Short Term Effects on Bone Quality Associated with Consumption of Soy Protein Isolate and Other Dietary Protein Sources in Rapidly Growing Female Rats

JIN-RAN CHEN,*,†,[1] ROHIT SINGHAL,*,† OXANA P. LAZARENKO,*,† XIAOLI LIU,*,† WILLIAM R. HOGUE,‡ THOMAS M. BADGER,†,§ AND MARTIN J. J. RONIS*,†,[1]

Department of Pharmacology and Toxicology, †Arkansas Children's Nutrition Center, ‡Department of Orthopedics, and §Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72202

Beneficial effects of soy protein consumption on bone quality have been reported. The effects of other dietary protein sources such as whey protein hydrolysate (WPH) and rice protein isolate (RPI) on bone growth have been less well examined. The current study compared effects of feeding soy protein isolate (SPI), WPH and RPI for 14 d on tibial bone mineral density (BMD) and bone mineral content (BMC) in intact and ovariectomized (OVX) rapidly growing female rats relative to animals fed casein (CAS). The effects of estrogenic status on responses to SPI were also explored. Tibial peripheral quantitative computerized tomography (pQCT) showed all three protein sources had positive effects on either BMD or BMC relative to CAS ($P < 0.05$), but SPI had greater effects in both intact and OVX female rats. SPI and E2 had positive effects on BMD and BMC in OVX rats ($P < 0.05$). However, trabecular BMD was lower in a SPI+E2 group compared to a CAS+E2 group. In OVX rats, SPI increased serum bone formation markers, and serum from SPI-fed rats stimulated osteoblastogenesis in *ex vivo*. SPI also suppressed the bone resorption marker RatLaps ($P < 0.05$). Both SPI and E2 increased alkaline phosphatase gene expression in bone, but only SPI decreased receptor activator of nuclear factor-κB ligand (RANKL) and estrogen receptor gene expression ($P < 0.05$). These data suggest beneficial bone effects of a soy diet in rapidly growing animals and the potential for early soy consumption to increase peak bone mass. Exp Biol Med 233:1348–1358, 2008

Key words: soy; estradiol; bone mineral density; dietary protein

Introduction

Osteoporosis, the most common chronic bone disease, is a major public health problem worldwide and is characterized by deterioration of skeletal architecture, resulting in increased bone fragility and risk of fractures. Although osteoporotic bone loss can be caused by variety of factors, such as nutrient deficiencies or chronic alcohol intake, sex steroid deficiency-induced bone loss, especially in women post-menopause, is considered primary osteoporosis and its consequences include seriously increased risk of morbidity and mortality as the result of hip fractures (1). The current standard treatments for prevention of osteoporosis include adequate calcium and vitamin D intake, hormone replacement therapy (HRT), and selective estrogen receptor modulators (SERMs) such as raloxifene. Recently, the Women's Health Initiative has advised that HRT not be regularly prescribed (2) due to undesired serious cardiovascular side effects which outweigh the evidence of benefit for osteoporotic fractures. Since osteoporosis is difficult to treat, early prevention of this disease is becoming of greater interest. In particular, building adequate bone during periods of rapid growth in early development is critical to increase peak bone mass and reduce the effects of bone loss later in life.

The potential of nutritional approaches for the prevention of multifactorial chronic diseases has been recognized (3). For example, soy foods and soy protein isolates are reported to prevent cardiovascular disease and also rescue bone loss in both adult humans and in animal models of osteoporosis (4–6). There are epidemiological studies of the elderly in Asian nations demonstrating that the incidence of hip fracture is much lower in China compared with that in Western populations (7). The reason for lower incidence of hip fracture in Asian countries may be explained by high consumption of soy products throughout life in comparison

This study was supported by ARS CRIS #6251-51000-005–03S.

[1] To whom correspondence should be addressed at Arkansas Children's Nutrition Center, Slot 512–20B, 1120 Marshall Street, Little Rock, AR 72202. E-mail: RonisMartinJ@uams.edu and chenjinran@uams.edu

Received February 22, 2008.
Accepted June 5, 2008.

DOI: 10.3181/0802-RM-63
1535-3702/08/23311-1348$15.00

to Western populations (8). Although the mechanisms of soy effects on bone are unclear, researchers have begun to examine the ability of soy foods consumed from a young age to reduce the risk of osteoporosis via attainment of increased peak bone mass (9).

The skeletal effects of soy have been attributed to potential estrogenic actions related to its high content of isoflavone phytoestrogens such as genistein and daidzein (10–12). These isoflavones may have both estrogenic and antiestrogenic activities in mammalian tissue and the preferential binding of isoflavones to estrogen receptor β suggests that they may act as SERMs (13). Soy has been selected by many postmenopausal women as a natural alternative to estrogen therapy (14). Experimental studies have demonstrated effects of genistein and daidzein on bone cells (15). However, the evidence for a positive effect of dietary isoflavone consumption on bone health is mixed (16). Although the majority of studies concerning soy effects on bone have focused on phytoestrogens, soy also contains many other phytochemicals, proteins and peptides (17) and the relative concentrations and forms of these dietary factors are significantly affected by processing of soy beans into different products. Further studies are required on skeletal effects of different soy food products consumed during different developmental windows.

In addition to published studies of effects of soy on bone, there are also literature reports suggesting beneficial skeletal effects associated with consumption of whey proteins isolated from cow's milk. A functional role for milk whey proteins has been described not only in regulation of bone remodeling, but also in protection against osteoporosis (18). Moreover, a recent study demonstrated significant increases in bone mineral density and improvement of bone metabolism in healthy young women consuming whey basic protein fractions (19). In contrast to a study of soy protein on bone recently conducted by Spence *et al*., where soy protein and soy isoflavones did not alter calcium metabolism (20), whey protein has been suggested to exert beneficial effects on bone through enhancement of calcium absorption thereby increasing bone mineral content (21). It is known that children who avoid drinking cow's milk have low dietary calcium intakes and poor bone health (22, 23), but the role of whey proteins in this phenomenon is unclear.

Unlike soy products or whey protein, rice products or rice protein isolates have not been examined for effects on bone health. Rice products are a major part of the diet in some Asian countries, and are consumed in greater quantities than soy. Rice products have been reported to provide health benefits in the prevention of cancer (24), inflammation (25), and reductions in serum cholesterol (26).

Since early consumption of different protein sources might have the potential to affect attainment of peak bone mass, the purpose of present study was to compare the short-term effects of consumption of three protein sources—soy protein isolate (SPI), whey protein hydrolysate (WPH) and rice protein isolate (RPI)—relative to a standard protein source casein (CAS) in AIN-93G based diets on bone density and mineralization in rapidly growing intact female rats. In addition, studies were conducted to investigate whether the effects of SPI feeding on bone mimic those of the classical estrogen 17β-estradiol and if the effects of SPI are affected by endogenous estrogen status in age-matched young ovariectomized rats.

