Effect of dietary calcium intake and protein source on calcium utilization and bone biomechanics in the spontaneously hypertensive rat

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The effects of dietary calcium level and protein source on in situ paracellular Ca absorption, and subsequent Ca utilization for bone mineralization and biomechanical strength were examined in spontaneously hypertensive (SHR) and normotensive Wistar Kyoto (WKY) rats fed 20% casein and soy protein isolate diets containing different levels of calcium (2.0, 0.5 and 0.05% wt/wt). Rats were meal fed for 10 weeks after which calcium absorption was measured by the disappearance of ^{45}Ca from the ligated ileal loop. Femoral deposition of ^{45}Ca, and femur and tibia mineral composition were also examined. Calcium utilization was estimated from femur and tibia biomechanical measurements. The greater ($P < 0.05$) bone mineralization observed in SHR compared to WKY in animals fed high and adequate Ca-containing diets was not attributable to the relative efficiencies of paracellular calcium absorption and had little effect on femur biomechanical force parameters. In both Ca replete SHR and WKY, the greater bone mineralization observed in casein-fed compared with soy-fed counterparts was reversed when animals were fed calcium deficient diets. Calcium absorption in situ was affected equally in both SHR and WKY ($P < 0.05$) by differences in dietary calcium intake as well as protein source. Similarly, in both animal strains, bone biomechanical strength parameters were correlated with bone mineralization, which in turn was influenced more by dietary calcium than protein source.

Keywords: SHR; bioavailability; dietary calcium level; paracellular absorption; bone biomechanics

Introduction

Dietary calcium intake is often considered to be a primary factor in bone mineralization and metabolism.[1-3] Epidemiological studies have indicated that bone density is positively correlated with past calcium and milk consumption patterns.[2,3] Other studies have shown a potential for increased calcium intake and calcium supplementation in the treatment of osteoporosis.[2,4-6]

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Another dietary factor to be considered in relation to overall dietary calcium intake is the bioavailability of calcium. Calcium absorption is characterized by carrier-mediated (transcellular) and passive (paracellular) mechanisms, which are affected differently by dietary and physiological factors.[7,8] Earlier studies have established that calcium absorption occurs mainly in the distal segment of the small intestine, or ileum, by a paracellular pathway involving passive transport at normal intake levels.[9,10] This is particularly true in the non-lactating rat, which does not have a detectable active Ca transport system in the jejunum or ileum, regardless of the level of Ca intake.[8] Dietary factors that influence paracellular calcium absorption include the level of intake[11] and its solubility in the intestinal chyme.[12,13] The latter factor is the basis of calcium bioavailability that can be influenced by food constit-

uents. The absorption of calcium from plant protein sources, such as soy, is noted to be inferior to that of animal derived proteins, namely casein, due to the presence of potential inhibitors such as fiber and phytate.[14] Alternatively, a positive effect of casein on calcium bioavailability has been attributed to the production of post-digestion casein phosphopeptides (CPP).[15] CPP have been reported to increase the proportion of soluble ionized calcium available for absorption from the distal small intestine.[15]

Despite these findings, few studies have attempted to relate intestinal calcium bioavailability to utilization. Using a slope-ratio test, Forbes et al. did not show a difference in bone calcification with normal rats fed soy, as compared to casein diets that were either deficient or adequate in calcium level.[16] In a previous study from this laboratory with rats fed a normal level of calcium, differences in paracellular calcium absorption measured in situ were not correlated with bone mineralization and biomechanical parameters.[13] A major limitation of these studies in concluding an association between calcium bioavailability and bone utilization is the small amount of bone remodelling in the skeleton of the normal rat. In the spontaneously hypertensive rat (SHR), Izawa et al. reported a decreased femoral density and mineralization similar to that of osteoporosis.[17] This observation coincides with the disturbed intestinal calcium absorption and metabolism in the SHR, not seen in the normotensive Wistar-Kyoto (WKY) rat.[18-22]

The objectives in the present study were to examine the effect of varying levels of dietary calcium and sources of dietary protein on in situ paracellular calcium absorption. Additional measurements of bone mineralization and biomechanical properties were taken to evaluate the significance of diet-related changes in calcium bioavailability from the ileum with subsequent skeletal utilization. These results were compared in the osteoporosis-prone SHR and its genetically related normotensive WKY counterpart.

Materials and methods

Animals and diets

Four-week-old male SHR and normotensive WKY rats (Charles River, Montreal, Canada) were each divided into six experimental groups (six animals per group). Animals were individually housed in stainless-steel cages with controlled temperature (25° C) and lighting (14:10 hr light:dark cycle). Dietary groups included 20% casein or soy protein isolate (ICN Biochemicals, Cleveland, OH, USA) containing a high level of dietary calcium (2.0% wt/wt); a medium level of calcium (0.5% wt/wt); and a low level of calcium (0.05% wt/wt), respectively. Dietary phosphorus contents by analysis were 0.54% (wt/wt) for all casein diets and 0.50% (wt/wt) for all soy protein diets. Diets contained (g/100 g): casein or soy protein isolate, 20.0; D.L. methionine, 0.3; cornstarch, 15.0; fiber, 5.0; vegetable oil, 5.0; Ca-free mineral mix, 3.5; vitamin mixture, 1.0; choline bitartrate, 0.2; calcium carbonate, 4.98, 1.23, or 0.11 for respective diets; and sucrose to 100 g.

