Effects of Estrogen on Mineral Metabolism in Postmenopausal Women as Evaluated by Multiple Assays Measuring Parathyrin Bioactivity

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Data on the effect of estrogen on immunoreactive parathyrin (iPTH) in postmenopausal women are conflicting. We administered estrogen or placebo to 21 postmenopausal women for 12 weeks and measured PTH bioactivity (BioPTH), using the renal cytochemical bioassay. Before treatment, there was a negative correlation between nephrogenous cAMP and the tubular maximum for urinary phosphate excretion and a positive correlation between values measured by a midregion-specific PTH RIA and those measured in an immunoassay for intact PTH. Values measured by the midregion-specific RIA were also positively correlated with nephrogenous cAMP. BioPTH values were not correlated with other indices of PTH activity but were increased compared with values for younger subjects. After estrogen treatment there was no change in BioPTH activity despite an early decrease in serum osteocalcin and a later increase in nephrogenous cAMP. PTH concentrations measured by midregion-specific or intact RIAs were unchanged, but sample size may have been insufficient to exclude the possibility of significant changes in these values. The effects of estrogen on mineral metabolism in postmenopausal women are time-dependent. Early effects are independent of PTH, and later effects are variably associated with increased PTH activity.

Additional Keyphrases: immunoactive parathyrin · bioactive parathyrin · nephrogenous cAMP · tubular maximum for PO42− excretion · osteoporosis · effects as a function of duration of treatment · effect on osteocalcin, other analytes in serum · double-blind, placebo-controlled trial · relative sensitivities of assays · hyper- and hypoparathyroidism

Estrogen prevents bone loss (1) and inhibits bone resorption (2) in postmenopausal women, but the mechanism of these effects is not established. Previous studies have shown increased concentrations of immunoreactive parathyrin (iPTH) in serum after months of estrogen administration to postmenopausal subjects with osteoporosis, attributed to a secondary hyperparathyroidism from the mild decrement in serum calcium resulting from estrogen-mediated inhibition of bone resorption (3).4 We recently showed that a two-week course of estrogen caused no increase in PTH activity as measured with four distinct radioimmunoassays (RIAs), in urinary cAMP, or in the tubular maximum for urinary phosphate excretion (TmPO4), despite a modest increase in serum 1,25-dihydroxyvitamin D (1,25(OH)2D) and a decrease in serum osteocalcin, urinary calcium, and hydroxyproline (4).

We have now investigated the effects of 12 weeks of estrogen treatment in postmenopausal women, measuring bioactive PTH (bioPTH) by use of the renal cytochemical bioassay (5, 6) in a double-blind, placebo-controlled trial. We have compared these results with PTH activity determined by a midregion-specific RIA and an immunoradiometric assay of intact PTH (7), nephrogenous cAMP, and TmPO4. We have also evaluated the relative sensitivities of these assays in this one group of homogeneous subjects with normal parathyroid function.

Materials and Methods

Twenty-one healthy postmenopausal women without fractures, including six with surgically induced menopause, were studied after informed consent was obtained. Their ages ranged from 25 to 63 years, and they were taking no medication. The subjects consumed an 800-mg calcium diet daily for two weeks before the initial visit and throughout the study. Preprandial 2-h urine samples and preprandial blood samples were collected at each visit to the Endocrinology Research Laboratory.1 The women were randomly assigned to receive either ethinyl estradiol (20 μg/day, n = 8; 50 μg/day, n = 8) or placebo (n = 5). Treatment was started at the second visit (t = 0), a week after the initial visit (t = −1), and was continued for 12 weeks. Subsequent visits were after one, two, four, eight, and 12 weeks of medication (t = 1, 2, 4, 8, 12) and then one and four weeks after stopping medication (t = +1, +4). Four women were unable to finish the full 12 weeks of estrogen treatment because of vaginal spotting. Two women taking the 20-μg dose stopped after eight weeks. One woman taking the 50-μg dose stopped after four weeks, another after 10 weeks. Data from the final visits on estrogen were pooled for calculations (t = FINAL).