Materials and Methods

Animal Care, Experiment Protocol, and Diets. *Experiment One.* Time-impregnated female Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) arrived on gestational day 4 and were individually housed in an Association for Assessment and Accreditation of Laboratory Animal Care-approved animal facility at the Arkansas Children's Hospital Research Institute with constant humidity and lights on from 06:00–18:00 hrs at 22°C. All animal procedures were approved by the Institutional Animal Care and Use Committee at UAMS. Pregnant rats were fed AIN-93G diets made with casein as the sole protein source (27) and their offspring weaned at post natal day 21 (PND21) and fed the same diet until PND55. In order to see whether endogenous sex hormones will modulate the effects of soy and other diets on bone in young rapidly growing animals, half the female offspring were ovariectomized (OVX). All animals (both intact and OVX) were immediately sub-divided into 8 groups ($n = 6$–8) and fed one of 4 pelleted AIN-93G diets made with casein (CAS), SPI, WPH or RPI proteins. The diets were made to be isonitrogenous and isocaloric and the calcium and phosphorus levels were adjusted to match the casein-based diet. The composition of each diet has been published previously (24, 28, 29). All diets were made according to the AIN-93G diet formula (27), except that corn oil replaced soy oil and the protein source was either CAS (Fonterra, Santa Rosa, CA), WPH 917 (Fonterra, Santa Rosa, CA), RPI made according to a proprietary method similar to that described by Morita *et al*. (30) (a gift from Riceland Foods, Inc., Stuttgart, AR) or SPI (Clinical blend 670, a gift from Solae, Du Pont, St. Louis, MO). The percentage of protein content of each diet was 20%. Amino acids were added to each diet to equalize the essential amino acids among diets. After 14 d of *ad libitum* feeding, the rats were sacrificed and blood, femur and tibia were collected. Formalin fixed tibia bones were scanned to determine and compare bone mineral density and bone mineral content from each group using peripheral quantitative computerized tomography (pQCT) as described below.

Experiment Two. Twenty-four female Sprague-Dawley rats on PND30 were fed pelleted AIN-93G diets made with either CAS or SPI from PND30 till PND69. On PND55, rats were OVX and both CAS and SPI groups were further divided into groups receiving polyethylene glycol vehicle or 17β-estradiol (E2). E2 was administered by Alzet

Table 1. Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) Primer Sequences

Gene	Forward primer	Reverse primer
RANKL	TGGGCCAAGATCTCTAACATGA	TCATGATGCCTGAAGCAAATG
ALP	TGAATCGGAACAACCTAGCTGA	TTCCACTAGCAAGAAGAAGCCTTT
Osteocalcin	AAGCCCAGCGACTCTGAGTCT	GCTCCAAGTCCATTGTTGAGGTA
ERα	TCTGACAATCGACGCCAGAAT	ACAGCACAGTAGCGAGTCTCCTT
ERβ	TGTGCCAGCCCTGTTACTAGTC	CATGACCAAACGCCGTAATG
18S	CCTGTAATTGGAATGAGTCCACTTT	ATACGCTATTGGAGCTGGAATTACC
GAPDH	TGAGGTGACCGCATCTTCTTG	TGGTAACCAGGCGTCCGATA

mini osmotic pump (model # 2004, AlzaCorp., CA) implanted subcutaneously. The final dose of E2 was 5 μg/kg/day. All animals were killed at PND69; blood and tibia bone tissues were collected.

In both experiments, rats were killed by injection with 100 mg Nebutol/kg body weight (Avent Laboratories), followed by decapitation, and the serum, femur and tibia bone, and bone marrow were collected. Formalin fixed tibia bones were scanned to determine and compare bone quality from each group using pQCT as described below.

Rat Tibia pQCT Scanning. pQCT scans were performed on individual bones (left tibia) from each rat. Scanning was done with a STRATEC XCT 960M unit (XCT Research SA, Norland Medical Systems, Fort Atkins, WI) specifically configured for small bone specimens. Software version 5.4 was used along with thresholds of 570 mg/cm^3 to distinguish cortical bone and 214 mg/cm^3 to distinguish trabecular from cortical and sub-cortical bone. *Ex vivo* pQCT analyses on each bone was conducted. Tibial bone mineral density (BMD) and bone mineral content (BMC) were automatically calculated by installed software with version 5.4 as mentioned above and color images generated. The CVs for these measurements were $<2\%$. The position for pQCT scanning was defined at a distance from the proximal tibia growth palate corresponding to about 7% of the total length of the tibia. Distance between each scanning was 1 mm; a total of 5 scans (5 slices) was carried out. Data were collected from the average of 3 continuous slices. pQCT data in Experiment 1 were an average of slice 1, 2, 3, and pQCT data in Experiment 2 were from an average of slice 2, 3, 4 where the largest changes in trabecular bone density were observed.

Serum Bone Turnover Markers. The serum bone formation marker alkaline phosphatase (ALP) was measured by a colorimetric assay using the time-dependent formation of p-nitrophenolate from para-nitrophenyl phosphate (PNPP) (31). The protocol used 5 μl of serum in a total volume of 1.5 ml containing 0.05 mmol/L PNPP, 2 mmol/L $MgCl_2$, and 10 mmol/L L-phenylalanine (to inhibit any circulating intestinal ALP activity). The serum bone formation marker osteocalcin and the serum bone resorption marker RatLaps were measured by Rat-MID™ Osteocalcin ELISA and RatLaps™ ELISA, respectively, from Nordic Bioscience Diagnostics A/S (Herlev Hovedgade 207, 2730 Herlev, Denmark).

Real-Time Reverse Transcription-Polymerase Chain Reaction. Tibial bone total RNA was extracted using TRI Reagent (MRC Inc., Cincinnati, OH) according to the manufacturer's recommendations followed by DNase digestion and column cleanup using QIAGEN mini columns. Reverse transcription was carried out using an iScript cDNA synthesis kit from Bio-Rad (Hercules, CA). Real-time RT-PCR was carried out using SYBR Green and an ABI 7000 sequence detection system (Applied Biosystems, Foster City, CA). Primers for rat receptor activator of nuclear factor-κB ligand (RANKL), alkaline phosphatase, osteocalcin, estrogen receptor alpha and beta were designed using Primer Express software 2.0.0 (Applied Biosystems). Their sequences are all listed in Table 1.