Animals were fed ad libitum until they reached 100 g body weight, after which meal feeding was initiated. Over a 2 week period, animals were trained to consume the diets within a 6-hour period daily (9 a.m. to 3 p.m.). Deionized water was made available to animals ad libitum. Daily feed intakes and weekly body weight gains were recorded throughout the experiment. Animals were cared for in accordance with the principles of the Canadian Council on Animal Care.

In situ paracellular calcium absorption measurements

Calcium absorption was measured from an in situ ligated ileal loop in animals that were 14 weeks of age, as previously reported.[13,23] On the morning of the experiment, rats were allowed access to their respective diets for a 1.5-hour period. Rats were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg body weight), and the ileum was ligated 1.5 hours following removal of the diets. Following exposure of the small intestine, an ileal loop was made between two ligations at points 8 cm and 20 cm from the ileocecal junction.[13] In an attempt to standardize the injected dose of ^{45}Ca with the intestinal calcium contents, and thus yield similar intestinal ^{45}Ca specific activities, 15 μCi total dose was chosen for animals fed the high calcium diets; 5.4 μCi for animals fed the medium calcium diets; and 0.375 μCi ^{45}Ca ($^{45}CaCl_2$, 18.2 mCi/mg Ca, ICN Biomedical Inc., Irvine, CA, USA) for animals fed the low calcium diets. The injection volume was kept constant (300 μL, 37° C), and the loop carefully massaged to ensure uniform distribution of the radioactivity in the contents of the ligated ileal loop. The absorption of ^{45}Ca was determined by calculating the percentage of dose absorbed. The amount absorbed was the amount of original dose less the amount remaining in the ligated loop after 1 hour.[15] ^{45}Ca was measured with an LKB-1215 Liquid Scintillation Counter (Wallac Oy, Turku, Finland). In separate experiments it was determined that 92 ± 3% of total radioactivity present in the intestinal loop was recovered. The measurement of ^{40}Ca in the ileal contents was performed by atomic absorption (Perkin Elmer-306 atomic absorption spectrophotometer; Perkin Elmer, Norwalk, CT, USA) following wet ashing with HCl/HNO_3,[24] and dilution with 0.5% $LaCl_3$.

Plasma minerals

Plasma minerals were measured from blood samples obtained by cardiac puncture. Plasma calcium and magnesium were determined by atomic absorption spectrophotometry in the presence of 0.5% $LaCl_3$. Plasma phosphorus was measured by the colorimetric method of Itaya and Ui.[25]

Bone biomechanics

After sacrifice, femur and tibia bone samples were excised, cleansed of adhering soft tissue, and epiphyses removed. The left femora, and tibiae were subjected to biomechanical three-point bending analysis using an Instron Universal Testing Machine (Model 1122, Instron Corp., Canton, MA, USA). Femora and tibiae were bent until failure occurred, by lowering a centrally placed point at a constant crosshead speed (1.0 mm/min).

The time-force deformation data from testing the femora and tibiae in three-point bending were monitored using the JCL 6000 Chromatography Data System (Jones Chromatography Ltd., Littleton, CO, USA), which was interfaced with the Instron through an IBM AT-compatible personal com-

puter. Sample run time was 3 minutes, at a sampling rate of five signals per second. Calibration of the Instron signal was performed using known weights of 1.0 and 2.0 kg. Data were analyzed by transforming the millivolt signal output into kg force.

Biomechanical three-point bending allows a number of whole bone properties to be determined for both femora and tibiae. These parameters included bioyield, the force at which there occurs the first damage to the bone tissue, expressed in Newtons (N); and peak force, the maximum force applied during the bending procedure (N), in the femora; and maximum bending stress (σ), a normalized, force value that takes into consideration bone size (N/mm^2)[26] in tibiae. This latter parameter was calculated using the formula described by Ortoff and Oxlund:[26]

$$\sigma = \frac{8 \times \text{Maximum Bending Load (L} - 1)\ D}{\pi(D^4 - d^4)}$$

where L is the distance between the supporting points (13 mm), and D and d are the outer and inner diameters of the bone (mm).

Bone mineralization

The deposition of ^{45}Ca in the femur, an endpoint index of ^{45}Ca intestinal absorption,[27] was determined from the right femur immediately after sacrificing the rat. The mineral content of femora and tibiae were determined using the right femur and tibia. Femur and tibia bone samples were dried (100° C, 72 hr), and then ashed (550° C, 24 hr). The bone ash was then solubilized in 3 mL, 4 mol/L HCl and aliquots taken for radioactivity and mineral content analyses. Femora were analyzed for ^{45}Ca radioactivity, ^{40}Ca and P, whereas tibiae were analyzed for Ca and Mg content. Aliquots of solubilized bone ash were diluted with 0.5% $LaCl_3$ for calcium and magnesium analyses by atomic absorption spectrophotometry. Solubilized bone ash, diluted with deionized water, was used for phosphorus determination by the colorimetric method of Chen et al.[28]

Statistical analyses

All data are expressed as mean ± SEM. The significance of all treatment differences was determined by one-way analysis of variance (ANOVA; SPSS Inc., Chicago, IL, USA). Where differences did exist, the Student-Newman-Keuls multiple range test was used to identify the sources of the differences at a $P < 0.05$ level of significance. Two-way ANOVA was used to detect interactions between animal strain, dietary calcium intake, and protein source (MANOVA, SPSS). Linear regression and correlation coefficients were calculated by the method of least squares (SPSS).

