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Calcitropic Hormones and IGF-I Are Influenced by Dietary Protein

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Elderly men and women with protein deficiencies have low levels of circulating IGF-I, and it is likely this contributes to reduced bone formation and increased bone resorption. We hypothesized that calcitropic hormones are involved in this effect and are affected by dietary protein. We therefore investigated the influence of a low-protein diet on the PTH-1,25-dihydroxyvitamin D3 \([1,25(OH)_2D_3]\) axis and IGF-I in rats, using pamidronate to block resorption that normally contributes to mineral homeostasis.

We fed 6-month-old Sprague Dawley female rats isocaloric diets containing 2.5% or 15% casein for 2 wk. Pamidronate was then administered sc (0.6 mg/kg) for 5 d. Blood samples were collected at different time points. Serum 1,25(OH)_2D_3, IGF-I, PTH, calcium, and phosphorus were determined in all rats; vertebral bone strength and histomorphometric analysis were performed in rats subject to the longest low-protein diets. We found 2 wk of low protein increased PTH levels, decreased 1,25(OH)_2D_3, calcium, and IGF-I, suggesting that increased PTH compensates for low-protein-induced decreases in 1,25(OH)_2D_3. Pamidronate augmented the increased PTH after 8 wk of low protein and prevented the 1,25(OH)_2D_3 decrease. IGF-I remained low. Protein malnutrition induced decreases in relative bone volume and trabecular thickness, which was prevented by pamidronate. Maximal load was reduced by protein restriction, but rescued by pamidronate. In summary, the low protein diet resulted in hyperparathyroidism, a reduction in circulating levels of IGF-I, and reduced 1,25(OH)_2D_3 despite hyperparathyroidism. Blocking resorption resulted in further increases in PTH and improved microarchitecture and biomechanical properties, irrespective of vitamin D status or protein intake. (Endocrinology 152: 1839–1847, 2011)

Malnutrition or undernutrition, particularly protein malnutrition, is frequently observed among the elderly (1). Animal studies have shown that isocaloric low-protein intake is associated with decreased bone mass and bone strength in adult female and male rats (2–5). A reduced protein intake is also associated with major alterations of bone microarchitecture (6) and decreased intrinsic bone tissue quality in rats (6). These modifications of bone are related to a decreased bone formation and an increased bone resorption leading to a negative bone balance and to bone loss.

It is likely that these alterations in bone formation may be due, at least in part, to decreases in the serum concentrations of IGF-I (3). IGF-I is an important factor that stimulates bone formation and allows for the maintenance of bone mass (7–9). Indeed, an increase in bone formation markers has been observed after IGF-I treatment in postmenopausal women (10). Basic and clinical studies suggest that dietary proteins influence both the production and action of IGF-I (3, 11–14). Indeed, elderly men and women with protein deficiencies have low levels of circulating IGF-I that return to normal after a correction of protein intake (11). The mechanisms underlying this reduction in circulating IGF-I may include a resistance to the actions of GH at the hepatic level (12, 15) and to an increase in the metabolic clearance rate of IGF-I (16). Furthermore, protein restriction has been shown to induce osteoblast resistance to IGF-I (3).
In addition to the observed decrease in bone formation, low protein intake also induces an increase in bone resorption (2, 4). This may be related, in part, to a depression of the gonadotrophic axis generated by protein deprivation. In fact, the normal estrous cycle was not detected in protein-deprived female rats, suggesting either estrogen deficiency or a resistance to estrogen (2). In addition to sex hormone deficiencies, other mechanisms are involved in the increased bone resorption after protein deficiency, including the effect of circulating or locally released cytokines (17, 18). In particular, TNFα has been shown to play a role in bone loss caused by protein deficiency. Blocking TNFα activity prevented the increased bone resorption induced by an isocaloric low-protein diet, without a concurrent modification of bone formation (17). The effects of chronic protein deficiency on the PTH-1,25 dihydroxyvitamin D₃ [1,25(OH)₂D₃] axis are not clear. Previous studies have shown that the PTH-1,25(OH)₂D₃ axis is influenced by dietary protein (19–23). Indeed, an acute, yet moderately low, reduction in dietary protein led to a decline in intestinal calcium absorption, secondary hyperparathyroidism, and an increase in 1,25(OH)₂D₃ in healthy young women (19, 22) although the effect on 1,25(OH)₂D₃ under more severe protein depletion remains to be investigated. It should be noted, however, that a study performed in rodents showed that 8 wk of protein restriction caused a reduction in both intestinal calcium absorption and in serum 1,25(OH)₂D₃ concentrations in growing rats (23).

