Estrogen Therapy Enhances Calcium Absorption and Retention and Diminishes Bone Turnover in Young Girls With Turner's Syndrome: A Calcium Kinetic Study

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Using stable tracers of calcium, we have previously shown a significant increase in calcium absorption and retention in prepubertal boys treated with exogenous testosterone. To investigate the effects of estrogen replacement on measures of calcium absorption, retention, and bone turnover, we studied a group of seven hypogonadal girls with Turner's syndrome (mean ± SE age, 12.5 ± 0.7 years). At baseline, ^{42}Ca intravenously (IV) and ^{44}Ca orally were administered, and blood and urine samples were collected for approximately 130 hours. Estrogen therapy was begun as oral ethinyl estradiol (4 or 20 μg/d) or intramuscular depot estradiol given over 4 weeks, after which an identical study was repeated. Analysis of calcium enrichment in blood and urine was performed using mass spectrometry methods. After estrogen therapy, there was a significant increase in calcium absorption ([Va] $P = .03$) and total calcium retention ([Vbal] $P = .04$), similar to the effects of testosterone in boys. Bone accretion (Vo^+) decreased after estrogen therapy ($P = .004$), as did resorption ($[Vo^-]$ $P = .004$). The overall rate of whole-body calcium turnover (Vt) was significantly decreased after estrogen administration ($P = .04$). These findings were opposite of those observed in prepubertal boys treated with testosterone. The contribution of bone resorption to whole-body turnover (E) also decreased after estrogen therapy ($P = .05$). These changes were associated with increased levels of 1,25-dihydroxyvitamin D after therapy with estrogens ($P = .05$). We conclude that estrogen supplementation is significantly anabolic for calcium metabolism by markedly increasing calcium absorption and retention and diminishing the estimated whole-body calcium turnover in girls with severe hypogonadism and Turner's syndrome. Further studies assessing the dietary calcium and/or vitamin D intake and bone mineral density of hypogonadal girls whose estrogen replacement is intentionally delayed will further define the need for calcium or vitamin D supplements in the peripubertal years in this condition.

THE EFFECTS OF SEX STEROIDS on bone physiology are complex. During active linear growth in childhood and throughout different reproductive stages in life, sex hormones undoubtedly influence the size, shape, and peak mass of the human skeleton.[1] In the female, estrogen plays a pivotal role in suppressing cancellous bone remodeling and maintaining a balance between osteoclastic (degradation) and osteoblastic (synthesis) cell activity, hence maintaining bone mass in adult females.[2] Estrogen is the treatment of choice to prevent bone loss after menopause,[3,4] and hypoestrogenemia is associated with reduced bone mineral density in a variety of clinical states in the female.[5,6] In conditions of pathologic hypogonadism such as Turner's syndrome, adult females have been found to have marked reductions in bone mineral density compared with controls,[7,8] and young untreated girls with this syndrome have diminished radial bone mineral content.[9] The latter is significantly improved by early administration of estrogenic hormones.[9]

Using nonradioactive tracers of calcium, techniques have been developed that allow a more precise characterization of changes in calcium absorption and retention and the dynamics of calcium movement between different body compartments and bone.[10,11] With these tools, we have previously shown significant increases in calcium absorption and retention after short courses of testosterone therapy in prepubertal boys.[12] In normal girls, calcium retention appears to be at a peak before and early in puberty,[13] immediately preceding the peak height velocity of growing females. Increasing evidence strongly suggests that the ovary is an active endocrine organ even in early childhood, and that dysfunction of the prepubertal gonad can have significant effects in the dynamics of the growth hormone (GH) and insulin-like growth factor-I (IGF-I) axis before puberty.[14] Hence, we designed these studies to investigate if the hypogonadism of young girls with Turner's syndrome alters measures of calcium and bone kinetics, and how those measures are affected by administration of different doses of estrogen.

SUBJECTS AND METHODS

Subjects

These studies were approved by the Nemours Children's Clinic Research Committee and the Baptist Medical Center Institutional Review Board. Seven girls with Turner's syndrome (45 XO and related karyotypes) were studied after provision of informed written consent. All subjects were in good health and had stopped any hormonal supplementation for at least 6 weeks before the study. Their clinical characteristics are summarized in Table 1.