In *Ex Vivo* Osteoblast Differentiation Cell Culture. Bone marrow cells were harvested from intact casein diet control rats according to a method described previously (32). Cells were seeded at a density of 3×10^6 cells per well in six-well cell culture plates and 1×10^6 cells per well in 24-well cell culture plates in the presence of minimal essential medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT) and 1 mM ascorbyl-2-phosphate (Sigma-Aldrich, St. Louis, MO), 4 mM L-glutamine, and 100 U/ml each of penicillin and streptomycin (Sigma-Aldrich). This condition is known as osteogenic medium and is able to drive osteoblast differentiation and used as positive control in this study. Cells were cultured with 7.5% FBS without osteogenic medium as negative control. Cells were also cultured in 5% FBS plus either 2.5% 0.45 μm filtered serum from SPI-fed intact female rats or 2.5% 0.45 μm filtered serum from CAS-fed intact female rats. All cell cultures were continued for 10 days and every 3 days half of culture medium was replaced by freshly made medium. At the end of the experiment, alkaline phosphatase staining was performed in 24-well plates according to a method described by the manufacturer (Sigma-Aldrich). Cell RNA was isolated and real-time RT-PCR was performed as described above.

Data and Statistical Analyses. Data were expressed as mean ± SD. One-way analysis of variance (ANOVA) was utilized to analyze diet effects and two-way ANOVA for analysis of SPI/CAS+E2 interactions followed by Student-Newman-Keuls post hoc analysis for multiple

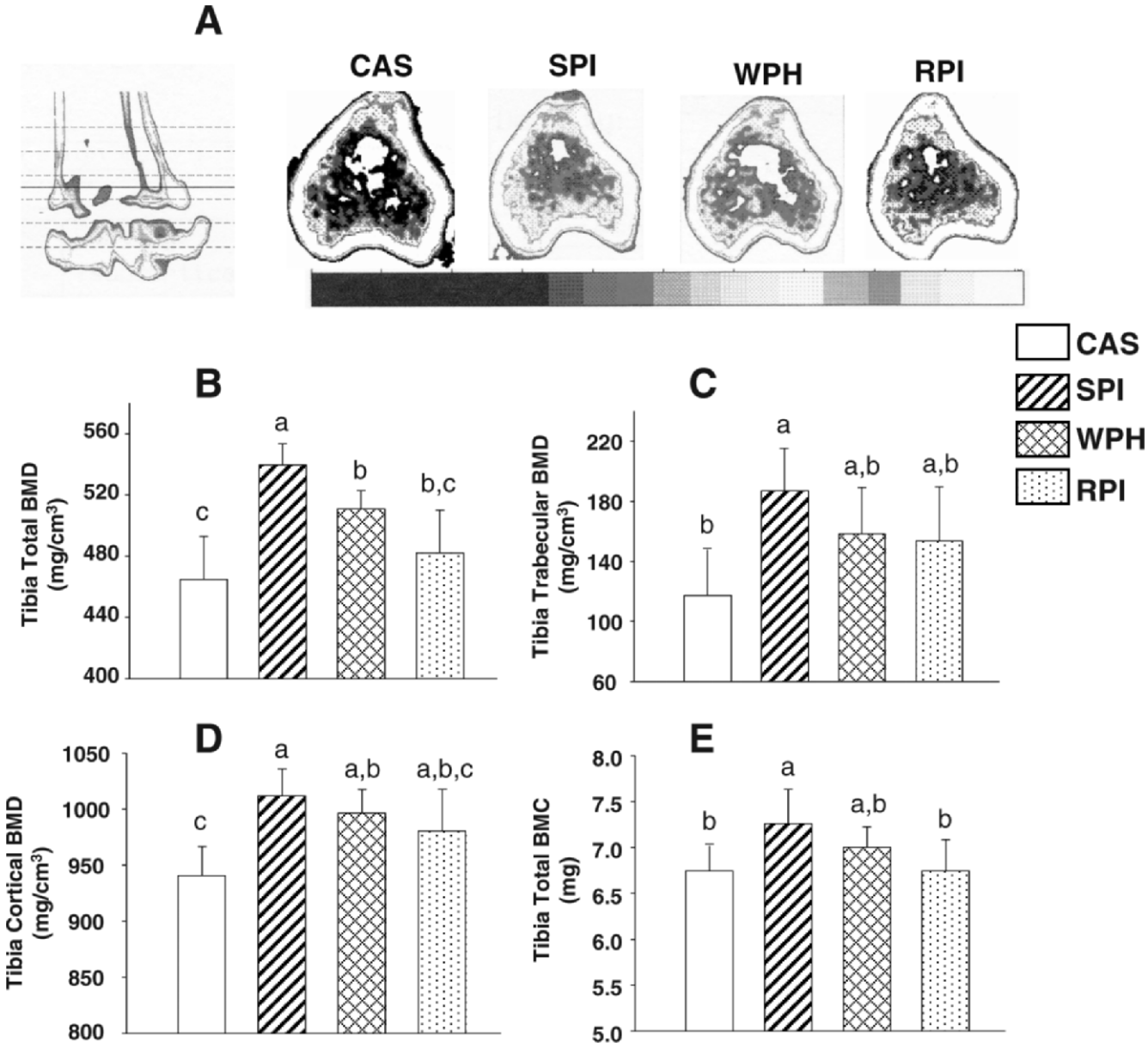

Figure 1. Effects of CAS, SPI, WPH and RPI diets on bone parameters in intact growing female rats (PND55). Rats were fed AIN-93G diets *ad libitum* made with CAS, SPI, WPH or RPI as the sole protein source for 14 d. A, Formalin fixed tibia from each group were scanned by pQCT. Representative pictures of slice 4 from five consecutive slices of a pQCT scan of rat tibia *ex vivo* from the different diet treatment groups are shown. Color changes from white to blue, yellow, red, to gray and black represent decreases in bone density, except white color in the center of each picture showing no tissue content (see detail in Materials and Methods). B, Comparison of total BMD among diet groups. C, Tibial trabecular BMD from each diet group. D, Tibial cortical BMD from each diet group. E, Tibial total BMC from each diet group. Open bars, CAS; diagonal bars, SPI; hatch bars, WPH; and dot bars, RPI. Data are expressed as mean ± standard deviation ($n = 6$/group). Means with different letters differ significantly from each other at $P < 0.05$, a > b > c as determined by one-way ANOVA followed by Student-Newman-Keuls post hoc analysis for multiple pairwise comparisons. A color version of the figure is available in the online journal.

pair-wise comparisons between treatment groups. Values were considered statistically significant at $P < 0.05$.