Results

There were no significant differences in final body weight, feed intake, or feed efficiency ratio (FER) between SHR and WKY rats fed the same diet (*Table 1*). Both SHR and WKY rats fed the 0.05% Ca diets exhibited a lower final body weight ($P \leq 0.05$), feed intake ($P \leq 0.05$), and FER ($P < 0.01$) compared to animals fed the 0.5% and 2.0% Ca diets. Dietary protein was observed to affect animal growth characteristics at the 2.0% dietary Ca level only; soy fed animals exhibited a lower ($P \leq 0.05$) feed intake and FER, which corresponded to a lower body weight gain than observed in those fed casein. This observation was common for both SHR and WKY animals.

Plasma minerals are presented in *Table 2*. For both SHR and WKY rats, dietary protein source was not observed to have an effect on plasma minerals in animals fed the high and medium calcium levels. Plasma total Ca was decreased ($P < 0.01$) in animals fed the 0.05% Ca casein diet only; conversely, plasma P was increased ($P < 0.05$) in these animals, compared with counterparts fed the 0.05% Ca soy diet. Plasma Mg was not affected by dietary treatment. These observations were common to both SHR and WKY animals.

Intestinal ^{40}Ca content was not different between SHR and WKY animals fed similar diets (*Figure 1a*). Ileal ^{40}Ca content was not affected by dietary protein source, but was positively correlated with dietary calcium intake ($r = 0.845$, $P < 0.01$). Intestinal ^{45}Ca specific activities were similar within the respective

Table 1 Body weight gain and food intake of experimental animals*

	Initial body wt.† (g)		Final body wt.‡ (g)		Feed intake§ (g)		Feed efficiency ratio¶	
Diet	SHR	WKY	SHR	WKY	SHR	WKY	SHR	WKY
2.0% Ca								
Casein	116 ± 2[a]	125 ± 4[a]	284 ± 6[a]	309 ± 11[a]	863 ± 38[a]	838 ± 10[a]	0.132 ± 0.015[ab]	0.157 ± 0.019[ab]
Soy	110 ± 4[a]	118 ± 2[a]	262 ± 7[ab]	257 ± 8[ab]	793 ± 25[ab]	750 ± 14[b]	0.146 ± 0.015[ab]	0.102 ± 0.013[bc]‖
0.5% Ca								
Casein	116 ± 4[a]	119 ± 5[a]	307 ± 9[a]	259 ± 8[ab]	982 ± 15[a]	844 ± 19[a]	0.173 ± 0.015[a]	0.180 ± 0.008[a]
Soy	108 ± 3[a]	108 ± 6[a]	282 ± 5[a]	252 ± 14[b]	888 ± 19[a]	850 ± 27[a]	0.177 ± 0.007[a]	0.184 ± 0.016[a]
0.05% Ca								
Casein	119 ± 6[a]	125 ± 4[a]	239 ± 13[b]	249 ± 9[b]	730 ± 11[b]	693 ± 13[b]	0.104 ± 0.014[b]	0.092 ± 0.016[c]
Soy	118 ± 6[a]	122 ± 5[a]	260 ± 9[b]	253 ± 9[b]	800 ± 22[b]	718 ± 15[b]	0.120 ± 0.010[ab]	0.111 ± 0.012[bc]

*Data are expressed as mean ± SEM; SHR, Spontaneously hypertensive rats ($n = 36$); WKY, Wistar Kyoto rats ($n = 36$).
†5 weeks of age.
‡14 weeks of age.
§Cumulative intake from 5–14 weeks of age.
¶Feed efficiency ratio = feed intake (g)/body weight gained (g).
[a,b,c]Means sharing the same letter within a column are not significantly different at ($P < 0.05$).
‖Significant ($P < 0.05$) difference between animal strains for individual treatment.

Table 2 Effect of dietary protein source on plasma minerals of rats fed different levels of calcium*

Diet	Ca		Mg		P	
	SHR	WKY	SHR	WKY	SHR	WKY
			(mg/dL)			
2.0% Ca						
Casein	11.3 ± 0.3[a]	10.4 ± 1.4[a]	2.0 ± 0.2[a]	2.1 ± 0.4[a]	8.8 ± 0.3[b]	8.9 ± 0.3[b]
Soy	10.1 ± 1.1[a]	10.3 ± 0.5[a]	2.2 ± 0.5[a]	2.1 ± 0.1[a]	9.6 ± 0.8[b]	8.6 ± 0.4[b]
0.5% Ca						
Casein	9.7 ± 0.4[a]	9.4 ± 0.7[a]	1.9 ± 0.1[a]	2.0 ± 0.2[a]	9.3 ± 0.5[b]	9.0 ± 0.4[b]
Soy	10.6 ± 0.6[a]	9.9 ± 0.4[a]	2.1 ± 0.3[a]	2.5 ± 0.5[a]	8.7 ± 0.7[b]	8.4 ± 0.5[b]
0.05% Ca						
Casein	5.9 ± 0.7[b]	6.6 ± 0.6[b]	1.8 ± 0.2[a]	2.4 ± 0.2[a]	11.8 ± 0.7[a]	12.5 ± 1.3[a]
Soy	10.3 ± 0.5[a]	9.8 ± 0.6[a]	2.4 ± 0.2[a]	2.4 ± 0.1[a]	8.6 ± 0.8[b]	8.6 ± 0.9[b]

*Data are expressed as mean ± SEM: SHR, Spontaneously hypertensive rats (n = 36); WKY, Wistar Kyoto rats (n = 36) at 14 weeks of age.