Bisphosphonates, antosteoporotic drugs that inhibit bone resorption, are commonly prescribed. By reducing bone turnover and increasing bone mineral content and bone strength, they also are effective in preventing various types of experimental osteoporosis, such as that induced by immobilization (24), ovariectomy, orchidectomy, corticosteroid administration, or a low calcium diet (25). The inhibition of bone resorption by the bisphosphonate, pamidronate, prevented the alteration of microarchitecture and diminution of mechanical properties induced by isocaloric low protein intake in both male and female adult rats (5). A significant improvement in both bone mineral density and bone microarchitectural parameters were also observed after pamidronate administration under a low-protein diet despite low bone formation. The underlying mechanism for this phenomenon remains obscure, especially when bisphophonates are normally considered to reduce both bone resorption and formation, although an indirect effect of the bisphosphonate on hormonal status and calcium metabolism is suspected.

In this study, the objectives were therefore to better understand the influence of an isocaloric low-protein diet on the PTH-1,25(OH)₂D₃ axis and second, to investigate the response of the PTH-1,25(OH)₂D₃ axis and the somatotrophic axis when bone resorption is decreased by pamidronate administration in rats subjected to an isocaloric low-protein diet. We hypothesize that a low-protein diet will lead to hormonal changes in parathyroid secretion and circulating levels of IGF-I and reduced 1,25(OH)₂D₃ despite hyperparathyroidism. Furthermore, we hypothesize that blocking resorption, a known side effect of a low-protein diet, will improve the bone quality without necessarily improving the hormonal status of the rats.

Materials and Methods

Animals and diet

All experimental designs and procedures received the approval of the Animal Ethics Committee of Geneva University Faculty of Medicine. In all experiments, animals were 6-month-old Sprague Dawley female rats (Charles River Laboratories, L’Arbresle, France). They were housed individually at 21°C with a 12-h light, 12-h dark cycle and were strictly pair fed with isocaloric synthetic diets provided by Provimi Kliba AG (Kaiseraugst, Switzerland) containing 15% or 2.5% casein, 0.8% phosphorus, 1% calcium, 70–80% carbohydrates, and 5% fat throughout the experimental period. Demineralized water was available ad libitum. The protein content of the control group receiving 15% casein was chosen according to recommendations found in the literature (26). The amount of protein in the diet of the low-protein group was chosen according to previous results obtained in female rats (2, 4). Because the minimal protein intake necessary to maintain normal bone homoeostasis in adult rats is 5%, a restriction to 2.5% corresponds to a 50% reduction of the minimal amount of protein intake for healthy adult animals, a reduction that is often observed in hip fracture patients (27).

Experimental design

The study consisted of two experiments. The same procedures were followed until the beginning of the treatment period in all experiments. After 1 wk of equilibration on a diet containing 15% casein, the rats were divided into four groups; two groups were pair fed an isocaloric diet containing 2.5% casein, and the remaining two groups received 15% casein until the end of the study. Subcutaneous administration of pamidronate was commenced 2 wk after the beginning of protein restriction for one of the low-protein diet (2.5% casein) groups and one of the 15% casein groups. The other rats were injected with vehicle at the same time. To investigate the effects of isocaloric low-protein diet and/or pamidronate treatment according to the duration of protein deprivation, blood samples were collected in each experiment at different time points. Serum 1,25(OH)₂D₃, IGF-I, PTH, calcium, and phosphorus were determined.

Baseline data or acute response to low-protein diet

The effects of 2 wk of low-protein intake on biochemical parameters were assessed in baseline blood samples collected at time point \( t = 0 \) h. Blood samples were taken from the tips of the tails. Using this method, blood quantities were not sufficient to determine all biochemical parameters of interest for each rat. For this reason, blood samples were randomly pooled from three rats in each group fed a normal or low-protein diet to determine serum IGF-I and...
1,25(OH)$_2$D$_3$ concentrations, allowing for five specimens per group for each of these parameters.