Results

Effects of SPI, WPH and RPI on Intact Rat Tibial Bone Mineral Density (BMD) and Content (BMC). Intact growing female rats were fed AIN-93G diets which were made with CAS SPI, WPH and RPI as the sole protein source described in the Materials and Methods section for 14 d. No diet effects were observed on either food intake or body weight gain (data not shown). Upon euthanasia, tissues were collected and formalin fixed. Left tibia was scanned using pQCT. Representative position matched pQCT slices from tibia of rats fed different dietary proteins are shown in Figure 1A. Total, trabecular and cortical BMD and total BMC of the tibia were all increased ($P < 0.05$) in rats fed with SPI compared with rats fed CAS (Fig. 1B–E). When the WPH-fed animal group was

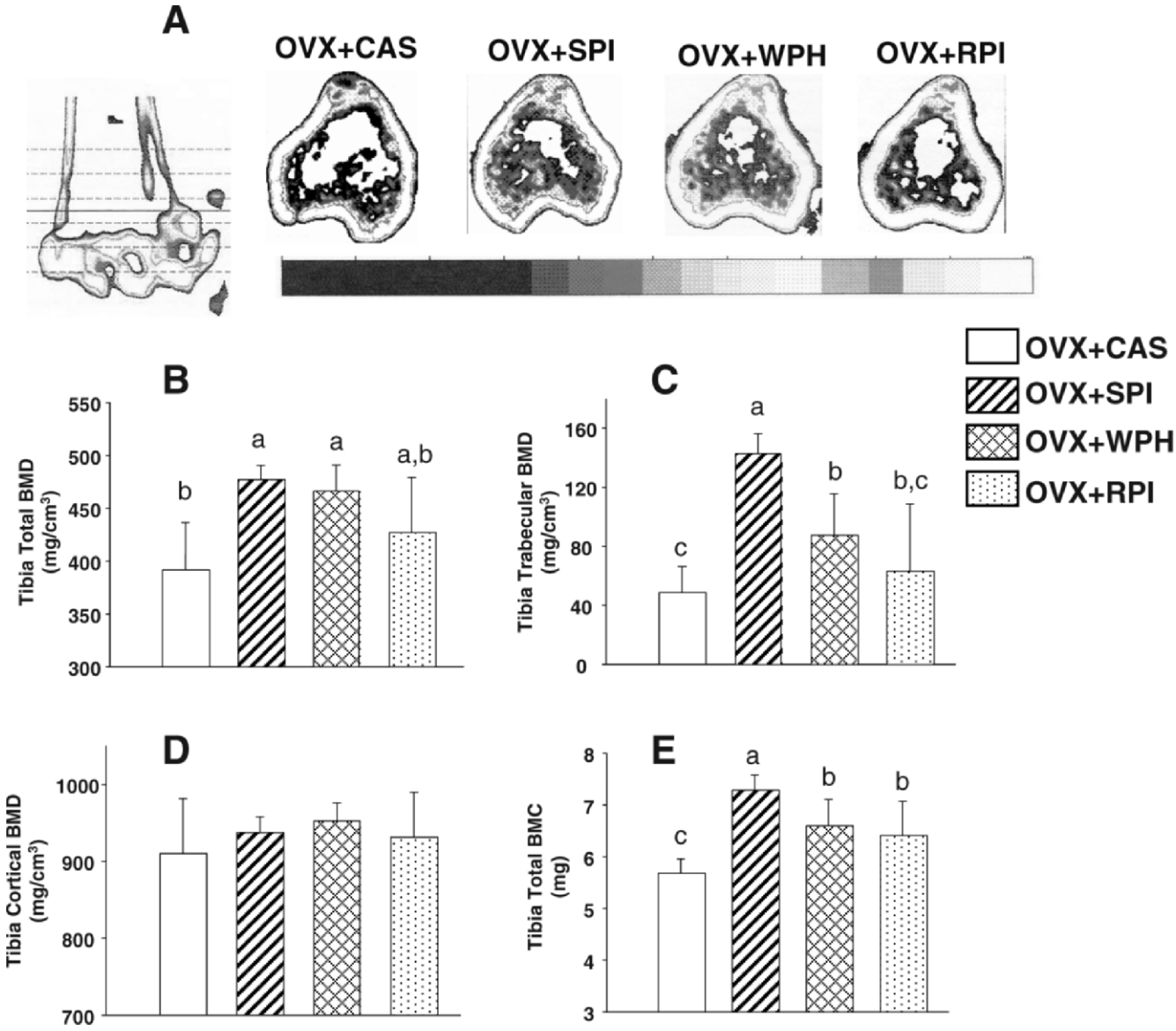

Figure 2. Effects of CAS, SPI, WPH and RPI diets on ovariectomized female tibial BMD and BMC. Female rats (PND55) were ovariectomized and fed AIN-93G diets *ad libitum* containing CAS, SPI, WPH and RPI immediately after surgery for 14 d. A, Tibia bones were taken under euthanasia from each group and were scanned by pQCT. Representative pictures of slice 4 from five consecutive slices of a pQCT scan of rat tibia *ex vivo* from the different diet treatment groups are shown. Color changes from white to blue, yellow, red, to gray and black represent decreases in bone density (see detail in Materials and Methods section). B, Comparison of total BMD among diet groups. C, Tibial trabecular BMD from each diet group. D, Tibial cortical BMD from each diet group. E, Tibial total BMC from each diet group. Open bars, CAS; diagonal bars, SPI; hatch bars, WPH; and dot bars, RPI. Data are expressed as mean ± standard deviation ($n = 6$/group). Means with different letters differ significantly from each other at $P < 0.05$, a > b > c as determined by one-way ANOVA followed by Student-Newman-Keuls post hoc analysis for multiple pairwise comparisons. A color version of the figure is available in the online journal.

compared with CAS-fed animals, we found total tibial BMD was increased in WPH-fed animal group ($P < 0.05$). We did not find any differences in pQCT parameters presented in Figure 1 when RPI-fed animal group was compared with CAS control group. Moreover, in Figure 1B, when all four groups were compared together, SPI had significantly greater effect on total BMD ($P < 0.05$).

