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Changes in Bone Turnover in Young Women Consuming Different Levels of Dietary Protein*

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ABSTRACT

Although high protein diets are known to increase urinary calcium excretion and induce negative calcium balance, the impact of dietary protein on bone turnover and fractures is controversial. We therefore evaluated the effect of dietary protein on markers of bone turnover in 16 healthy young women. The experiment consisted of 2 weeks of a well balanced diet containing moderate amounts of calcium, sodium, and protein followed by 4 days of an experimental diet containing one of three levels of protein (low, medium, or high). On day 4, serum and urinary calcium, serum PTH, 1,25-dihydroxyvitamin D, serum osteocalcin, bone-specific alkaline phosphatase, and urinary *N*-telopeptide excretion were measured. Urinary calcium excretion was significantly higher on the high than on the low protein diet. Secondary hyperparathyroidism occurred on the low protein diet. Urinary *N*-telopeptide excretion was significantly greater during the high protein than during the low protein intake (48.2 ± 7.2 *vs.* 32.7 ± 5.3 nM bone collagen equivalents/mM creatinine; $P < 0.05$). There was no increase in osteocalcin or bone-specific alkaline phosphatase when comparing the low to the high diet, suggesting that bone resorption was increased without a compensatory increase in bone formation. Our data suggest that at high levels of dietary protein, at least a portion of the increase in urinary calcium reflects increased bone resorption. (*J Clin Endocrinol Metab* **84:** 1052–1055, 1999)

OSTEOPOROSIS is a major public health problem in the United States, and the role of diet in both its prevention and pathogenesis is an area of current investigative interest. It has been recognized for more than a half a century that dietary protein intake impacts calcium metabolism (1). High levels of dietary protein increase urine calcium excretion (2), and it is estimated that when protein intake doubles, urinary calcium increases by approximately 50% (3). At reduced levels of dietary protein, we have recently observed a fall in both urinary calcium excretion and intestinal calcium absorption with the appearance of secondary hyperparathyroidism (4, 5).

Although changes in mineral metabolism clearly attend alterations in dietary protein intake, the long term implications for skeletal health are uncertain. Cross-sectional studies evaluating bone mineral density as a function of historical protein intake have yielded conflicting results (6–11). Studies examining the association between protein intake and fracture incidence, however, have generally observed a positive association (12, 13). We have reported a significant cross-cultural association between dietary animal protein intake and the age-adjusted incidence of hip fracture (14). None of these data provides direct evidence for a causal relationship between protein intake and fracture.

Over 25 yr ago, Wachman and Bernstein (15) proposed that the skeleton played a role in acid-base homeostasis. They suggested that skeletal stores of base in the form of calcium salts could be called upon to neutralize endogenous acid loads generated from high protein diets. The long term result would be accelerated rates of skeletal loss (16, 17). Further, the diminished ability of the kidney to excrete fixed acid loads with aging may lead to increasing dependence on buffering from bone with a deleterious long term effect on skeletal mass (18). If this hypothesis is correct, one would expect to see changes in bone turnover and, more specifically, an increase in bone resorption with the long term ingestion of high protein diets. Biochemical studies addressing this question are limited. Schuette and colleagues (19) and Chan and Swaminathan (20) reported that urinary hydroxyproline excretion increased with increasing levels of dietary protein intake, whereas Shapses *et al.* (21) found no impact of increasing levels of dietary protein on bone turnover. We therefore prospectively examined the effect of dietary protein on markers of bone turnover in healthy young women studied at low, medium, and high levels of protein intake.

Subjects and Methods

The details of the study design have been reported previously (4). Briefly, the protocol included three cycles, each of which consisted of 2 weeks of an adjustment diet followed by 4 days of the experimental diet

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and 3 days of an *ad libitum* diet. During the adjustment period, subjects modified their usual intake to contain moderate amounts of protein, sodium, calcium, and caffeine. The next 4 days constituted the experimental period, during which the subjects received all of their food from the Yale General Clinical Research Center's (GCRC) metabolic kitchen. The experimental diet was controlled in calcium, sodium, and phosphorus while containing one of three levels of dietary protein: low, medium, or high. Fasting blood and urine samples were collected on the mornings of days 0 and 4 of each experimental period. The cycle of a 2-week adjustment period and a 4-day experimental period was repeated two more times until all subjects received each of the three levels of dietary protein in random order. This study was approved by human investigation committees at both Yale University and the University of Connecticut.