BioPTH was measured with a renal cytochemical bioassay method similar to that previously reported (5, 6) with minor modifications (8, 9). This assay procedure is based on the dose-dependent response of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activity to PTH in cultured cells of the renal cortex from guinea pigs. Kidneys were excised from the animals, decapsulated, and the medullary region was removed, leaving cortical tissue for use in the assay. Renal cortical segments (5–7 mm in all dimensions) were maintained in organ culture in Trowell's T-8 medium containing 25 mmol of Hepes per liter at 37 °C for 5 h in an atmosphere of O2/CO2 95/5 by vol. Subsequently, individual segments were stimulated for 6 min at 37 °C in the same atmosphere with graded doses of human PTH(1−84) (code 79/500; National Institute for Biological Standards and Controls, London, U.K.) as reference standard, and with 100-fold dilutions of coded samples of patients' plasma. All dilutions were performed with T-8 medium containing, per liter, 10 mL of outdated blood-bank plasma previously
stripped of PTH with 10 mg of QUSO-32 microfine silica (Philadelphia Quartz, via Calbiochem, La Jolla, CA) per milliliter. The stimulation of glucose-6-phosphate dehydrogenase activity was terminated by freezing the cortical segments rapidly in n-hexane on solid CO2. The frozen segments were stored at −70 °C for three to five days, at which time unified frozen 17-μm cryostat sections were prepared at −30 °C. These sections were thawed and reacted for glucose-6-phosphate dehydrogenase activity (5). The intensity of the formazan reaction product was measured within cells of the distal convoluted tubules by microdensitometry (Zeiss) at 589 nm with an 8-μm mask. The mean absorbance value from each incubation sample (PTH standard or unknown) was calculated from absorbance measurements taken from 11 to 20 different fields within the same section. In a separate assay, the dose–response relationship of PTH in plasma of patients selected without conscious bias was confirmed by assay of serial dilutions of the samples. The interassay CV was 15%; the intra-assay CV, 9%. BioPTH was detectable in 116 normal men and women under age 42 with a range of 1.3–31 ng/L (mean 9.2, SE 1.9 ng/L). The bioPTH exceeded 50 ng/L in all 46 patients with primary hyperparathyroidism; mean bioPTH was 0.6 (SE 0.1) ng/L in six patients with hypoparathyroidism. The lowest detectable concentration by this assay was 0.25 ng/L. There was no direct effect of estrogen on glucose-6-phosphate dehydrogenase activity in the assay.

Serum iPTH was measured with two different RIAs. RIA1 was a homologous midregion-specific assay in which we used an antisera, generated in a goat, against crude native PTH (generously supplied by Dr. Lawrence Mallette) and 125I-labeled Tyr43-(44–68)-human PTH as tracer (Diagnostic Systems Laboratories, Inc., Webster, TX). The technical details of the assay were as previously described (10), with minor modification (4). The interassay CV was 7%, and the intra-assay CV was 5%. iPTH was detectable in serum from all 161 normal subjects tested, with a range of 160 to 1000 ng/L (mean 490, SE 20 ng/L). In nine patients with hypoparathyroidism iPTH was undetectable, and 40 patients with primary hyperparathyroidism and normal renal function had serum iPTH values ranging from 1200 to 3400 ng/L. The detection limit, as previously defined (8), was 110 ng/L in each assay run.

The second RIA (RIA2) was a two-site immunoradiometric assay for the measurement of biologically intact PTH 1–84 (Allegro Intact PTH; Nichols Institute, San Juan Capistrano, CA) and technical details were as previously described (7). The assay involved the use of a goat polyclonal antibodies to human PTH, one specific for PTH-(39–84) immobilized onto plastic beads and the other specific for PTH-(1–84) radiolabeled for detection. The normal range was 10–55 ng/L, with a detection limit of 1 ng/L.

Serum osteocalcin was measured by RIA, with use of a rabbit antibody to bovine osteocalcin and 125I-labeled bovine osteocalcin as tracer (ImmunoNuclear, Stillwater, MN); assay characteristics were as described previously (4). We determined nephrogenous cAMP by RIA of plasma and urine, using an acetylation step (Biomedical Technologies, Inc., Cambridge, MA), and calibrated it as urinary cAMP corrected for glomerular filtration minus plasma cAMP (11). Serum calcium was measured in an Astra-8 Analyzer (Beckman Instruments, Palo Alto, CA) and serum phosphorus, alkaline phosphatase (EC 3.1.3.1), and albumin were determined in an SMA 12/60 continuous-flow analyzer (Technicon Instruments, Tarrytown, NY). Urinary calcium was measured in a Techtron automatic absorption spectrophotometer, and urinary phosphorus was determined colorimetrically. The TmPO4,FFR was calculated by using the nomogram of Walton and Bijvoet (12).