Effects of SPI, WPH and RPI on OVX Rat Tibial Bone Mineral Density (BMD) and Content (BMC). In order to ascertain whether there were effects of the three diets on bone in the absence of endogenous estrogens, we ovariectomized female rats belonging to the same litters as the intact rats. OVX rats were given the same four different diets as given to the intact animals for 14 days. Tibial pQCT scans were also performed on formalin fixed samples from the OVX female rats. Figure 2A illustrates representative scans from position marched slices of one tibia of each diet group. It should be noted that the pQCT data from Figure 1 (intact rats) and Figure 2 (OVX rats) were from same scan slice (slice 2) and therefore they are comparable. Indeed,

when CAS intact data from Figure 1C is compared to OVX+CAS data from Figure 2C, there was a significant decrease in trabecular BMD and total BMC in OVX control group relative to the intact control group ($P < 0.05$). These data indicated dramatic bone loss after OVX even after as short an interval as 14 d in young female growing rats. Total tibial BMD, trabecular BMD and total tibial BMC from the OVX SPI group were all higher than those rats from the OVX CAS group ($P < 0.05$) (Fig. 2B, C, E). Similarly, total tibial BMD, trabecular BMD and total tibial BMC were significantly higher in the OVX WPH group compared to OVX CAS group. There were no significant differences in tibial BMD when OVX RPI was compared to OVX CAS. However, in the OVX RPI group, significant difference was found in total BMC when compared to the OVX CAS group ($P < 0.05$) (Fig. 2E). Interestingly, the OVX SPI group had significant higher trabecular BMD and total BMC compared to either OVX WPH or OVX RPI groups ($P < 0.05$) (Fig. 2C, E), and these values were not statistically different from those of intact female rats fed SPI (Fig. 1C, E). These data suggest that SPI is superior to WPH and RPI in restoring OVX-associated bone loss. There were no significant differences in tibial cortical BMD among all four OVX rat groups fed different diets. This may be due to a short period of both OVX and diet consumption.

Interactions Between SPI-Feeding and Estradiol on Bone in the OVX Female. To further investigate the interactions of SPI and endogenous estrogens on bone, the effects of SPI-feeding were compared to those of the classical estrogen 17β-estradiol (E2) and to the effects of a combination of SPI-feeding and E2 replacement in OVX female rats. Plasma E2 values in the OVX+E2 group were 21.8 ± 4 pg/ml (within the normal physiological range) compared to 3.8 ± 1 pg/ml in the OVX group. No significant effects of SPI feeding were observed on plasma E2 in either the OVX or E2 replaced groups (data not shown). pQCT data are shown in Figure 3. Consistent with our data in Experiment 1, in the OVX SPI-fed group, total tibial BMD, trabecular BMD and total tibial BMC were all significantly higher than in the OVX CAS group ($P < 0.05$). Equally consistent to many previous findings that E2 can restore bone loss after OVX, all pQCT measures were increased in the OVX CAS+E2 group when compared to the OVX CAS group (Fig. 3A–D) ($P < 0.05$). However, E2 replacement to physiological levels had greater effects on tibial BMD and BMC compared to SPI feeding in OVX rats ($P < 0.05$). Interestingly, with the combination of SPI and E2 replacement, SPI-feeding reduced E2 effects on tibia trabecular BMD and tibia total BMC (Fig. 3B, D) ($P < 0.05$). We found a similar pattern of effects of CAS, SPI, CAS+E2 and SPI+E2 on total tibial BMD, but there was no statistical difference between OVX CAS+E2 and OVX SPI+E2 (Fig. 3A). Replacement of E2 was also able to increase tibial cortical BMD, consistent with data in Experiment 1. However, there was no effect of SPI on cortical BMD when compared to the OVX CAS control group (Fig. 3C).

Effects of SPI and E2 on Serum Bone Turnover Markers and Gene Expression in OVX Female Rats. Serum was collected at sacrifice from all 4 groups of OVX rats in Experiment 2. Serum osteocalcin and ALP activity were used as bone formation markers and serum RatLaps level as a bone resorption marker and were measured using ELISA. The data are shown in Table 2. Osteocalcin and ALP were increased ($P < 0.05$) and RatLaps was decreased ($P < 0.05$) in OVX animals fed with SPI diets as compared to OVX CAS-fed rats. In sharp contrast, serum bone formation markers and serum bone resorption markers were both decreased in the OVX CAS+E2 ($P < 0.05$) compared with the OVX CAS control group. Surprisingly, serum osteocalcin in the OVX SPI+E2 group was significantly higher than OVX CAS+E2 group, but were significantly lower than the OVX group fed SPI alone. Likewise, serum ALP values in the OVX SPI+E2 group were intermediate between those of the OVX SPI-fed group and the OVX CAS+E2 group. Clearly, SPI diet and E2 supplementation to OVX rats showed opposite effects on serum bone formation markers. On the other hand, the bone resorption marker RatLaps in the OVX SPI+E2 group was significantly higher than the OVX CAS+E2 group (Table 2). In total RNA extracted from tibial bone, (Fig. 4), ALP mRNA was significantly increased in the OVX SPI group compared to the OVX CAS group ($P < 0.05$), and osteocalcin mRNA tended to increase in the SPI group compared to CAS group but was not statistically significant (Fig. 4). Interestingly, in the OVX CAS+E2 group, both ALP mRNA and osteocalcin mRNA were increased in bone compared to the OVX CAS group (Fig. 4). The antagonistic effects of the combination of SPI diet +E2 on bone formation genes in OVX rats were consistent with what we observed on serum bone formation markers (Table 2, Fig. 4). RANKL mRNA expression in tibial bone was decreased in both the OVX SPI diet group and the OVX SPI+E2 group as compared to the OVX CAS group. There was no effect of E2 itself on RANKL, and RANKL mRNA expression in the OVX SPI+E2 group was higher than that observed in OVX SPI diet group ($P < 0.05$) (Fig. 4).

Effects of SPI and E2 on Estrogen Receptor Gene Expression in Bone Tissue. To elucidate if altered estrogen receptor expression might be involved in the antagonistic effects of SPI and E2 on bone in OVX female rats, we measured the two estrogen receptor forms ER alpha and ER beta (ERα, ERβ) mRNA expression by real time RT-PCR. Whereas CAS+E2 marginally suppressed ERα and had no statistical effects on ERβ gene expression, expression of both receptors was dramatically down-regulated by feeding SPI diet ($P < 0.05$). When SPI diet and E2 treatment was combined in OVX rats, ERα and ERβ gene expression levels were in between the OVX SPI and the OVX CAS+E2 group but remained lower than the OVX CAS group ($P < 0.05$) (Fig. 5). This pattern of effects