[a,b]Means sharing the same letter within a column are not significantly different at ($P < 0.05$). There were no animal strain differences between treatment means in rows.

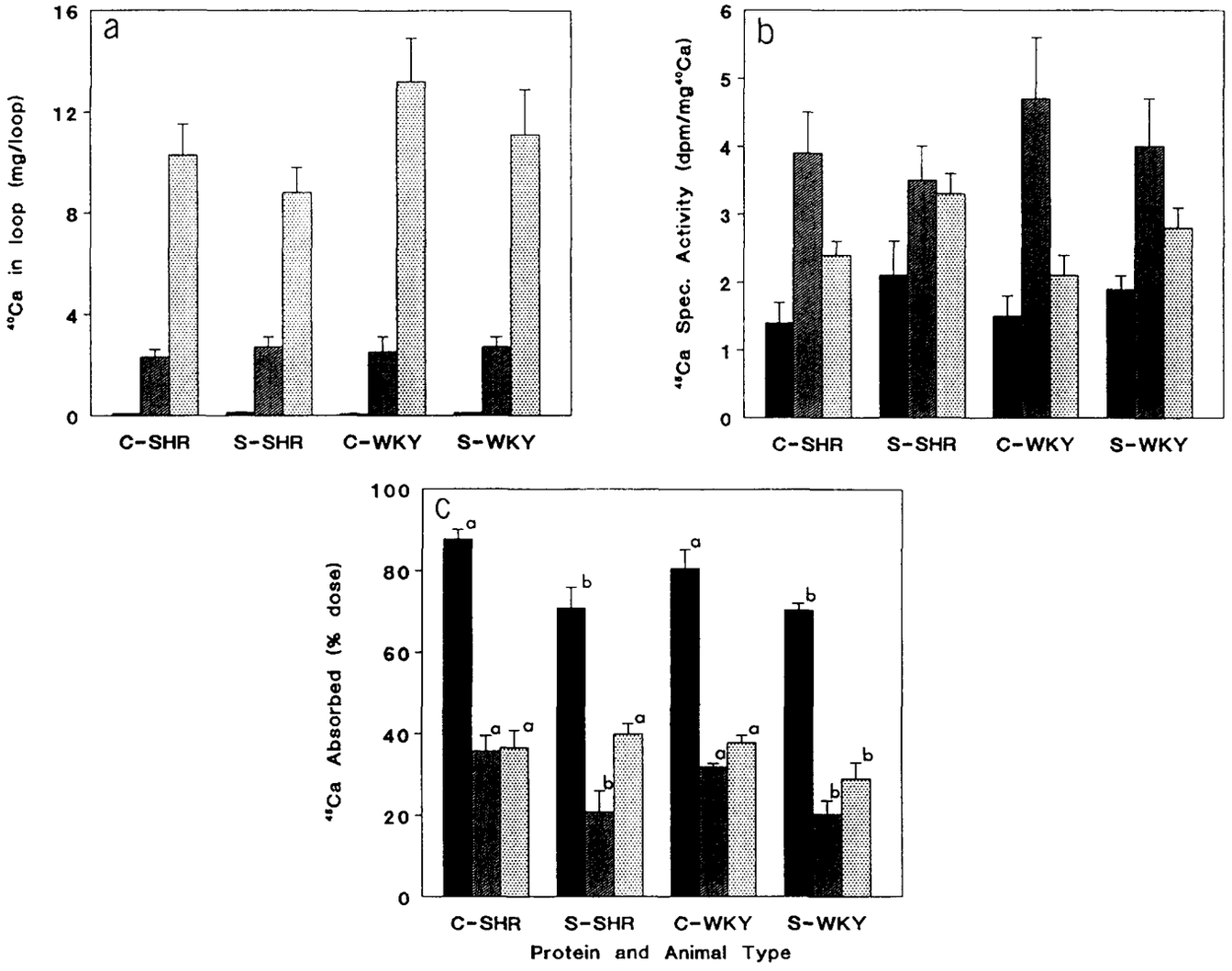

Figure 1 Effect of dietary calcium intake and protein source on calcium content and absorption from the ligated ileal loop of spontaneously hypertensive (SHR) and control Wistar-Kyoto (WKY) rats fed casein (C) and soy protein isolate (S) diets containing 0.05% ■, 0.5% ■, 2.0% ▨ calcium. (a) ^{40}Ca content of ligated ileal loop, actual values for 0.05% Ca fed animals were C-SHR = 0.08 ± 0.01, S-SHR = 0.14 ± 0.02, C-WKY = 0.07 ± 0.01 and S-WKY = 0.11 ± 0.01 (mg/loop); (b) ^{45}Ca specific activity of the ligated ileal loop; (c) ^{45}Ca absorbed from the ligated ileal loop.