Data are expressed as the mean ± SE. Overall group comparisons were made by using analysis of variance for repeated measures. In the presence of significant effects, individual groups were compared by using the Student–Neuman–Keuls multiple comparisons procedure; differences at particular time points between groups were compared by using Tukey's HSD ("Honestly Significant Difference") multiple comparisons procedure (13, 14). We used Student's paired t-test to compare results at the two pretreatment time points. We also performed power calculations to examine the ability of this study to detect differences of various magnitudes between the groups (15).

Results

The three groups were comparable with respect to age and years since menopause (54 ± 4 and 9 ± 2 y, 54 ± 3 and 8 ± 2 y, 51 ± 4 and 7 ± 2 y, respectively, for the placebo, 20-μg, and 50-μg estrogen groups). Several subjects adjusted their diets slightly to consume 800 mg of calcium daily, but there were no major changes in calcium intake as a result of this diet. Adequate equilibration was confirmed by the lack of differences in any measured variable between the initial two visits before starting estrogen. There were also no differences for any variable between the placebo and treatment groups before starting estrogen, except for alkaline phosphatase, which for unexplained reasons was slightly lower in the placebo group than in the group receiving 20 μg of ethinyl estradiol.

Changes in clinical chemical values for serum and urine (Table 1): The most striking effect of estrogen was a decrease in serum osteocalcin, noted after as little as one week of treatment. The decrement in serum osteocalcin was still noted one week after cessation of either dose of estrogen but was no longer present four weeks later. No comparable changes in alkaline phosphatase were seen. With estrogen treatment there was a downward trend in serum calcium and albumin and in urinary calcium excretion, none of which achieved significance. The serum phosphorus was significantly lower at the final time point in the 20-μg estrogen group than at the initial time point or than in the placebo group.

Serum iPTH, bioPTH, nephrogenous cAMP, and TmPO4,FFR (Table 2): All measurements of bioPTH were within the normal reference interval. Serum iPTH was slightly increased in baseline determinations in one subject measured with RIA1, in five subjects with RIA2, and in one subject with RIA1 and RIA2; serum calcium concentrations were within normal limits in all of these subjects.

There were no significant changes in bioPTH with estrogen treatment. The power of this test to detect a change of 21 pg/mL in bioPTH (100% of placebo mean) was 0.99. There were also no significant changes in midregion-specific iPTH (RIA1), intact iPTH (RIA2), or TmPO4,FFR with estrogen treatment. However, the power of this test to detect a change of 390 ng/mL (50% of placebo mean) in RIA1 was only 0.38; for RIA2, only 0.17. The power of this test to detect a change of 0.96 (25% of placebo mean) in TmPO4,FFR was 0.32.

The nephrogenous cAMP gradually increased with time in the 50-μg estrogen group and during the final week of treatment was significantly higher than (a) before treat-
ment in this group and (b) the equivalent time point in the placebo group.

Regression analysis of the immediate pretreatment data (t = 0) confirmed the significant negative association between nephrogenous cAMP and TmPO4/GFR (r = -0.45, P = 0.04). There was also a significant positive correlation between RIA1 and RIA2 (r = 0.45, P = 0.03) but only RIA1 was significantly correlated with nephrogenous cAMP (r = 0.45, P = 0.04). BioPTH determination did not correlate with any other indices of PTH activity. There were no significant correlations between the indices of PTH activity and age, years since menopause, years since estrogen, serum osteocalcin or alkaline phosphatase, or urinary calcium excretion.

Discussion

After estrogen replacement, there was no evidence for a change in PTH activity as measured by bioassay despite an early decrease in serum osteocalcin, a later increase in nephrogenous cAMP, and changes in prepubertal urinary calcium excretion and serum 1,25(OH)2D as previously reported (4). The power of the test for bioPTH indicated that there was <1% chance that a change of 21 pg/mL would have been missed because of small sample size. An increase

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<th>Table 1. Effects on Some Clinical Chemical Results for Postmenopausal Women of Placebo (P, n = 5) or Treatment with Estrogen (20 μg/day, n = 8, or 50 μg/day, n = 8)</th>
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Values are the mean (and SE).

* P < 0.05 compared with t = 0.
** P < 0.01 compared with placebo.
*** P < 0.01 compared with placebo.
** P < 0.05 compared with t = FINAL.
+1: one week after stopping estrogen.  
+4: four weeks after stopping estrogen.