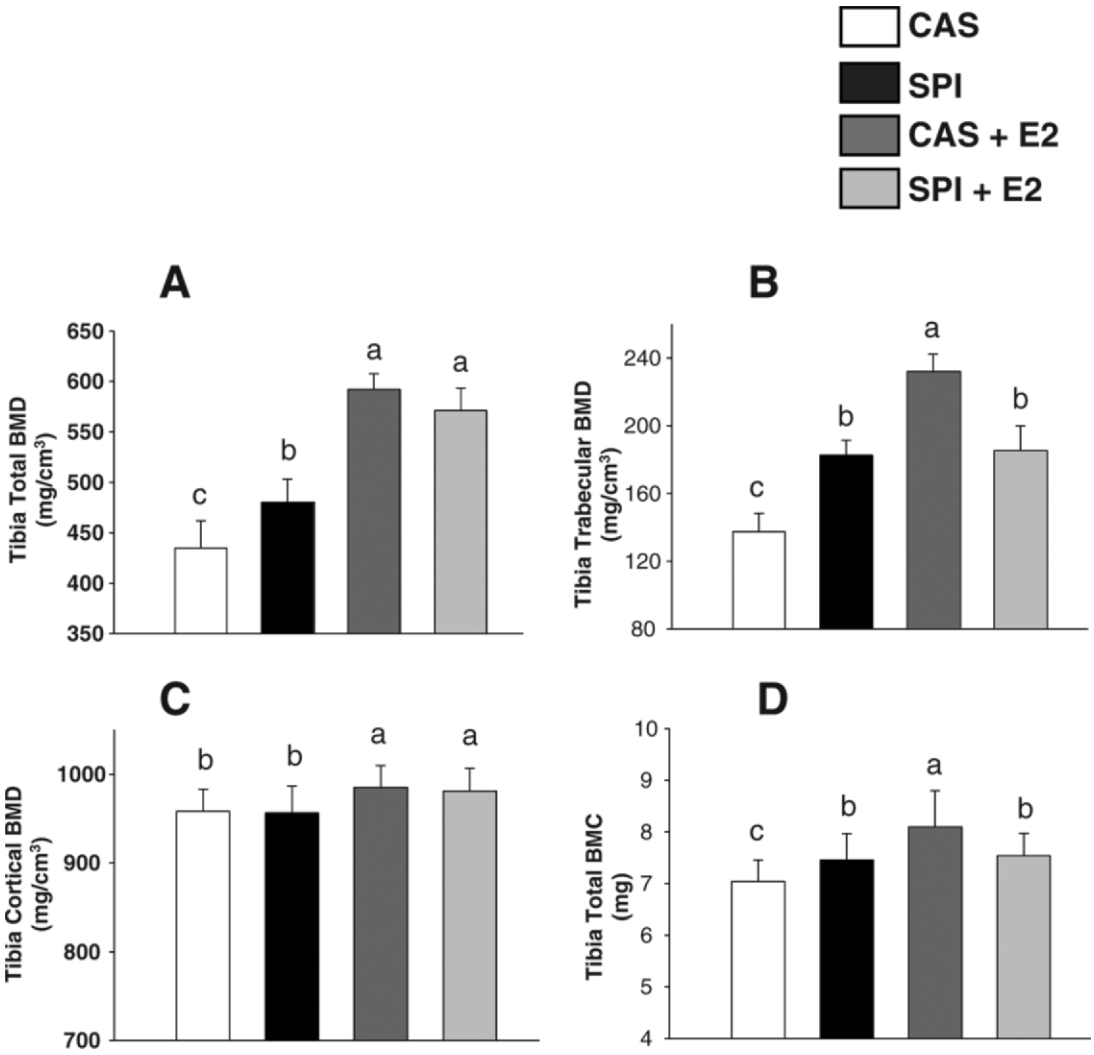

Figure 3. Effects of SPI, E2 and E2+SPI diet on ovariectomized female tibia BMD and BMC. Female rats PND55 were ovariectomized and given CAS or SPI diets, E2 by subcutaneous mini pump or E2 combined with SPI diets for 14 d. Tibial bones were taken under euthanasia from each group and were scanned by pQCT. A, Comparison of total BMD among different groups. B, Tibia trabecular BMD from each treatment group. C, Tibia cortical BMD from each treatment group. D, Tibia total BMC from each treatment group. White bars, CAS; black bars, SPI; dark gray bars, E2; and light gray bars, E2+SPI. Data are expressed as mean ± standard deviation ($n = 6$/group). Means with different letters differ significantly from each other at $P < 0.05$, a > b > c as determined by two-way ANOVA followed by Student-Newman-Keuls post hoc analysis for multiple pairwise comparisons.

of SPI+E2 was similar to the effect of SPI+E2 on RANKL mRNA expression in OVX rats (Fig. 4).

Serum from Soy-Fed Intact Rat Was Able to Stimulate Osteoblast Differentiation in *Ex Vivo* Cell Culture. Our *in vivo* data indicated that SPI appears to stimulate osteoblastic bone formation in addition to suppressing osteoclastic bone resorption. This supports our hypothesis that the effect of soy diet on bone in the rapidly growing young female is anabolic. To further test our hypothesis, we aspirated bone marrow cells from femur of intact CAS diet control rats and treated cells with serum from either SPI-fed intact rats or CAS-fed intact rats. Osteoblast differentiation was evaluated by alkaline phosphatase cell staining as well as alkaline phosphatase and osteoclacin gene expression. Compared to serum from CAS-fed control intact rats, 2.5% serum from SPI-fed intact rats dramatically promoted osteoblast differentiation as indicated in Figure 6A by alkaline phosphatase staining after 10 days of culture. Increased gene expression of alkaline phosphatase and osteocalcin from cells treated with 2.5% serum from SPI-fed intact rats was observed ($P < 0.05$) (Fig. 6B). Surprisingly, 2.5% serum from SPI-fed intact rats had greater effects on osteoblast differentiation than the positive control, an osteogenic medium standardized for osteoblast differentiation in vitro cell culture (32).

Discussion

Three dietary protein sources (soy, milk and rice) have been recently receiving attention for their potential roles in prevention of chronic diseases, such as cardiovascular

Table 2. Serum Bone Formation and Bone Resorption Markers[a]

	CAS	SPI	CAS+E2	SPI+E2
Osteocalcin (pg/ml)	490.2 ± 92.0[c]	662.1 ± 81.0[a]	370.6 ± 47.1[d]	532.2 ± 123.6[b]
ALP(μU/min)	0.227 ± 0.03[b]	0.277 ± 0.04[a]	0.099 ± 0.04[c]	0.177 ± 0.08[b]
RatLaps (ng/ml)	40.6 ± 2.5[a]	33.0 ± 3.5[b]	24.5 ± 3.0[c]	28.0 ± 4.7[b]

[a] All serum bone formation and bone resorption markers were measured by ELISA. Data are expressed as mean ± standard deviation ($n = 6$/group). Mean ± SEM with different letters differ significantly from each other at $P < 0.05$, a > b > c > d as determined by two-way ANOVA followed by Student-Newman-Keuls post hoc analysis for multiple pairwise comparisons.