### Four weeks after pamidronate treatment

In this experiment, 40 rats were divided into four groups of 10 rats each. Pamidronate was administered daily (0.6 mg/kg/d) for 5 d. The dose and treatment schedule of pamidronate was selected based on previous studies showing optimal effects on bone mass and mechanical properties (5, 28, 29). Blood samples were collected by aortic puncture under general anesthesia 4 wk after the 5-d pamidronate treatment period finished.

### Eight weeks after pamidronate treatment

Thirty-six rats were divided into four groups of nine rats each. Pamidronate was again administered daily (0.6 mg/kg/d) for 5 d; 8 wk after the 5-d pamidronate treatment period ended, blood samples were collected by aortic puncture under general anesthesia. After death the lumbar spine was removed for microtomographic (μCT) histomorphometry and mechanical testing.

### Biochemical determination

Intact plasma PTH was measured by ELISA using a kit from Immutopics (San Clemente, CA). 1,25(OH)$_2$D$_3$ in serum was determined by RIA using the ImmunoDiagnostics Systems γ-B 1,25-Dihydroxyvitamin D kit (Boldon, UK). Serum osteocalcin and IGF-I were measured by RIA using reagents from Biomedical Technologies (Stroughton, MA) for the former, and a kit from Nichols Institute (San Juan Capistrano, CA) after extraction by acid-ethanol and cryoprecipitation, for the latter. Calcium in serum was assayed by atomic absorption spectrometry with acid-ethanol and cryoprecipitation, for the latter. Calcium in

### Bone mechanical testing

The lumbar spine was excised immediately after death and frozen at −20 C overnight and then warmed to room temperature before mechanical testing. The L4 vertebrae were isolated from the lumbar spine at the level of the intervertebral discs. The vertebral pedicles were dissected out carefully to avoid any damage to the cortical shell. Because the caudal and cranial surfaces of the rat vertebral body are not parallel, 1 mm of the caudal and cranial parts of each vertebral body was embedded in methylmethacrylate cement (Technovit 4071; Heraeus Kulzer GmbH, Wehrheim, Germany) to ensure regular distribution of the compressive forces (31). Between each preparation step, the specimens were kept immersed in physiological saline solution. The mechanical resistance to failure was tested using a servo-controlled electromechanical system (Instron 5566; Instron Corp., High Wycombe, UK) with the actuator displaced at 2 mm/min. Displacement and load were simultaneously recorded every 0.01 sec. Maximal load (N) was directly obtained from the load-deformation curve; the stiffness (slope of the linear part of the curve, representing the elastic deformation, N/mm) and the energy absorbed by the bone tissue (area under the load-deformation curve before the bone breaks, in N x mm) were calculated. Reproducibility was 4.8% for vertebrae and was evaluated as the CV of pair sample measurements (L3–L4).

### Microtomographic histomorphometry by μCT

Parameters of mass and architecture of the vertebrae were investigated using μCT histomorphometry with a high-resolution μCT system (μCT 40; Scanco Medical AG, Bassersdorf, Switzerland) as previously described (29, 31). In summary, three-dimensional (3D) images of a vertebra were acquired with a voxel size of 20 μm in all spatial directions. No sample preparation was needed, and the vertebrae were secured in a cylindrical sample holder in air. The resulting gray-scale images were segmented using a low-pass filter to remove noise and a fixed threshold to extract the mineralized bone phase. The trabecular and cortical regions of the vertebral bodies were separated with semi-automatically drawn contours.

From the binarized images, structural indices were assessed. Relative bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp) were calculated by measuring the 3D distances directly (31, 32) in the trabecular network. Connectivity densities, based on Euler number (33) and the structure model index (SMI), were also calculated. The SMI quantifies the plate vs. rod characteristics of trabecular bone (32), and an SMI of 0 indicates a purely plate-shaped bone, whereas an SMI of 3 indicates a rod-like fashioned bone, and values in between stand for a mixture of plates and rods.

### Statistical analysis

Statistics were performed using the Statview software (Statview SE + Graphics 1; Abacus Concept, Berkeley, CA). Unpaired Student’s t test was performed to determine the effect of low-protein diet at baseline time (pretreatment). In all experiments, one way and two-way ANOVA were performed to determine the effects of treatment and diet. When the ANOVA indicated a significant difference among the groups, statistical differences between individual groups were evaluated with Fisher posttest. A value of $P < 0.05$ was considered significant for all statistical analyses. All results are expressed as means ± SEM.