Experimental Design

Each subject was studied twice. They were encouraged to consume a regular weight-maintenance diet for at least 3 days before and throughout the study. For 3 days before each study, careful food records were kept by the parents for analysis of nutrient and calcium intake. Each subject was admitted to the Clinical Research Unit of Wolfson Children's Hospital the afternoon before study day 1. At 6:00 PM, they consumed their evening meal. A mixture of milk or juice with a stable isotope of calcium (^{44}Ca 0.5 mg/kg) prepared 12 hours before the study was also consumed then, and a fractionated urine collection was begun and continued for the next 28 hours for measurement of calcium isotopic enrichment. Subjects were given a bedtime snack and then fasted until the study was completed the following morning. At 7:00 AM, two intravenous (IV) catheters were inserted, one on each forearm.

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Table 1. Clinical Characteristics of the Study Patients

Subject No.	Age (yr)	Weight (kg)	Body Mass Index	Height (cm)	Tanner Stage (breasts)	Race	Estrogen Dose	Karytope
1	11.9	29.5	16.8	132.6	I	White	Low	46XX isoq
2	12.9	48.7	23.3	144.6	I	White	Low	45XO
3	10.7	37.3	20.7	134.2	I	White	Medium	46XX isoq
4	11.9	37.6	21.7	131.6	I	White/Middle Eastern	Medium	45XO
5	16.5	51.5	29.9	130.7	II*	Black	Medium	45XO/46XX isoq
6	10.7	30.5	17.6	131.8	I	White	Medium	46XX isoq
7	12.8	36.7	18.4	141.4	I	White	High	45XO
Mean ± SE	12.5 ± 0.7	38.8 ± 3.2	21.2 ± 1.7	135.3 ± 2.1				

*This subject had arrested puberty for >2 years.

Time 0 was 8:00 AM. At −10 minutes, IV calcium tracer (^{42}Ca 0.6 mg/kg) was infused over 10 minutes. Blood samples were obtained at −10, 0, 5, 10, 15, 20, 30, 40, 60, 120, 180, 240, and 480 minutes for measurement of calcium isotopic enrichment. Serum GH was determined in samples every 20 minutes. Plasma IGF-I, IGF binding protein 3 (IGFBP3), 1,25-dihydroxyvitamin D, and estradiol concentrations were also measured. After the 4:00 PM sample, subjects were discharged home to complete the urine collections. Daily spot urine samples were obtained for the next 5 consecutive days and frozen at −70°C until analysis.

All subjects were then started on estrogen supplementation at different doses. Two subjects received oral ethinyl estradiol at a low dose of 100 ng/kg (~4 μg/d); four subjects received a medium dose of ethinyl estradiol 20 μg/d, and one subject received an intramuscular injection of 3 mg depot estradiol administered twice 4 weeks apart. Four weeks later, subjects were admitted to the Research Unit, and the study was repeated identically.

Assays

Plasma IGF-I, IGFBP3, and estradiol concentrations were measured by commercial immunoassay, and 1,25-dihydroxyvitamin D_3 levels were measured by radioreceptor assay after extraction and reverse-phase chromatography (Endocrine Sciences, Calabassas Hill, CA). GH was kindly determined by Dr Alan Rogol's laboratory at the General Clinical Research Unit in Charlottesville, VA, by radioimmunoassay.

Total calcium was determined in the urine by flame atomic absorption spectrometry, and isotopic enrichment was determined using a dual-filament thermal ionization quadruple mass spectrometer (Model THQ; Finnigan MAT, Bremen, Germany) as previously described.[10] Tracer enrichment were expressed as Δ% excess of natural abundance isotope ratios:

$$\Delta\%\ \text{excess} = \frac{\text{observed ratio} - \text{natural abundance ratio}}{\text{natural abundance ratio}} \cdot 100.$$

Isotopes

Calcium isotopes were obtained from Oak Ridge National Laboratory (Oak Ridge, TN) as calcium carbonate, dissolved in HCl solution, and infused as a $CaCl_2$ salt. Sterile solutions were prepared by the National Institutes of Health pharmacy and tested for pyrogenicity and sterility before use.