[a,b]Significant ($P < 0.05$) difference between protein treatment means.

dietary calcium treatment levels, thus indicating that differences in ileal calcium absorption could not be attributed to different isotopic dosage levels (*Figure 1b*). There was no difference in the in situ paracellular ^{45}Ca absorption between SHR and WKY rats fed the same diet (*Figure 1c*). Intestinal ^{45}Ca absorption was, however, lower ($P < 0.05$) in soy-fed animals than casein-fed counterparts at both the medium and low dietary calcium levels. Animals fed the 0.05% calcium level exhibited a greater absorption efficiency of the ^{45}Ca dose when compared with counterparts fed the 0.5% and 2.0% calcium levels. This result corresponded to a greater ($P < 0.05$) ^{45}Ca specific activity of femora in the 0.05% Ca-fed animals compared with counterparts fed the 0.5% and 2.0% calcium diets (*Figure 2*). The lower ($P < 0.05$) amount of ^{45}Ca deposited to the bone in animals fed the 2.0% calcium diets may have indicated a lower efficiency in deposition of the absorbed calcium to bone by these animals.

Femur mineralization is summarized in *Table 3*. Femur ash weight and calcium content were decreased ($P < 0.05$) in both SHR and WKY animals fed 0.05% calcium compared with counterparts fed 0.5% and 2.0% calcium levels. SHR animals had a greater ($P < 0.05$) femur ash weight and calcium content than WKY counterparts at both the 2.0% and 0.5% dietary calcium levels. Soy fed animals exhibited a lower ($P < 0.05$) femur calcium content than those fed casein at the 2.0% and 0.5% calcium levels. Femoral calcium content was correlated with dietary calcium intake ($r = 0.771$, $P < 0.001$). Thus, significant interactions were found to exist between calcium intake and animal strain ($F(2,61) = 7.33$, $P \leq 0.001$) and protein source ($F(2,61) = 9.43$ $P < 0.001$), respectively, for femur calcification. The femur Ca:P ratio was decreased ($P < 0.05$) in animals fed the 0.05% calcium diet when compared with those fed the 2.0% and 0.5% calcium diets. There were no animal strain or protein source effects on femur Ca:P ratio. These differences were confirmed in the tibiae of respective animals. In particular, the greater ($P < 0.05$) amount of Ca observed

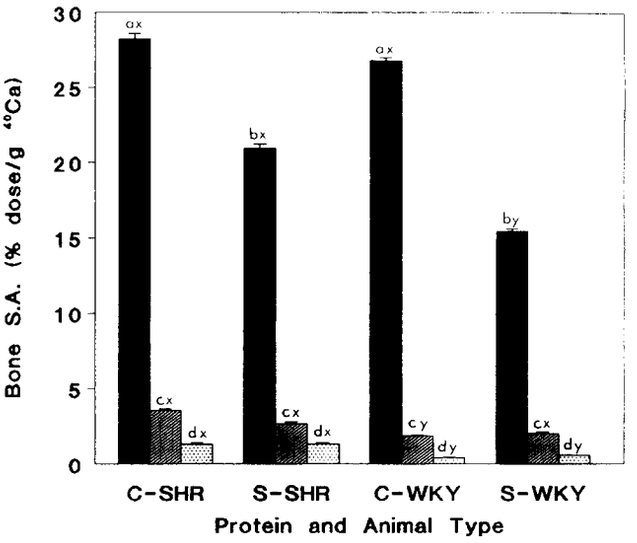

Figure 2 Femur ^{45}Ca specific activities in spontaneously hypertensive (SHR) and control Wistar-Kyoto (WKY) rats fed casein (C) and soy protein isolate (S) diets containing 0.05% ■, 0.5% ▩, 2.0% ▯ calcium. Actual values for 2.0% Ca fed animals were C-SHR = 1.29 ± 0.10, S-SHR = 1.32 ± 0.08, C-WKY = 0.41 ± 0.04 and S-WKY = 0.58 ± 0.03 (% dose/g ^{40}Ca).
[a,b,c]Significant ($P < 0.05$) difference between treatment means.
[x,y]Significant ($P < 0.05$) difference between animal strains.

in the femora of soy-fed Ca-deficient animals compared with casein-fed counterparts was also seen in the tibiae (soy range = 27–30 mg/bone; casein range = 19–23 mg/bone) of these animals, thereby indicating that the effect was common to other bone tissue.

Femur bone length and bone dry weight were not different in SHR and WKY animals fed the same diet (*Table 4*). Bone length was decreased ($P < 0.05$) in only those animals fed the 0.05% calcium casein diet. Bone dry weight was decreased ($P < 0.05$) in animals fed the 0.05% calcium diets compared with those fed the 0.5% and 2.0% calcium diets.

Femoral bioyield and peak force values were de-

Table 3 Femur mineral composition of rats fed experimental diets*

Diet	Ash wt. (g/bone)		Ca (mg/bone)		Ca/P ratio	
	SHR	WKY	SHR	WKY	SHR	WKY
2.0% Ca						
Casein	0.247 ± 0.006[a]	0.219 ± 0.009[a]†	99.60 ± 2.98[a]	86.25 ± 4.02[a]†	2.22 ± 0.05[a]	2.00 ± 0.20[a]
Soy	0.226 ± 0.008[a]	0.196 ± 0.007[a]†	88.10 ± 4.70[ab]	71.55 ± 5.01[b]†	2.06 ± 0.19[a]	1.91 ± 0.06[a]
0.5% Ca						
Casein	0.251 ± 0.011[a]	0.184 ± 0.006[a]†	93.09 ± 4.39[a]	64.50 ± 2.71[b]	2.17 ± 0.07[a]	2.11 ± 0.03[a]
Soy	0.218 ± 0.008[ab]	0.173 ± 0.011[ab]†	79.72 ± 0.83[b]	68.62 ± 2.36[b]	2.15 ± 0.03[a]	2.19 ± 0.04[a]
0.05% Ca						
Casein	0.067 ± 0.015[d]	0.056 ± 0.006[d]	27.75 ± 3.18[d]	27.98 ± 2.31[d]	1.36 ± 0.05[b]	1.44 ± 0.04[b]
Soy	0.095 ± 0.009[c]	0.108 ± 0.005[c]	39.00 ± 3.68[c]	43.11 ± 3.01[c]	1.39 ± 0.04[b]	1.38 ± 0.05[b]