disease, osteoporosis and cancer (4, 5, 24). With regard to bone effects, a number of studies have been conducted in female experimental animals and post-menopausal women using soy products or soy-associated isoflavones as an alternative to classic estrogens to restore bone loss stemming from sex steroid deficiency (33). However, there is more limited data on the effects of soy or milk and rice proteins on bone in young rapidly growing animals. The majority of adult bone is believed to be built in this rapid growth phase in both humans and animals. In our present study, we provide data which clearly indicate that consumption of dietary SPI promotes bone growth in both intact animals and in the absence of estrogens. It has been suggested that the effects of SPI on bone are due mainly to the isoflavone phytoestrogens genistein, and daidzein associated with the protein and the daidzein metabolite equol (34). We have previously reported that rats fed SPI have total plasma isoflavone concentrations in the 1–3 μM range and make large amounts of equol (35). However, we hypothesize that there are also other factors in SPI that may be equally important in bone health effects. There are over 100 phytochemicals associated with SPI and additional bioactive peptide and protein components (29, 36). WPH had similar effects on bone as SPI, but of lesser magnitude. Since WPH does not have phytochemicals such as isoflavones associated with it, the mechanisms are likely to differ substantially from SPI and probably involve bioactive peptides derived by hydrolysis of the parent whey proteins, which promote cell proliferation and collagen synthesis by osteoblasts (37), and cystatin, which has been shown to suppresses osteoclast-mediated bone resorption (38). On the other hand, except for modest effects on mineral content in ovariectomized rats, RPI had no positive effects on bone mineral density. We previously reported that RPI has a completely different phytochemical profile to SPI despite having similar anti-cancer, insulin-sensitizing, anti-atherosclerotic and hypocholesterolemic effects similar to SPI (24, 29).

It has been well documented that estrogen replacement therapy initiated soon after menopause and continued indefinitely will protect against bone fracture (39). However, this long-term hormone therapy is associated with serious adverse effects, including increased risk of cardiovascular disease and breast cancer (2). This has lead women to search for alternatives to traditional therapy (40). Early

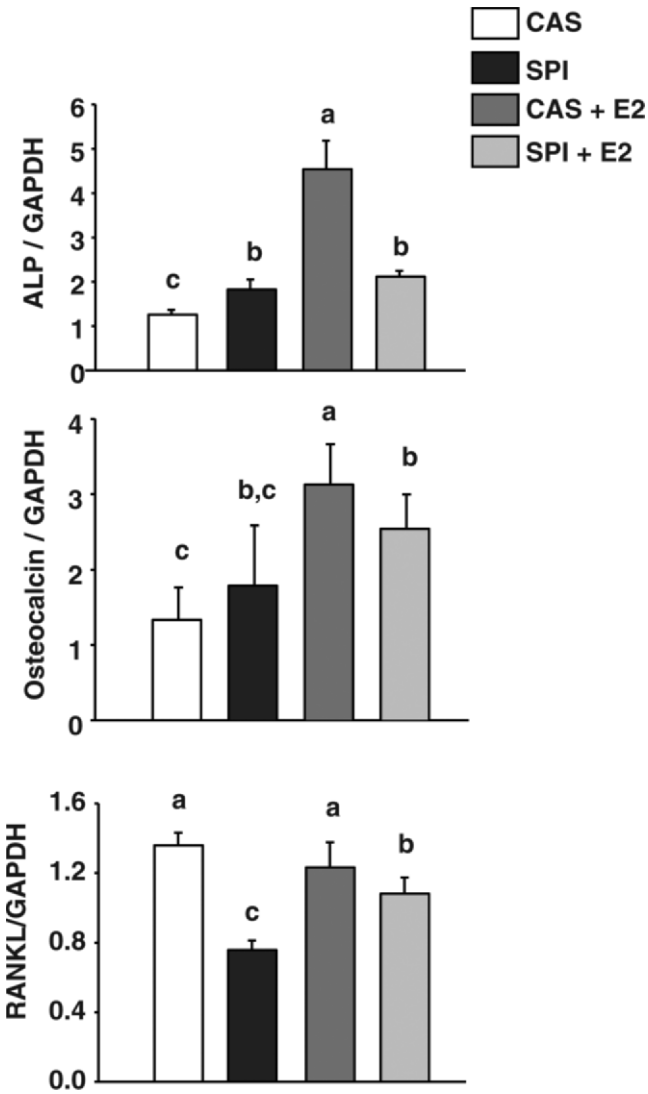

Figure 4. Effects of SPI, E2 and E2+SPI diet on bone forming and resorption responsive gene expression in ovariectomized female tibial bone. Tibial bone RNA was isolated and real-time RT-PCR was used to analyze each individual mRNA expression from each group. A, Alkaline phosphatase (ALP) mRNA. B, Osteocalcin mRNA. C, RANKL mRNA. Open bars, CAS; diagonal bars, SPI; hatch bars, WPH; and dot bars, RPI. Data are expressed as mean ± standard deviation ($n = 6$/group). All mRNA expression was normalized to the expression level of GAPDH mRNA. Means with different letters differ significantly from each other at $P < 0.05$, a > b > c as determined by two-way ANOVA followed by Student-Newman-Keuls post hoc analysis for multiple pairwise comparisons.

