in general, age had a greater effect on in vitro 1,25(OH)2D production than did ovariectomy, whereas in vivo loss of estrogen produced a more significant decline in [3H]1,25(OH)2D recovery than did advancing age. This discrepancy is perhaps not surprising given the apparent indirect effect of estrogen vitamin D metabolism. The isolation of the kidney using an in vitro technique could dampen any estrogen-mediated effect. In addition the in vivo finding of a more marked decrease in [3H]1,25(OH)2D recovery with estrogen loss than with increasing age is in agreement with findings in humans, indicating that estrogen deficiency is the more important contributor to bone loss (1-4).

The ability of exogenously administered β-estradiol to increase the level of recovery of [3H]1,25(OH)2D from the whole adult animal suggests that despite an apparent age-related decline in 1-hydroxylase activity, the enzyme does not become refractory to stimulation. Certainly, estrogen therapy was shown to be an effective treatment for bone loss in postmenopausal women (29, 30), and its success has been attributed to an increase in 1,25(OH)2D production (10, 11). In addition, it was shown that β-estradiol administration increases the transport of Ca across the intestinal brush border in the female rat (12).

It has been suggested that the age-related decrease in 1-hydroxylase activity is not due to protein breakdown but rather represents a shift in preference of a Ca source from exogenous dietary forms to that stored in bone (31). This may be an adaptive response to the decrease in Ca intake that usually occurs with age. The total potential for 1-hydroxylase activity would therefore remain unchanged and be able to respond to estrogen stimulation.

Note that β-estradiol treatment did not increase the recovery of tritium as 24,25(OH)2D in either the young ovariectomized or adult intact animals. This correlates well with the in vitro finding of no effect of ovariectomy on 24,25(OH)2D production in either young or adult animals and no effect in vivo in young animals. It is not clear why ovariectomy in adult animals did produce a significant decline in [3H]24,25(OH)2D recovery after [3H]25(OH)D administration. However, the bulk of the evidence indicates that the estrogen-related effects on 1,25(OH)2D production may be specific to the 1-hydroxylase enzyme and represent a discrete mechanism for modulating PTH-induced synthesis of the active form of vitamin D.

Results from the in vitro experiment do suggest a decline in 24-hydroxylase activity with age, however, although in vivo 24,25(OH)2D production appeared to be unchanged in the older animals. These results are in disagreement with Armbrecht et al (31) who found increases in 24-hydroxylase activity with advancing age. However, those investigators used male rats on diets that were either very high or very low in calcium. Thus differences in the sex of the animals and/or dietary regimen may account for the discrepancies in the results.

It remains to be determined whether estrogen acts directly at the parathyroid gland to stimulate release of PTH, with subsequent stimulation of 1-hydroxylase activity, or whether it works indirectly by decreasing bone resorption, thereby decreasing serum Ca levels that in turn trigger PTH secretion. Given either scenario, one might expect PTH levels to decline with age, at least after menopause, because of the decline in estrogen-stimulated production, and to be particularly low in osteoporotics. Unfortunately, studies measuring serum immunoreactive parathyroid hormone (iPTH) in young, old, and osteoporotic individuals have given conflicting results. One found age-related decreases in iPTH in women but not men (32) while others reported increases in both men and women (33, 34). Osteoporotics have been found to have low (35), normal (36), and high serum iPTH (37). Variations in the radioimmunoassay systems used may explain some of the discrepancies. In addition, osteoporosis itself may actually represent the end result of a number of different disorders.
AGE, ESTROGEN, AND VITAMIN D METABOLISM

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References