Subjects

Sixteen healthy women (aged 20–40 yr) were recruited to participate in the study. The average age was 26.7 ± 1.3 yr, and they had a body mass index (kilograms per m^2) of 22.3 ± 0.6. Exclusion criteria have been previously described (4). Throughout the study, subjects were free living and continued their usual activities at home, school, and work. Informed consent was obtained from each study participant.

Diets

During the 2-week adjustment period, subjects were instructed to self-select their diets to contain approximately 1 g protein/kg, 20 mmol calcium and 100 mmol sodium. Caffeine-containing beverages were limited to one per day, and alcohol was not permitted.

During the 4-day experimental period, subjects reported daily to the GCRC to receive their meals and record their body weights. Each subject began at an energy intake of 125–150 kJ/kg, which was adjusted in 840- to 1260-kJ increments (with simple sugars and fats) during the experimental period to maintain body weight within an average of 1% of the initial weight.

All experimental diets consisted of a variety of common foods that were prepared, weighed (to 0.1 g), and served from the GCRC metabolic kitchen under the supervision of the research dietitian. The experimental diets were individually calculated for each subject to contain one of three levels of protein, whereas other nutrients remained constant and controlled (19.8–20.3 mmol calcium, 26–38 mmol phosphorus, and 99–101 mmol sodium). Protein intake was low (0.7 g/kg), medium (1.0 g/kg), or high (2.1 g/kg). The source of calcium in the experimental diets was primarily dairy foods and a commercially available, chewable, form of calcium carbonate (Tums, SmithKline Beecham, Pittsburgh, PA). The macronutrient and mineral compositions of the experimental diets were previously reported (4).

Sample collection

Blood and urine samples were collected on days 0 and 4 of each experimental period. On days −1 and 3, subjects collected timed 24-h urine samples for determination of calcium, sodium, and creatinine excretion. On the mornings of days 0 and 4, fasting 2-h urine samples were obtained for determination of cAMP, creatinine, and type 1 collagen *N*-telopeptide concentrations. Blood was drawn at the midpoint of the 2-h period for the measurement of plasma cAMP, midmolecule PTH, intact PTH, 1,25-dihydroxyvitamin D, total and ionized calcium, phosphorus, creatinine, osteocalcin, and bone-specific alkaline phosphatase.

Assays

Urinary and blood calcium and creatinine, blood phosphorus, intact PTH-(1–84), midmolecule PTH, 1,25-dihydroxyvitamin D, and urinary sodium, nitrogen, and nephrogenous cAMP (NcAMP) excretion were determined as previously reported (4). The percent calcium reabsorption during the 2-h fasting urine specimen was calculated using the following formula: 100 − [(urinary calcium ÷ plasma calcium) × (plasma creatinine ÷ urinary creatinine) × 100].

Serum osteocalcin was measured using the method of Gundberg *et al.* (22), and bone-specific alkaline phosphatase was measured by differential binding to wheat germ lectin (Boehringer Mannheim, Mannheim, Germany) followed by colorimetric assay according to the method of Farley *et al.* (23). Urinary excretion of the cross-linked *N*-telopeptide of type 1 collagen was determined using a commercially available enzyme-linked immunosorbent assay (Osteomark, Ostex International, Inc., Seattle, WA). All six samples from a single subject were analyzed in duplicate in the same assay.

Statistical analyses

All values are presented as the mean ± SEM. Within each time point, repeated measures ANOVA was used to evaluate differences between dietary protein levels using SYSTAT for Windows, version 5.0 (Evanston, IL). $P < 0.05$ indicated statistical significance. When there was an overall effect of protein level, *post-hoc* orthogonal contrasts were then used to evaluate the differences at each time point. Because multiple contrasts were performed (low *vs.* medium, medium *vs.* high, and low *vs.* high), a Bonferroni correction was applied (24).

Results

Average body weight in the subjects fluctuated less than 1% from the beginning of the study to the end. All subjects remained healthy throughout the experiment. The mean 24-h urinary nitrogen excretion values on day 4 of the three experimental diets were 485 ± 36 mmol (low protein), 628 ± 43 mmol (medium protein), and 1092 ± 100 mmol (high protein), paralleling dietary protein intake and indicative of good subject compliance with the three dietary interventions.