### Results

#### Effect of low-protein diet on the PTH-1,25(OH)$_2$D$_3$ axis and IGF-I levels: baseline data or acute response to low-protein diet

After 2 wk of an isocaloric, low-protein diet ($t = 0$ h), serum PTH was significantly increased (+46%) in rats fed a low-protein diet compared with rats subject to a normal protein diet before pamidronate treatment (Table 1). Serum IGF-I showed a significant decrease (−15%) as a result of low-protein intake. Baseline serum calcium was significantly decreased (−5%) in rats fed a low-protein diet, whereas no significant changes in serum phosphorus were detected at baseline (Table 1). Serum 1,25(OH)$_2$D$_3$ showed a significant decrease (−68%) in rats fed a low-protein diet in baseline blood samples, (i.e. after 2 wk of protein restriction).

#### Four weeks after the 5-d pamidronate treatment

Rats were treated daily with pamidronate for 5 d. Four weeks after pamidronate treatment, PTH levels were modestly, but not significantly, raised in animals subject to low...
protein, and animals were treated with pamidronate regardless of protein intake (Fig 1A). In vehicle-treated rats, a marked and significant drop in circulating 1,25(OH)2D3 was observed in rats subject to low protein compared with those on the normal protein diet (Fig 1B). Serum 1,25(OH)2D3 values in pamidronate-treated rats on a low-protein diet were significantly increased compared with vehicle-treated rats on the same low-protein diet. When rats on the 15% casein diet were treated with pamidronate, a small, but not significant, increase in circulating 1,25(OH)2D3 was observed after the beginning of treatment (Fig 1B). As observed at baseline, protein deficiency induced a significant decrease in serum IGF-I independently of pamidronate treatment (Fig 1C). Pamidronate administration had no effect on serum IGF-I in rats on either diet compared with their respective vehicle-treated control groups (Fig 1C). A two-way ANOVA indicated that serum calcium was significantly decreased in both groups receiving low protein compared with those receiving normal (15%) protein, and there were no differences between these two low-protein groups regardless of treatment (Table 2).

Serum phosphorus levels were lowest in the pamidronate-treated rats fed a low-protein diet; however, this was not statistically significant from any of the other groups (Table 2).

### Eight weeks after the 5-d pamidronate treatment

In this experiment, rats were treated as in experiment 2, and blood samples were collected 8 wk after the beginning of pamidronate treatment. A two-way ANOVA revealed that a low-protein diet induced an increase in serum PTH, although one-way analysis revealed that this increase was only significant in pamidronate-treated animals (Fig 2A and Table 2). Serum PTH was not altered by pamidronate treatment in rats fed a normal protein diet; however, it was significantly increased by pamidronate in the low-protein groups (Fig 2A). Serum 1,25(OH)2D3 values were significantly decreased in vehicle-treated rats fed a low-protein diet compared with vehicle-treated rats fed a normal protein diet. We also observed the same trend in the serum 1,25(OH)2D3 responses to pamidronate treatment as in experiment 2, with increases observed in both protein groups; however, only in the low-protein group was this increase significant (Fig. 2B). Protein deficiency was also associated with a significant decrease of IGF-I independently of pamidronate treatment (Fig. 2C). As with the earlier 4-wk postpamidronate time point, no effect on serum IGF-I was observed after pamidronate administration. Calcium and phosphorus levels showed similar, yet insignificant, patterns to those responses observed in experiment 2 (Table 2).

### Table 1. Effect of low-protein intake on serum biochemical analyses after 2 wk of low-protein intake

<table>
<thead>
<tr>
<th>Casein diet</th>
<th>15% (n = 20)</th>
<th>2.5% (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTH (pg/ml)</td>
<td>347.3 ± 58.4 (19)</td>
<td>509.4 ± 60.2a (19)</td>
</tr>
<tr>
<td>1,25(OH)2D3 (pg/ml)</td>
<td>261 ± 6 (5)</td>
<td>82 ± 20a (5)</td>
</tr>
<tr>
<td>Calcium (mmol/liter)</td>
<td>2.97 ± 0.04 (20)</td>
<td>2.82 ± 0.04a (20)</td>
</tr>
<tr>
<td>Phosphorus (mmol/liter)</td>
<td>1.44 ± 0.05 (20)</td>
<td>1.36 ± 0.06 (18)</td>
</tr>
<tr>
<td>IGF-I (ng/ml)</td>
<td>990.3 ± 58.5 (5)</td>
<td>775.4 ± 42.3a (5)</td>
</tr>
</tbody>
</table>