*Data are expressed as mean ± SEM; SHR, Spontaneously hypertensive rats ($n = 36$); WKY, Wistar Kyoto rats ($n = 36$) at 14 weeks of age.
[a,b,c,d]Means sharing the same letter within a column are not significantly different at ($P < 0.05$).
†Significant ($P < 0.05$) difference between animal strains for individual treatment.

Table 4 Femur physical parameters of rats fed experimental diets*

Diet	Bone length (mm)		Bone dry wt. (g)	
	SHR	WKY	SHR	WKY
2.0% Ca				
Casein	30.39 ± 0.44[a]	30.10 ± 0.25[a]	0.400 ± 0.011[a]	0.360 ± 0.014[a]
Soy	29.98 ± 0.49[a]	29.54 ± 0.54[a]	0.365 ± 0.013[a]	0.331 ± 0.012[ab]
0.5% Ca				
Casein	32.00 ± 0.58[a]	30.78 ± 0.66[a]	0.383 ± 0.016[a]	0.296 ± 0.010[b]†
Soy	31.84 ± 0.04[a]	29.56 ± 0.59[a]	0.342 ± 0.011[a]	0.270 ± 0.014[bc]†
0.05% Ca				
Casein	26.96 ± 1.36[b]	24.24 ± 1.82[b]†	0.167 ± 0.026[c]	0.144 ± 0.008[d]
Soy	29.20 ± 0.25[a]	29.61 ± 0.35[a]	0.211 ± 0.010[b]	0.235 ± 0.007[c]

*Data are expressed as mean ± SEM; SHR, Spontaneously hypertensive rats (n = 36); WKY, Wistar Kyoto rats (n = 36) at 14 weeks of age.
[a,b,c,d]Means sharing the same letter within a column are not significantly different at ($P < 0.05$).
†Significant ($P < 0.05$) difference between animal strains for individual treatment.

creased ($P < 0.05$) in SHR and WKY fed the 0.05% Ca diets compared with counterparts fed 0.5% and 2.0% Ca (*Figure 3*). These parameters were strongly correlated with the decreased bone mineralization observed in both SHR and WKY rats ($r = 0.915$, $P < 0.001$ and $r = 0.923$, $P < 0.001$, respectively). An analysis of pooled data from all rats indicated that significant interactions between calcium intake and protein source existed with femur bioyield ($F(2,49) = 8.13$, $P < 0.01$) and peak force ($F(2,49) = 17.47$, $P < 0.01$).

Varying Ca intake was also shown to influence tibia Mg content and maximum bending stress (σ). SHR fed 2.0% Ca diets had lower ($P < 0.05$) tibia Mg content (range = 1.52–1.56 mg/bone), compared with WKY (2.3–2.4 mg/bone) counterparts, as well as the SHR (2.10–2.17 mg/bone) and WKY (2.16–2.24 mg/bone) animals fed the 0.5% Ca diets. Ca-deficient SHR and WKY similarly exhibited low tibia Mg contents (range = 1.42–1.58 mg/bone). A significant interaction between Ca intake and animal strain ($F(2,54) = 13.17$, $P < 0.01$) existed for tibia Mg content. The susceptibility to tibia fracture injury was shown to be greater in Ca-deficient animals (σ range = 10–34 N/mm^2) compared with Ca replete counterparts (56.2–79.2 N/mm^2) regardless of animal strain or dietary protein source. Of further interest was the greater susceptibility of Ca-deficient SHR to tibia fracture (σ range = 10.7–20.2 N/mm^2) than WKY (16.6–34.4 N/mm^2) counterparts, and the fact that both soy-fed SHR and WKY Ca-deficient animals exhibited a greater resistance to tibia injury (SHR σ 20.2 ± 2.9 N/mm^2; WKY σ 16.6 ± 1.2 N/mm^2).