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<th>Table 2. Effects of Placebo (P, n = 5) or Estrogen (20 μg/day, n = 8, or 50 μg/day, n = 8) Treatment on Indices of Parathyroid Function in Postmenopausal Women</th>
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Footnotes, t, same as in Table 1.

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of this magnitude in the placebo mean would still yield a bioPTH value well below that seen in patients with primary hyperparathyroidism.

Estrogen-replacement therapy yielded no evidence for changes in PTH activity measured by the two RIAs, or in TmPO4/GRF. However, the powers for the RIAs and the TmPO4/GRF test indicate that the sample size was insufficient to exclude the possibility of significant changes in these values.

Previous studies have also shown great variability in the response of iPTH to estrogen replacement in postmenopausal women. An increase in iPTH was found in a group of postmenopausal women with osteoporosis after 28 weeks of 1.25–2.5 mg of conjugated estrogen daily (3). Another group reported a significant increase in iPTH after 12 weeks of topical estrogen administered to normal postmenopausal women and a trend upward after oral ethinyl estradiol and conjugated estrogen (16). We previously reported a slight decrease in iPTH after two weeks of 50 μg of ethinyl estradiol daily but a trend upward in iPTH and urinary cAMP and downward in TmPO4/GRF after a similar eight-week course (4). Other studies, with duration of estrogen replacement ranging from a week to a year, have failed to show significant changes in iPTH (17–22), although in some there were changes in nephrogenous cAMP and TmPO4/GRF consistent with increased PTH activity (17,20). Comparison of these studies is difficult because of inconsistencies in the numbers and clinical features of subjects, type of estrogen preparation, duration of treatment, and performance of PTH RIAs.

Measurement of PTH by RIA has been complicated by the relative insensitivity of some early assays, the immunoheterogeneity of the peptide, and the predominant use of antisera that recognize biologically inert fragments of the hormone. Recent technological innovations have led to the development of sensitive immunoradiometric assays of intact PTH (7) and renal cytochemical bioassays of PTH (5, 6). The availability of these more-sensitive and specific assays led us to re-examine the effects of estrogen on mineral metabolism in postmenopausal women. This new generation of assays has been used to document increased concentrations of PTH with normal aging (23,24). Baseline values for bioPTH in our current group of postmenopausal women were increased compared with those in younger subjects: 20 (SD 1) ng/L vs 9.2 (SD 1.9) ng/L, respectively. A recent study of normal, healthy postmenopausal women (25) also revealed that concentrations of intact iPTH are greater than the upper limits of normal in more than 5% of subjects. Our previous experience with the assays used in the present study demonstrated a correlation between the renal cytochemical bioassay and the RIAs for midregion and intact PTH in normal subjects and patients with parathyroid disease (26), newborns (27), and a well elderly population (24). However, baseline bioPTH values did not correlate with the two RIAs in this study, possibly owing to a greater homogeneity in age and sex of our subject group and its smaller size.

The lack of change in bioPTH is not consistent with the increase in nephrogenous cAMP seen at the final time point. It is possible that nephrogenous cAMP is a more sensitive index of PTH bioactivity than bioPTH measured in the renal cytochemical assay. Changes in nephrogenous cAMP independent of PTH might also be explained by speculating direct effects of estrogen on the kidney. Estrogen has been shown to increase adenylate cyclase activity in vivo in chicken kidney in one study (28), but not in another (29). Estrogen receptors have been localised in rat proximal tubules in proximity to PTH receptors (30).

In summary, estrogen replacement in postmenopausal women does not affect bioPTH as measured with the renal cytochemical assay. Estrogen replacement does cause (a) an early decrease in serum osteocalcin, suggesting a rapid inhibition of bone turnover, and (b) a later increase in nephrogenous cAMP, which may be a more sensitive indicator of PTH bioactivity or may reflect PTH-independent effects on renal adenylate cyclase. Thus, estrogen has effects on bone and kidney that become evident at different times. Early effects are independent of PTH, and later effects are variably associated with increased PTH activity.

We thank Dr. Lawrence Mallette for his generosity in establishing the midregion PTH RIA and for his valuable review of this manuscript. Ms. Allison Capilan for her able technical help, and Mr. Stephen Baker for expert assistance with statistical analyses. This work was supported in part by the Roger Robinson Research Fund at Worcester Memorial Hospital.

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