Calculations

True fractional calcium absorption was calculated from the ratio of the integrals of decay of oral and IV tracers in urine:

$$\alpha = \frac{\int_0^t {}^{44}Ca}{\int_0^t {}^{42}Ca}, \text{ True calcium absorption is defined as } Va = Vu \cdot \alpha.$$

The mathematical models used to calculate calcium kinetics have been described previously.[10] To describe the time course of the tracer excess in serum and urine over the course of the study, a three-term sum of the exponential equation was used: $y = A_1e^{-a1t} + A_2e^{-a2t} + A_3e^{-a3t}$. The SAAM program was used to calculate the least-squares best-fit linear curve to the data and allowed the calculation of these coefficients.[15] In this equation, the coefficients (Ai) and exponents (ai) relate tracer excess y to time t. Total exchangeable pool size (TEP) and forward flow of calcium into bone (Vo^+) can be calculated from disappearance rates of IV tracers over time, $TEP = 1/A_3$ and $Vo^+ = (a_3 \times TEP) - Vu + Vendo$, where Vu is the total urinary calcium excretion rate and Vendo represents endogenous fecal excretion of the IV calcium tracer. The latter is estimated from previously published normative data.[16] Net calcium retention, Vbal, can be calculated as $Vbal = Va - (Vu + Vendo)$. Vbal is also expressed as $Vbal = Vo^+ - Vo^-$, where Vo^- represents a measure of bone resorption, calculated hence by subtraction. Vt represents the rate of whole-body calcium turnover and can be calculated as $Vt = Vo^+ - Vu + Vendo$. E represents the contribution of bone resorption to whole-body calcium turnover, $E = Vo^-/Vt$. Even though this model may yield higher values for the rate of calcium movement into the final pool (Vo^+) than a compartmental model,[17] since each patient served as her own control, the relative differences found after intervention are still robust.

Statistics

One- and two-tailed paired Student t tests were used to calculate differences in the various parameters before and after administration of estrogen. ANOVA was used to compare these data with previously reported data. Significance was established at P less than .05.

RESULTS

There were no qualitative changes in any of the parameters measured depending on the estrogen dose, and hence the data were grouped for analysis. There was a modest increase in body weight after estrogen therapy (38.8 ± 3.2 *v* 40.4 ± 3.2 kg, $P = .002$). Circulating IGF-I concentrations did not change significantly in this group of subjects after estrogen (201 ± 19 before *v* 231 ± 7 μg/L after, P = NS). However, mean 4-hour GH concentrations increased after treatment (2.3 ± 0.8 *v* 4.2 ± 1.1 μg/L, $P = .035$), as well as peak GH concentrations (6.7 ± 1.5 *v* 14.0 ± 3.3, $P = .046$). This was accompanied by a modest but significant increase in circulating IGFBP3 concentrations (2.8 ± 0.1 to 3.3 ± 0.2 mg/L, $P = .015$) (Fig 1). Plasma estradiol concentrations remained invariant (2.13 ± 0.65 before *v* 2.31 ± 0.37 pmol/L after). The level of 1,25-dihydroxyvitamin D_3 increased after estrogen treatment (55.0 ± 7.9 *v* 82.0 ± 10.9 pg/mL, $P = .05$, one-tailed test).