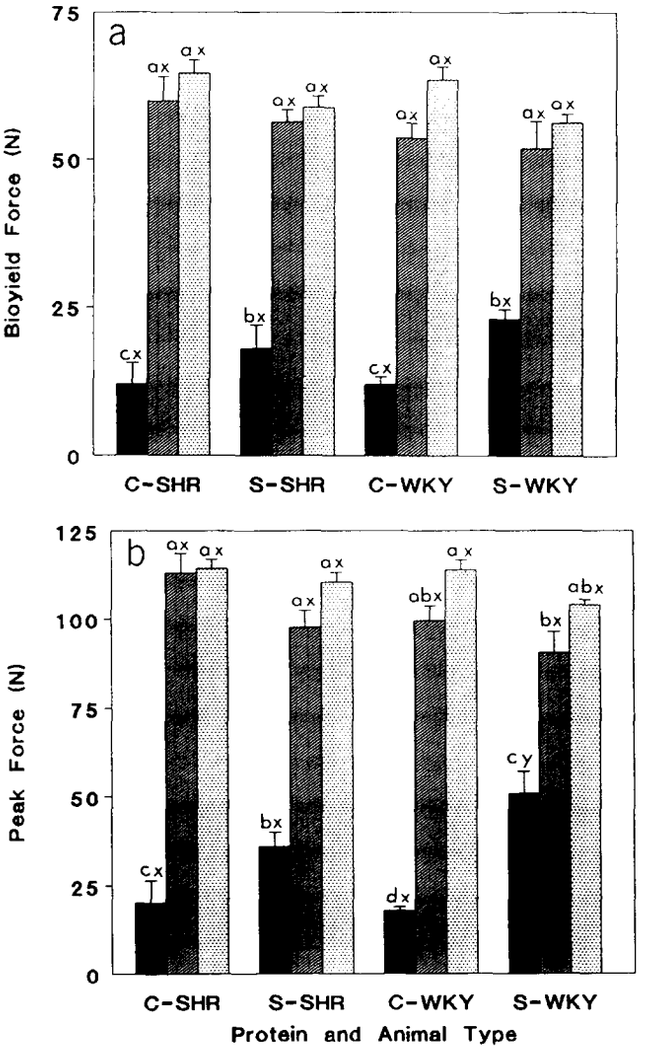

Figure 3 Femur biomechanical force parameters in spontaneously hypertensive (SHR) and control Wistar-Kyoto (WKY) rats fed casein (C) and soy protein isolate (S) diets containing 0.05% ■, 0.5% ■, 2.0% ▨ calcium. (a) femoral bioyield force; (b) femoral peak force.
[a,b,c,d]Significant ($P < 0.05$) difference between treatment means.
[x,y]Significant ($P < 0.05$) difference between animal strains.

Discussion

The lower plasma calcium and elevated levels of plasma phosphorus observed in casein-fed animals on a low calcium diet, but not in soy fed counterparts, suggests impaired parathyroid gland function in the mediation of bone resorption and renal phosphate excretion.[29] Hypocalcemia normally stimulates parathyroid hor-

mone (PTH) mediated bone resorption and vitamin D synthesis for increased intestinal calcium transport.[30,31] Because other investigators have cautioned against suggesting that diet-related bone loss can be solely attributable to changes in PTH levels,[32] further studies are required to characterize endocrine involvement as a possible mechanism for the variable protein effect on plasma calcium: phosphorus homeostasis.

Intestinal calcium absorption by SHR animals has been reported to be lower,[33] not significantly different,[18] as well as greater,[19,20,22] than in age-matched WKY controls. Adding to the controversy are the variety of techniques, namely in vitro tissue preparations,[18,22] in situ methodologies,[22] and balance studies[19–21,34] used to measure Ca absorption from the proximal intestine or the entire digestive tract in the SHR animals. In contrast, there is a paucity of information on Ca absorption from the distal small intestine in the SHR animals. In the present study, the disappearance of ^{45}Ca from the ligated ileal loop was not different between SHR and WKY animals with similar calcium intakes, thereby indicating that the difference in calcium metabolism between the genetically hypertensive SHR and normotensive WKY rats noted by others[21] was not due to a dissimilarity in ileal paracellular calcium absorption.

In animals fed low calcium diets, the low calcium content of the ileal loop combined with the high efficiency of ^{45}Ca disappearance are indicative of the increased net calcium flux, previously noted from in vitro and in vivo studies with the ileum of calcium-deficient rats.[11,35] These investigators also demonstrated that animals fed high calcium diets exhibited a decreased intestinal permeability in calcium flux from the mucosa to serosa as lumenal calcium concentration increased. Thus, the ^{45}Ca disappearance data obtained from the in situ ligated ileal loop technique used here, confirms that net ileal calcium flux in calcium replete animals is lower than that of calcium deficient counterparts, and independent of apparent sensitivities in overall calcium homeostasis. This observation is particularly important in calcium-deficient animals, because the distal small intestine has added physiological significance in regulating calcium homeostasis.[35]

Soy protein sources have been reported to result in a lower calcium bioavailability than animal proteins, namely casein, due to the activity of potential inhibitors such as fiber and phytates.[14] Differences in protein digestibility between casein and relatively structured soy proteins have been previously reported not to influence dietary calcium utilization in normal rats fed an adequate calcium level.[13] Alternatively, casein and casein phosphopeptides may promote and enhance calcium absorption from the lower small intestine by increasing the relative amount of bioavailable calcium.[15] Naito et al. consistently demonstrated a greater proportion of soluble calcium in the digesta of casein-fed rats compared with counterparts fed other dietary proteins or amino acid mixtures.[12,15,23,36,37] In the present study, the greater disappearance of ^{45}Ca dose from the ligated ileal loop of casein-fed animals at the medium and low dietary calcium levels suggests an enhanced calcium bioavailability from the distal small intestine, which was not apparent when calcium was fed in excess of requirements.