Changes induced in mineral metabolism and calcitropic hormones

Changes observed in mineral metabolism and calcitropic hormones were presented in detail previously (4). Briefly, baseline measures of these parameters did not differ among the three protein levels. By day 4, urinary calcium was significantly decreased during the low protein diet compared to that during the medium protein diet (low, 108 ± 14 mg/day; medium, 129 ± 14 mg/day; $P < 0.05$) and was significantly increased during the high protein diet compared to that during the medium protein diet (medium, 129 ± 14 mg/day; high, 196 ± 19 mg/day; $P < 0.0005$). Urinary sodium did not differ among any of the three experimental diets. The glomerular filtration rate rose as dietary protein increased (low, 85 ± 5; medium, 95 ± 5; high, 107 ± 6 mL/min). Fasting calcium reabsorption was significantly reduced during the high protein diet compared to the medium and low protein diets (high protein diet, 98.99 ± 0.14%; medium protein diet, 99.35 ± 0.10%; low protein diet, 99.42 ± 0.88%; $P < 0.05$).

As previously reported (4), secondary hyperparathyroidism developed during the low protein diet. By day 4 of the low protein diet, circulating levels of midmolecule PTH were 2.4-fold higher than the mean values during the medium protein diet (37.7 ± 1.9 *vs.* 15.5 ± 1.0 nmol/L; $P < 0.0001$). In every subject consuming the low protein diet, the value for midmolecule PTH on day 4 exceeded the upper limit of normal (20 nmol/L). Serum levels of intact PTH, 1,25-dihydroxyvitamin D, and NcAMP were also significantly increased within 4 days of consumption of the low protein diet ($P < 0.005$ compared to the medium diet), consistent with an increase in bioactive PTH. In contrast, mineral metabolism and calcitropic hormones remained stable during the medium protein diet. Subjects consuming the high protein diet

showed no change on day 4 in circulating levels of PTH, 1,25-dihydroxyvitamin D, or NcAMP excretion. However, as reported previously (4), there was a slight suppression of the PTH-1α-hydroxylase axis when subjects continued the high protein diet for 14 days.

Changes induced in bone turnover

Baseline values of osteocalcin and urinary *N*-telopeptide excretion did not differ among the dietary treatments (data not shown). In response to increasing dietary protein, urinary *N*-telopeptide excretion rose progressively, such that by day 4 the value was significantly greater during the high *vs.* the low protein diet (Table 1). The frequency distribution of *N*-telopeptide excretion during the low and high protein diets is shown in Fig. 1. As can be seen, the frequency distribution of *N*-telopeptide excretion was shifted to the right (*i.e.* toward higher values) on the high protein diet. Of the 16 women, 12 increased their *N*-telopeptide excretion, with the increase ranging from 28–327% between the low and high protein diets. The mean day 4 urinary *N*-telopeptide excretion during the low diet was lower, although not significantly so, compared to the medium protein intake. In contrast to the rise in urinary *N*-telopeptide excretion, there was no difference in day 4 mean serum osteocalcin values during any of the three diets. Day 4 values of bone-specific alkaline phosphatase were higher during the low compared to the medium protein diet, but were not different between the low and high or medium and high protein intakes.

Discussion

We assessed bone turnover and mineral metabolism in 16 young healthy women as they consumed low, medium, and high levels of dietary protein in otherwise well balanced, controlled, and nutritionally complete diets. As we have previously reported and has been observed by others, elevations in urinary calcium excretion were observed during the high protein diet (2). Depressed urinary calcium excretion was found on the low protein diet and was accompanied by secondary hyperparathyroidism (4, 5). When comparing the low with the high dietary protein intake, we found that urinary *N*-telopeptide excretion was significantly greater at the high level of dietary protein intake. Further, there was a progressive rise in *N*-telopeptide excretion with increasing levels of dietary protein. In contrast, there was no increase in day 4 levels of osteocalcin or bone-specific alkaline phosphatase when comparing the low to the high diet, suggesting that resorption was increased without evidence for a compensatory increase in formation, at least by these biochemical criteria. The magnitude of increase in bone resorption (32.7 $\pm$ 5.3 *vs.* 48.2 $\pm$ 7.2; mean difference, 15.5 mM bone collagen equivalents/mM creatinine; 47% increase) was not as great as the difference in *N*-telopeptide excretion seen in pre- *vs.* postmenopausal Australian women (36.9 $\pm$ 1.6 *vs.* 86.0 $\pm$ 7.0; mean difference, 49.1 pmol bone collagen equivalents/nmol creatinine; 133% increase) (25). Nonetheless, we observed these changes in young healthy premenopausal women who are estrogen sufficient and in whom acid-base handling by the kidney is completely normal. Whether postmenopausal women respond in a quantitatively different manner is of considerable interest and is currently under investigation. In addition, we do not know whether protein-induced changes in bone turnover persist long term or if the findings of this study apply to other ethnic groups, such as African-Americans.