Table 2 shows the changes in calcium kinetic parameters in these girls before and after administration of estrogen. Va and Vbal could not be calculated in one subject due to difficulties in

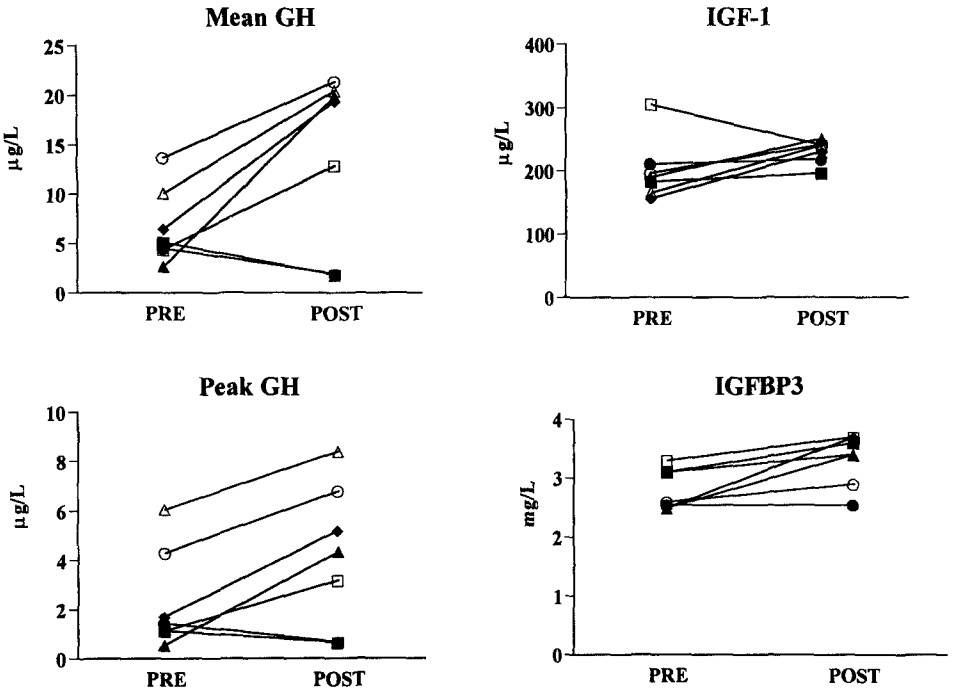

Fig 1. Changes in mean GH, peak GH, IGF-I, and $IGFBP_3$ concentrations in subjects studied before (pre) and after (post) estrogen therapy.

urine collection, but her calcium kinetic parameters were calculated and used in the analysis. Data are expressed as the mean ± SE.

After estrogen therapy, there was a significant increase in calcium absorption (Va) and calcium retention (Vbal) in these hypogonadal girls (Fig 2). This is similar to what we observed in prepubertal boys treated with testosterone.[12] There was a mild (10%) but significant reduction in bone accretion (Vo^+, $P = .04$) and a 25% reduction in bone calcium resorption (Vo^-, $P = .004$). There was an overall reduction in estimates of whole-body calcium turnover (Vt, $P = .04$) and in the contribution of bone resorption to whole-body turnover (E, $P = .005$) (Fig 3).

DISCUSSION

Contrary to the lack of effect of estrogens on measures of whole-body protein metabolism in prepubertal girls,[18] estrogenic hormones significantly increase measures of calcium absorption (Va) and retention (Vbal) in young hypogonadal girls. Both estimates of bone accretion (Vo^+) and resorption (Vo^-) diminish after estrogen, as well as the estimate of whole-body calcium turnover rates, expressed here as Vt. Specifically, the contribution of bone calcium resorption to whole-body calcium turnover (E) is diminished after estrogen therapy. These findings are congruent with the changes reported previously in normal girls in early puberty[13,19] and with the diminution in bone turnover rates observed after estrogen treatment in a variety of animals and humans.[1] However, these responses are clearly different from those of prepubertal boys treated with testosterone, who showed no significant decrease in bone turnover after testosterone therapy.[12] Males are typically taller and have a greater cortical bone width and total bone mass than females, and hence this dichotomy in the rates of bone turnover in response to changing sex steroid concentrations in the two sexes suggests that the rapidly expanding bone mass in the male continues to require an increased bone turnover rate as compared with that in females exposed to an increasingly enriched estrogen milieu.