Values are means ± SEM. a P < 0.05 vs. 15% group as evaluated by Student’s t test. The numbers in parentheses represents the number of animals. For 1,25(OH)2D3 blood was pooled from five rats.
**TABLE 2.** Serum biochemical analyses in rats fed either a normal or a low-casein diet and pamidronate or vehicle treated

<table>
<thead>
<tr>
<th>Time after treatment</th>
<th>Protein intake and treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15% + Veh</td>
</tr>
<tr>
<td>Pamidronate</td>
<td></td>
</tr>
<tr>
<td>4 wk</td>
<td>Calcium (mmol/liter)</td>
</tr>
<tr>
<td></td>
<td>Phosphorus (mmol/liter)</td>
</tr>
<tr>
<td>8 wk</td>
<td>Calcium (mmol/liter)</td>
</tr>
<tr>
<td></td>
<td>Phosphorus (mmol/liter)</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Veh, Vehicle.

\(^a\) P < 0.05 vs. baseline (15% casein, vehicle-treated) group as evaluated by ANOVA. \(^b\) P < 0.05 vs. 2.5% casein diet, vehicle-treated group.

**Effect of pamidronate treatment on microarchitecture and bone strength in lumbar vertebrae of rats fed an isocaloric normal or low-protein diet**

Parameters of bone microarchitecture were studied in the lumbar vertebrae of rats killed 8 wk after the beginning of pamidronate treatment. Protein deprivation resulted in alterations of most microarchitectural parameters. Administration of pamidronate was associated with a significant increase in BV/TV and Tb.Th independently of protein intake, although the increases observed in both BV/TV and Tb.Th were more marked (+35% and +25%, respectively) compared with those increases observed in rats subjected to normal protein (+22% and +14%). In fact, alterations in BV/TV and Tb.Th induced by low-protein intake were fully prevented by pamidronate administration (Table 3 and Fig. 3), and there was even a significant increase in trabecular thickness compared with vehicle-treated rats fed a normal protein diet (Table 3 and Fig. 3).

Alterations observed in the microarchitecture were associated with changes in mechanical properties. Pamidronate treatment resulted in a significant increase in maximal load and energy in rats fed a normal and low-protein diets as assessed by two-way ANOVA analysis.

Maximal load was also significantly increased in the low-protein-fed, pamidronate-treated rats compared with their low-protein, vehicle-treated control group (Table 4). Stiffness was modestly increased by pamidronate treatment although the value was not quite significantly different from the 15% protein control group.

**Discussion**

In previous studies, we demonstrated that the bisphosphonate, pamidronate, was able to induce an increase in trabecular bone volume and an overall improvement in microarchitecture, perhaps due to a positive bone balance, in animals fed a low-protein diet. It is unlikely that a direct effect on bone is a plausible explanation for this action of the bisphosphonate. We therefore hypothesized that a modulation of the PTH-1,25(OH)\(_2\)D\(_3\) axis by the low-protein diet may be involved. In the current study, we observed changes in the PTH-1,25(OH)\(_2\)D\(_3\) axis after a dietary protein restriction in adult female rats. Specifically, we found a decreased serum 1,25(OH)\(_2\)D\(_3\) and an increased serum PTH. These changes were observed after only 2 wk of isocaloric low-protein intake and persisted for up to 10 wk. As expected, the somatotrophic axis was also influenced by the isocaloric low-protein diet as shown by a decrease in IGF-I. This is in agreement with previous studies in which we were able to demonstrate that protein depletion resulted in similar variations of IGF-I, which in turn were associated with depressed bone formation (2, 3).

Because protein depletion affects circulating levels of IGF-I, it should also be pointed out that the effects of IGF-I on muscle growth, development, and maintenance are highly significant (34); many studies report that IGF-I stimulates muscle cell growth and proliferation (35) as well as an essential lack of muscle function in mice lacking IGF-I, in which embryonic development is impaired and pups do not survive after birth due to an inability to breathe (36) or are severely growth impaired (37, 38).