Potential differences in the distribution of extracellular calcium by SHR animals reported by others[21,38,39] may also have been observed in the present study. The overall greater ^{45}Ca activities in the bone of SHR, compared with WKY animals, supports the observations of other workers who suggested that SHR animals have an altered distribution of calcium between extracellular and intracellular compartments.[38,39] Although the acute bone ^{45}Ca specific activities paralleled the intestinal disappearance of ^{45}Ca, it should be noted that unlike the results of Sato et al.,[15] an associated increase in ^{45}Ca deposition to bone in casein fed animals was not observed. It is important to recognize that acute deposition of Ca radiolabel to bone may not accurately reflect the true effects of increased bioavailability on calcium utilization in bone metabolism, due to confounding effects of the exchange of ^{45}Ca for ^{40}Ca on the surface of bone, rather than deposition into osteoid tissue.[40]

Significant interactions between Ca intake with animal strain and with protein source for femur calcium content were found in this study. The enhanced femur ash weight and calcium content of SHR animals fed the 2.0% and 0.5% calcium diets are contrary to the suggestions of Izawa et al.,[17] who found that SHR are prone to the development of reduced bone density, similar to that seen in osteoporosis. The discrepancy in these observations may be due to the much greater age of the animals in the former study (26 weeks) as compared with our study (14 weeks). Signs of age-related osteoporosis would not be expected in growing animals whose skeleton shows very little bone remodelling. An interesting finding in the present study was the difference between the effects of casein and soy protein on relative bone calcification in animals fed medium and high calcium diets. The observed effect of protein source on bone mineralization was reversed in animals fed calcium-deficient diets, which coincided with the relative differences in plasma calcium and phosphorus noted in these animals. These results strongly suggest that casein and soy protein have differing effects on the mechanisms regulating calcium homeostasis in calcium-deficient rats. The fact that we could not differentiate a significant effect of dietary protein source on bone calcification in the WKY rat agrees with previous studies in normal rats.[13,16] Hence, significant interactions between calcium intake and protein source with bone mineralization were manifested only in the SHR model, pointing to an important affiliation between dietary[12,13] and physiological factors[17,21] in the overall regulation of Ca absorption and utilization in bones.

In this study bone biomechanical measurements were also used as indices of dietary calcium utilization from diets varying in calcium level and protein source in SHR and WKY rats. All three biomechanical tests were successful in showing an increased susceptibility

to bone injury in calcium-deficient animals. The peak force parameter, characterizing the maximum force required to break femora, was particularly sensitive in identifying a potential reduced susceptibility to injury in casein-fed animals. However, it is clear that the biomechanical parameters could not identify any differences associated with either animal strain or dietary protein source when calcium intake was adequate. That both femur and tibia calcium content were associated with dietary calcium intake, and furthermore, that specific bone biomechanical force parameters were correlated with the calcium content of these bones, corroborate previous findings that bone strength is dependent on dietary calcium intake[41,42] and bone mineral content.[43] The significant interactions obtained between dietary calcium intake and protein source, with femoral and tibia biomechanical force parameters, is a further indication that the bioavailability of dietary mineral salts may have a role in regulating the hardness and rigidity characteristics of bones. This observation however, does not appear to hold true in calcium-deficient animals, whereby soy-fed animals consistently exhibited a decreased susceptibility to femoral and tibial injury. This effect was particularly noticeable in the SHR group of animals, which, together with the relative differences noted in both plasma and bone mineralization parameters, strengthens the conclusion that a difference exists in the calcium metabolism between SHR and WKY calcium-deficient rats fed soy protein.

Antagonistic mineral interactions occur with a dietary excess or deficiency of one particular mineral, such as in the high or low calcium diets used in the present study.[44] In particular, when calcium and magnesium are not balanced in a diet, respective bioavailabilities and utilization can be affected.[44–46] Indeed, a significant interaction between dietary calcium intake and animal strain was found to exist for tibia Mg content in the present study. This can be explained on the basis that Mg absorption declines with age in the SHR, but not in age-matched WKY counterparts.[47] Also, an increased Mg requirement has been indicated when high levels of calcium are fed.[44,48] The decreased bone Mg content observed in the SHR fed high calcium diets for a long period of time is likely the result of decreased intestinal Mg absorption in these animals.[47]

Our results with animals fed diets deficient in calcium support previous findings that showed that adaptation to a low calcium diet does not necessarily prevent disturbed bone mineralization and metabolism.[49] Similarly, the fact that bone calcification and biomechanical strength were not increased in animals fed the high calcium diets is additional evidence of homeostatic mechanisms controlling calcium and bone metabolism.[29] These same mechanisms may be at work in calcium supplemented osteoporotic patients whereby dietary calcium supplementation results in suppressed PTH secretion and bone remodelling, improved Ca balance, and protection of bone mass.[50]

In summary, the use of the SHR model demonstrated a potentially important interaction between the level of calcium intake and dietary protein source with bone mineralization and biomechanical strength parameters, which was not seen in WKY counterparts. This observation was made despite the fact that ileal paracellular calcium absorption was equivalent in both rat stains. The interaction between dietary calcium intake and protein source in SHR only, suggests that the SHR may have some value in evaluating the importance of specific dietary components to bone biomechanical and mineral composition parameters. Histological studies describing the contribution of unmineralized osteoid tissue are required to confirm the suggestion that biomechanical differences in bone are explained by differences in calcium intake. This work demonstrates that varying dietary calcium intake has a more pronounced effect on calcium utilization and may dominate potential differences due to animal strain or dietary protein sources.

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