Changes in calcium absorption and kinetics were accompanied by a significant increase in circulating levels of vitamin D_3 (1,25-dihydroxyvitamin D_3). This modulation of calcium transport by estrogens observed in our patients after therapy may be secondary to an increase in the 1α-hydroxylation of vitamin D, since in humans states of relative hyperestrogenemia (like pregnancy) are associated with increased serum 1,25-dihydroxyvitamin D levels,[20-22] whereas a decrease in estrogen concentrations after luteinizing hormone–releasing hormone analog therapy reduces the levels of this vitamin.[23] In Turner's syndrome, there appears to be relative impairment of vitamin D metabolism, with a lack of increase in vitamin D after a low-calcium diet, but it is unclear if such an effect is due to the estrogen deficiency, the syndrome itself, or both.[24]

Despite the observed changes in vitamin D_3 after estrogen therapy, the precise mechanism of estrogen's effects on the movement of calcium from the gut into different body pools in the hypogonadal youngsters reported here is not fully elucidated in this experimental design. Estrogen's effects on calcium and bone physiology are mediated in part through estrogen's interaction with its cytoplasmic receptor, triggering a complex cascade of transcriptional and regulatory responses involving the release of soluble cytokines and growth factors by the osteoblast,[1] leading ultimately to increased bone matrix synthesis and decreased resorption. Estrogen stimulates maturation of cartilage on the growth plate, and this hormone is thought to be predominantly responsible for the epiphyseal fusion, even in the male.[25] Since estrogen receptors have not been demonstrated in the growth plate,[1] it is not certain if these effects of estrogen on growth are direct or mediated through the associated increase in GH and IGF-I production observed during puberty.[26,27] Similar to previous reports, in the present model there were significant

Table 2. Calcium Kinetic Parameters in Girls Treated With Estrogen

Subject No.	Absorption			Retention		Resorption		Accretion		Turnover		TEP		Resorption/Turnover	
	αD_1	αD_2	Va D_1	Vbal D_1	Va D_2	Vbal D_2	$Vo^- D_1$	$Vo^- D_2$	$Vo^+ D_1$	$Vo^+ D_2$	Vt D_1	Vt D_2	D_1	D_2	E D_1	E D_2
1	—	—	—	—	—	—	49.4	31.1	88.1	74.2	90.1	76.5	469	245	0.55	0.41
2	0.27	0.41	12.2	7.7	20.8	15.3	93.0	68.9	100.8	83.4	105.2	88.9	292	218	0.89	0.77
3	0.10	0.80	4.3	2.3	25.6	23.8	77.9	49.2	80.1	72.8	82.2	74.6	343	311	0.95	0.66
4	0.20	0.23	4.6	2.6	5.8	1.9	37.2	28.9	39.7	30.8	41.8	34.7	109	103	0.89	0.83
5	0.58	0.53	7.4	5.3	9.5	7.8	46.4	41.8	51.7	49.6	53.9	51.4	136	174	0.86	0.81
6	0.49	0.86	17.8	14.5	30.9	28.1	63.3	56.1	77.8	84.2	81.1	87.0	179	444	0.78	0.65
7	0.26	0.45	6.6	3.6	13.8	10.2	60.8	44.0	72.1	62.2	74.6	65.2	270	210	0.83	0.70
Mean ± SE	0.32 ± 0.07	0.55 ± 0.10	8.8 ± 2.1	6.0 ± 1.9	17.7 ± 4.0	14.5 ± 4.1	61.1 ± 7.3	45.7 ± 5.3	72.9 ± 7.9	65.3 ± 7.3	75.5 ± 8.1	68.3 ± 7.4	257 ± 48	244 ± 41	0.82 ± 0.5	0.69 ± 0.5
P		.04*			.03	.048		.004		.043		.04		NS		.005

NOTE. All units are mg/kg · d, except α and E (no units) and TEP (mg/d).
*One-tailed test.