Further studies are therefore necessary to investigate the role of IGF-I on muscle homeostasis in rats fed a normal or low-protein diet and treated with pamidronate.

We also investigated the response of the PTH-1,25(OH)\(_2\)D\(_3\) axis to treatment with pamidronate. The results show that administration of pamidronate accentuated the PTH increase observed in rats fed a low-protein diet; however, pamidronate treatment returned 1,25(OH)\(_2\)D\(_3\) values to normal in the low-protein group. IGF-I was not influenced by pamidronate treatment and remained depressed in rats subject to an isocaloric low-protein diet.
One of the aims of the current study was to investigate the effect of protein deprivation on the PTH-1,25(OH)₂D₃ axis. PTH plays a major role in the regulation of serum calcium concentrations by promoting bone resorption, by reducing renal calcium excretion, and, indirectly, by stimulating intestinal calcium absorption (39). PTH secretion is principally determined by serum calcium concentrations. In response to a reduction in serum calcium, PTH secretion increases rapidly to return serum calcium concentrations to normal values. In our study, we found that a reduction in protein intake was associated with a secondary hyperparathyroidism. This was observed after only a short time period of protein restriction in the initial experiment, as well as after more chronic protein deprivation as in the other experiments, i.e., after 6 and 10 wk. Some studies in humans also report

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![FIG. 2. Serum PTH (A), 1,25(OH)₂D₃ (B), and IGF-I (C) in 6-month-old rats fed either normal-casein diet (15%) or low-casein diet (2.5%), 8 wk after pamidronate administration. Treatment was administered sc after a 2-wk equilibration on either a low- or a normal-protein diet. Values are means ± SEM. *, P < 0.05 vs. 15% casein diet; †, P < 0.05 vs. 2.5% casein diet.

![FIG. 3. 3D µCT reconstructions of images of vertebrae. Parameters of mass and microarchitecture of the vertebrae were investigated using microtomographic histomorphometry with a high-resolution µCT system (µCT 40; Scanco). 3D images of the vertebrae were acquired with a voxel size of 20 µm in all spatial directions. Images show vertebrae from vehicle-treated rats receiving 15% protein (A), pamidronate (APD)-treated rats receiving 15% protein (B), vehicle-treated rats receiving 2.5% protein (C), and pamidronate (APD)-treated rats receiving 2.5% protein (D).](image)

### TABLE 3. Effect of pamidronate on bone microarchitecture in lumbar vertebrae of rats fed different amounts of protein 8 wk after administration

<table>
<thead>
<tr>
<th>Casein diet</th>
<th>15% + Veh</th>
<th>15% + pamidronate</th>
<th>2.5% + Veh</th>
<th>2.5% + pamidronate</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV/TV (%)</td>
<td>25.8 ± 1.8</td>
<td>31.5 ± 1.7a</td>
<td>19.9 ± 1.8</td>
<td>26.9 ± 0.1b</td>
</tr>
<tr>
<td>Tb.N (1/mm)</td>
<td>3.17 ± 0.101</td>
<td>3.29 ± 0.10</td>
<td>3.03 ± 0.09</td>
<td>3.00 ± 0.07</td>
</tr>
<tr>
<td>Tb.Th (mm)</td>
<td>0.08 ± 0.003</td>
<td>0.09 ± 0.002a</td>
<td>0.071 ± 0.002a</td>
<td>0.089 ± 0.002a,b</td>
</tr>
<tr>
<td>Tb.Sp (mm)</td>
<td>0.299 ± 0.01</td>
<td>0.281 ± 0.11</td>
<td>0.32 ± 0.011</td>
<td>0.311 ± 0.008</td>
</tr>
<tr>
<td>SMI</td>
<td>0.158 ± 0.142</td>
<td>-0.087 ± 0.135</td>
<td>0.633 ± 0.209a</td>
<td>0.26 ± 0.084</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Veh, Vehicle.