Experimental studies directly examining the effect of animal protein on bone turnover are limited. Scheutte *et al.* (19) studied 16 young men during 12 days of low (50 g/day) or high (150 g/day) levels of protein intake with dietary calcium fixed at 500 mg/day. She reported that urinary hydroxyproline increased from 21.4 to 28.2 mg/24 h (a 32% increase) in response to the increase in dietary protein. In contrast, Shapses *et al.* (21) studied 7 young men and 8 young women and found that urinary hydroxyproline, pyridinoline, and deoxypyridinoline did not increase when dietary protein was increased from 0.44 to 2.71 g/kg. After 5 days on each diet, mean values for each urinary metabolite were not different on the two diets. However, in this study, dietary calcium was increased from 423 to 1589 mg/day concomitant with the increase in dietary protein, potentially obscuring an effect of dietary protein on bone resorption.

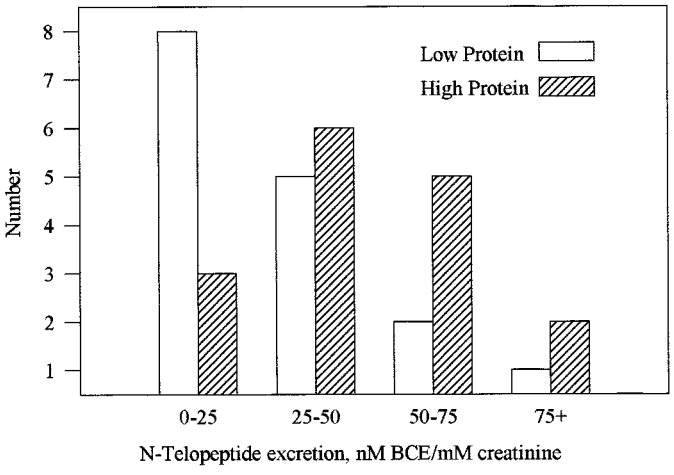

FIG. 1. Frequency distribution of *N*-telopeptide excretion on day 4 of the low and high protein diets.

TABLE 1. Effect of dietary protein on markers of bone turnover

Markers of bone turnover	Protein		
	Low	Medium	High
Osteocalcin (ng/mL)	7.6 $\pm$ 1.4	6.9 $\pm$ 1.2	5.7 $\pm$ 0.8
Bone-specific alkaline phosphatase (IU/L)	69.4 $\pm$ 8.8[a]	46.4 $\pm$ 5.8	57.2 $\pm$ 7.8
N-Telopeptide (nM bone collagen equivalent/mM creatinine)	32.7 $\pm$ 5.3[b]	43.5 $\pm$ 7.0	48.2 $\pm$ 7.2

Values are the means of day 4 $\pm$ SEM.
[a] Significantly different from the medium protein diet, $P < 0.005$.
[b] Significantly different from the high protein diet, $P < 0.05$.

Dietary protein may exert a continuum of effects on skeletal homeostasis involving both intestine and bone. Combined with our previous studies, the current data suggest the following model to explain the induced changes in mineral metabolism observed during low, medium, and high levels of protein intake. A medium protein intake induces no change in mineral homeostasis, intestinal calcium absorption, or bone turnover rates. At high levels of dietary protein, urinary calcium excretion is elevated despite normal intestinal calcium absorption and in the face of slight suppression of the PTH-1α-hydroxylase axis (4, 5). As reported in this study, high levels of dietary protein are associated with increased rates of bone resorption without an increase in markers of formation. Taken together, these data suggest that at high levels of dietary protein, at least a portion of the increase in urinary calcium may reflect increased bone resorption rates. Recent work by Sebastian *et al.* (26) suggesting a reduction in markers of bone resorption in postmenopausal women supplemented with alkali is consistent with this idea. As noted in the current study, bone resorption rates were slightly, albeit not statistically significantly, lower during the low compared to the medium protein intake. These skeletal effects, in combination with reduced intestinal calcium absorption (5), probably explain the secondary hyperparathyroidism observed during the low protein diet. Our findings raise the possibility that at low levels of dietary protein, skeletal calcium accretion may be impaired. In this context it is of interest that Chiu *et al.* (27) reported reduced skeletal bone mass in long term vegans. Long term prospective studies are needed to further explore various components of this hypothesis. Further, indirect markers of bone resorption and formation may not precisely reflect what is occurring at the level of skeletal tissue. More direct methods, such as bone histomorphometry and calcium kinetic studies, will be required to precisely quantitate the relative contributions of altered bone metabolism and intestinal calcium absorption to the observed changes in whole body mineral homeostasis induced by different levels of dietary protein intake.

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