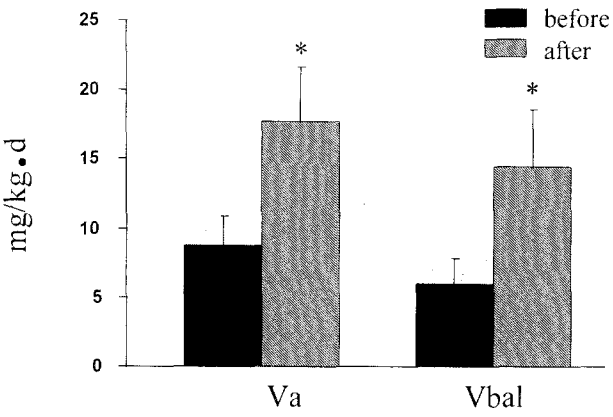

Fig 2. Estimates of calcium absorption (Va) and retention (Vbal) in subjects before and after estrogen treatment for 4 weeks. *$P \leq .04$.

increases in circulating mean and peak GH concentrations. IGFBP3 also increased after estrogen treatment, but not IGF-I. It is possible that estrogen and GH may act together in their anabolic roles in cortical and trabecular bone independently of IGF-I. The changes in bone turnover rates reported here are congruent with the significant increases in carboxy-terminal propeptide of type I collagen, a marker of osteoblast function, reported by us in these same patients after estrogen treatment.[28]

Peak bone mass of premenopausal women is determined to a significant extent by bone accretion during puberty.[29,30] More recently, Abrams et al[17] have shown that the rate of calcium flow into bone (Vo^+) peaks before menarche and diminishes subsequently. One of the strongest predictors of bone mineral density and content is body weight, height, and proper pubertal development,[31] and hence any delays in achieving peak bone mass may well have long-lasting consequences in the adult mineralization of bone and the ultimate risk of osteoporosis.[9,32]

Proper calcium supplementation, even before puberty, will likely have an impact on bone mineral density in the adult as well.[11,33] The development of a highly sensitive estrogen bioassay has clearly shown that estrogen production in the prepubertal female is significantly greater than in the prepubertal male,[34] suggesting that the prepubertal ovary is actively producing minuscule amounts of estrogen that might be paramount for the maintenance of at least normal GH production.[14]

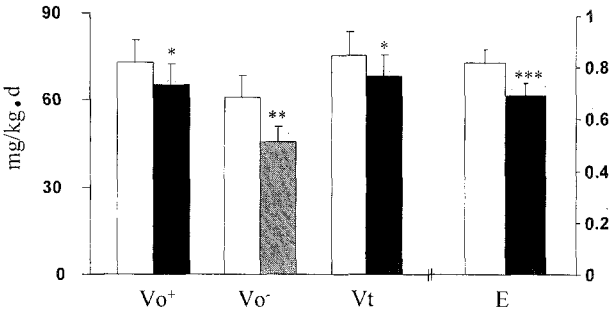

Fig 3. Changes in calcium kinetics after estrogen therapy. Vo^+, calcium accretion into bone; Vo^- resorption; Vt, whole-body calcium turnover; E, contribution of bone resorption to whole-body calcium turnover. *$P \leq .04$, **$P < .004$, ***$P < .005$.

Very small doses of estrogen have been shown to promote linear growth without undue advancement of skeletal maturation,[35] which suggested the need for a more physiologic approach to estrogen replacement therapy in hypogonadal girls at an earlier date.[36] Since girls with Turner's syndrome are very short, GH therapy has been customarily used and an intentional delay in the timing of estrogen supplementation advocated in an attempt to promote linear growth. Current practice guidelines for the supplementation of youngsters with hypogonadism such as Turner's syndrome recommend not starting estrogen therapy before the age of 12 years, and no recommendation for calcium supplementation is included.[37] Due to the significant anabolic effects of estrogen supplementation in the severely hypogonadal girls reported here, careful studies involving the routine assessment of dietary calcium intake and bone mineral density in these patients need to be performed to determine if calcium supplementation during the critical years of prepuberty and puberty is justified.

In conclusion, in girls with Turner's syndrome and hypogonadism, estrogen therapy for 4 weeks significantly increases calcium absorption and retention, and affects the contribution of bone resorption to whole-body calcium turnover and significantly increases vitamin D_3 levels. The dietary calcium and/or vitamin D intake of girls with Turner's syndrome should be individually assessed in the prepubertal years, especially in youngsters whose estrogen replacement is intentionally delayed in an attempt to promote linear growth.

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