a P < 0.05 vs. baseline (15% casein, vehicle-treated) group as evaluated by ANOVA. b P < 0.05 vs. 2.5% casein diet, vehicle-treated group.
that a moderate reduction of protein intake was associated with secondary hyperparathyroidism assessed after 4 and 14 d (19, 21). It is likely that a reduction of protein intake induces secondary hyperparathyroidism by decreasing intestinal calcium absorption (22). Indeed, impaired intestinal calcium absorption causes a transient fall in the circulating calcium concentration, which in turn stimulates the release of PTH. In our experiments, we did not assess intestinal calcium absorption; however, it is very possible that the acute decreased serum calcium observed in rats subject to low protein may reflect a reduction of intestinal calcium absorption. The fact that this secondary hyperparathyroidism was observed when we looked at animals 10 wk after the beginning of protein restriction (representing the greatest duration of protein deprivation), suggests that the secondary hyperparathyroidism observed after a short-term reduction of protein in the diet (19, 21), and in our experiment 1, may continue to persist for the duration of protein deprivation. Because long-term elevation of PTH results in an increased bone turnover, bone loss, and increased fracture risk (40), chronic secondary hyperparathyroidism observed under protein deprivation may contribute to bone loss under these conditions.

In the current experiments, 1,25(OH)2D3 was significantly decreased in rats subjected to a low-protein diet. This was previously observed in a study performed in growing rats subjected to low protein, and the authors suggested it was possible that this decrease in circulating 1,25(OH)2D3 was instrumental in the concurrently observed decrease in intestinal calcium absorption (23). However, effects of protein reduction in rats may not be reflected in humans because protein deprivation was associated with an elevation of 1,25(OH)2D3 in studies performed in young women (19, 21). It should be noted that in the human studies, decreases in dietary protein were not severe, averaging around a 20% reduction, and as such, the effect of more severe protein depletion on 1,25(OH)2D3 remains to be investigated. It is generally accepted that under physiological conditions, the production of 1,25(OH)2D3 is directly proportional to PTH concentrations. In our study, however, the duration and severity of protein deprivation were more important determinants of changes in 1,25(OH)2D3, and this may partially explain the different 1,25(OH)2D3 response we observed. The mechanism underlying the decreased circulating levels of 1,25(OH)2D3 in this situation may be related to a decrease in serum IGF-I. Via its renal action, IGF-I influences the production of 1,25(OH)2D3, because it can increase renal 1α-hydroxylase activity, which in turn elevates serum 1,25(OH)2D3 concentrations (41–43). Through this mechanism, the effect of reduced protein intake on 1,25(OH)2D3 reported in our experiments and in a previous study (23) may be mediated, at least in part, by decreased circulating IGF-I levels. We observed decreases in IGF-I levels in all rats fed an isocaloric low-protein diet, regardless of the time periods that rats were subject to the diet and regardless of pamidronate treatment (Table 1 and Figs. 1C and 2C). These findings are in agreement with previous studies showing that low-protein intake induces a depressed somatotrophic axis (2, 3, 5). IGF-I is important for the formation of bone and the maintenance of bone mass (7–9). The decreased levels of IGF-I observed in this study suggest an important role for IGF-I in the pathogenesis of bone loss after protein malnutrition. It would be of interest to investigate whether treatment with IGF-I might reverse the changes seen in 1,25(OH)2D3, bone volume, trabecular thickness, and bone strength in future studies, because an increase in bone formation markers has been observed after IGF-I treatment in postmenopausal women (10). IGF-I may also be involved in the changes observed in the PTH-1,25(OH)2D3 axis after protein deprivation as discussed later. This may underlie the decrease in intestinal calcium absorption seen with protein undernutrition as has been observed previously in growing rats (23). This secondary hyperparathyroidism induced by the decreased protein intake may be at least partially responsible for the increased bone resorption observed in animals fed a low-protein diet.

The second aim of the present study was to investigate whether administration of pamidronate would alter the increased parathyroid secretion induced by the low-protein diet considering the ability of this bisphosphonate to block the bone resorption that would normally occur in response to decreased protein, as we have previously re-

### TABLE 4. Effect of pamidronate on bone strength in lumbar vertebrae of rats fed different amounts of protein 8 wk after administration

<table>
<thead>
<tr>
<th>Casein diet</th>
<th>15% + Veh</th>
<th>15% + pamidronate</th>
<th>2.5% + Veh</th>
<th>2.5% + pamidronate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximal load (N)</td>
<td>198 ± 21</td>
<td>301 ± 27</td>
<td>184 ± 25a</td>
<td>270 ± 22a,b</td>
</tr>
<tr>
<td>Stiffness (N/mm)</td>
<td>661 ± 106</td>
<td>738 ± 84</td>
<td>692 ± 32</td>
<td>765 ± 48</td>
</tr>
<tr>
<td>Energy (N × mm)</td>
<td>47 ± 11</td>
<td>101 ± 20a</td>
<td>35 ± 9</td>
<td>76 ± 11</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Veh, Vehicle.

a P < 0.05 vs. baseline (15% casein, vehicle-treated) group as evaluated by ANOVA. b P < 0.05 vs. 2.5% casein diet, vehicle-treated group.
ported. We observed that after 4 wk, an increase in PTH was observed in those animals fed a low-protein diet, and in animals receiving pamidronate. After 8 wk, this secondary hyperparathyroidism returned to normal in animals on the normal protein diet receiving pamidronate; however, in those fed a low-protein diet, the levels remained high, particularly in the animals on the low-protein diet that also received pamidronate. This indicates that bisphosphonates are able to induce a pronounced and prolonged hyperparathyroidism in rats subject to compromised diet intake.

Administration of pamidronate also resulted in a significant increase in BV/TV and trabecular thickness independently of the protein intake 8 wk after the pamidronate treatment. These parameters were more substantially increased in the low-protein diet group than in rats fed a normal protein diet, although an increase was observed in both treatment groups. Similar effects were observed for changes in biomechanical parameters, with pamidronate inducing a significant increase in maximal load independently of protein intake. Pamidronate treatment in the low-protein group resulted in a maximal load that was significantly higher than in the 15% casein, vehicle-treated group. These changes in the microarchitecture and biomechanics of bone suggest that the decrease of bone resorption prevents the detrimental alterations induced by a low-protein diet and results in a positive bone balance. It is unlikely that the mechanism underlying this positive effect of pamidronate on bone balance is related to somatotrophic axis because IGF-I was unchanged in pamidronate-treated groups when compared with vehicle-treated rats. These changes in the microarchitecture and biomechanics of bone suggest that the decrease of bone resorption prevents the detrimental alterations induced by a low-protein diet and results in a positive bone balance. It is unlikely that the mechanism underlying this positive effect of pamidronate on bone balance is related to somatotrophic axis because IGF-I was unchanged in pamidronate-treated groups when compared with vehicle-treated groups regardless of the amount of protein in the diet. The inhibition of bone resorption by bisphosphonates enhances intestinal calcium absorption (44). The mechanism underlying this enhanced calcium uptake is related, but not restricted, to an increase in PTH levels, a rise in renal clearance of calcium, and thus increased intestinal calcium absorption in rats fed a normal protein diet (44). In our study, 4 wk after the administration of pamidronate, the increase in PTH was no longer significant, and it returned to normal levels by 8 wk after pamidronate treatment. 1,25(OH)2D3 levels also returned to normal, or to values higher than control, after pamidronate treatment was administered in protein-unrestrained rats at both time points investigated. By administering pamidronate, we were able to block the contribution of bone resorption to calcium homeostasis. Bisphosphonate administration resulted in a transient increment of PTH when animals were fed a normal protein diet and a permanent secondary hyperparathyroidism in rats fed a low-protein diet. Together with an inhibition of bone resorption, this increased PTH may lead to a positive bone balance and an improvement of bone mass and microarchitecture. As expected, biomechanical parameters were increased 8 wk after pamidronate treatment when compared with vehicle-treated rats subject to the same protein intake. This observation is in agreement with a previous study performed in this laboratory although the duration of the treatment in the present study was shorter and represented only the initial phase of the process. It may be of value to investigate the effects of our current findings on the fibroblast growth factor 23 axis, to reveal any involvement of fibroblast growth factor 23 on vitamin D status.

In conclusion, in the current study, we observed that a low-protein diet resulted in secondary hyperparathyroidism independently of the vitamin D status. This may be due to the low circulating levels of IGF-I caused by the reduced protein intake, which in turn induced low concentrations of circulating 1,25(OH)2D3 levels and thus reduced calcium absorption in the gut, leading to an increased production of PTH. Blocking the resorption that would normally occur to correct these biochemical changes caused by the low protein resulted in an increased 1,25(OH)2D3 and PTH. We also observed improvements in bone microarchitecture and biomechanical properties, which are normally killed to restore calcium homeostasis in situations of compromised nutrition in the absence of bisphosphonate treatment.

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