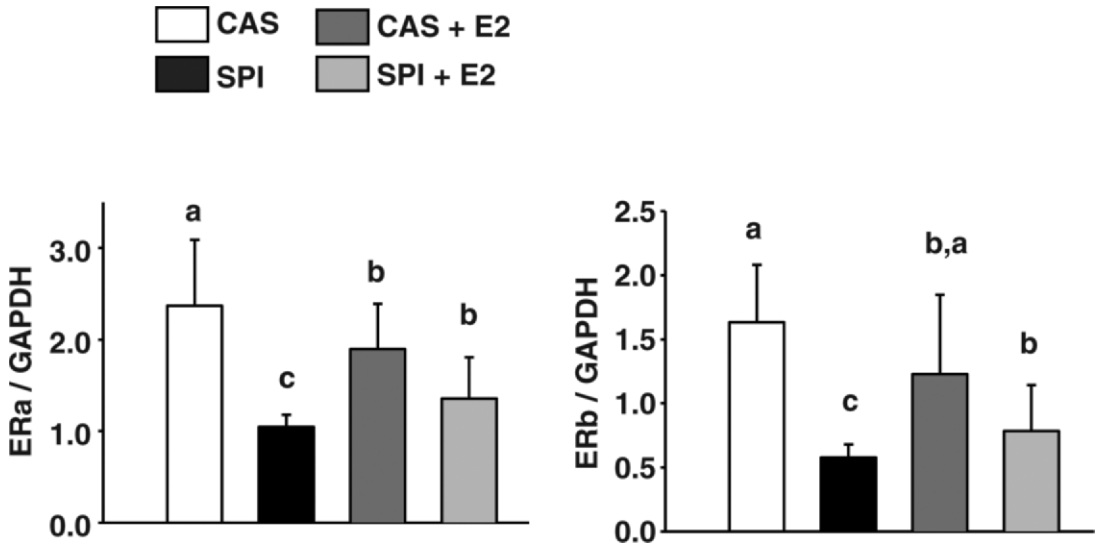

Figure 5. Effects of SPI, E2 and E2+SPI diet on estrogen receptor gene expression in ovariectomized female tibial bone. Tibia bone RNA was isolated and real-time RT-PCR was used to analyze each individual gene expression from each group. A, Estrogen receptor alpha mRNA. B, Estrogen receptor beta mRNA. Open bars, CAS; diagonal bars, SPI; hatch bars, WPH; and dot bars, RPI. Data are expressed as mean ± standard deviation ($n = 6$/group). All mRNA expressions were normalized to the expression level of GAPDH. Means with different letters differ significantly from each other at $P < 0.05$, a > b > c as determined by two-way ANOVA followed by Student-Newman-Keuls post hoc analysis for multiple pairwise comparisons.

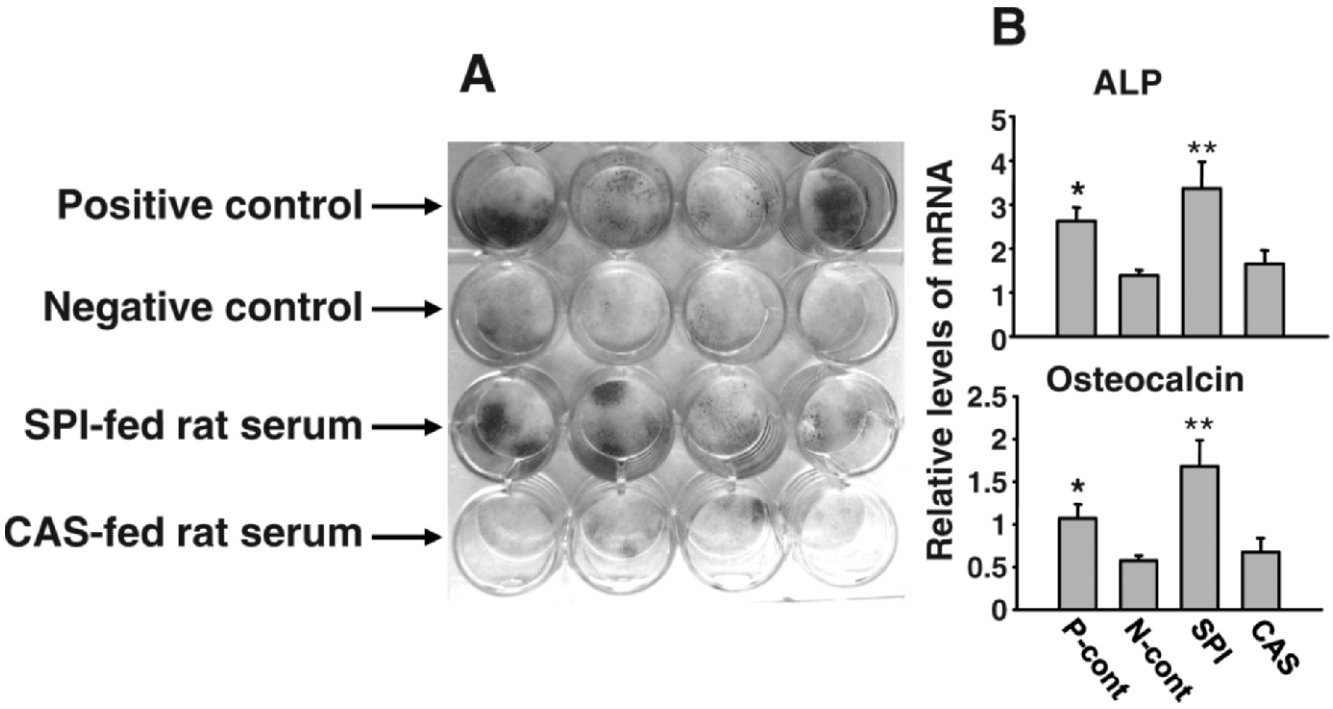

Figure 6. Serum from SPI-fed intact rats promotes bone marrow stromal osteoblast differentiation. Bone marrow cells were aspirated from CAS-fed intact control rat femur. Cells were seeded in to 6- and 24-well plates with cell density 3×10^6 and 1×10^6 cells per well respectively. Cells were cultured with 5% FBS plus 2.5% SPI-fed rat serum or 2.5% CAS-fed rat serum from 10 days. Every 3 days, half culture medium was replaced by new medium. Cells were cultured in α-MEM with10% FBS and 1 mM ascorbyl-2-phosphate (A2P) as positive control, and α-MEM with7.5% FBS without A2P as negative control. A, Pictures show stromal osteoblast differentiation in 24-well plates with quadruplicates. Cells were stained with alkaline phosphatase staining kids and pink color indicates positive. B, Cell RNA was extracted from cells cultured in 6-well plates, and ALP and osteoclacin gene expression were accessed by real-time PCR. Data represent mean ± standard deviation from triplicates and * $P < 0.05$ positive control versus negative control. ** $P < 0.05$ SPI versus CAS. A color version of the figure is available in the online journal.

consumption of soy products may have the potential to increase peak bone mass and may also prevent bone loss during the menopausal transition. Soy-associated isoflavones are estrogen-like substances structurally and functionally similar to 17β-estradiol (41). Depending on the tissue in which they act and the level of endogenous estrogens, isoflavones may exert either estrogenic or antiestrogenic effects (42). In addition soy products contain many other phytochemicals and the complex mixture in SPI may have very different properties than isolated isoflavones. In this report, we have for the first time compared the bone effects of endogenous 17β estradiol with SPI and the combination of E2 and SPI in ovariectomized female rats. Interestingly, treatment with a combination of SPI and E2 for a short period of time showed no additive, but actually antagonistic, effects on both bone mineral density and content. Serum bone formation and resorption markers were consistent with pQCT bone density data. In agreement with Tivesten *et al.*, we demonstrated that 17β-estrodiol treatment reduced serum markers of both bone resorption and bone formation indicating reduced bone turnover (43). In sharp contrast, soy protein isolate increased serum bone formation markers, but decreased bone resorption markers supporting the hypothesis that soy protein isolates influence the rodent bone in a significantly different manner than does estradiol. This was also supported by our in *ex vivo* osteoblast differentiation data. The ability of SPI to affect bone formation and osteoblast activity indicates that it possesses specific bone anabolic properties. The bioactive SPI components responsible for these effects remain to be identified. Moreover, to clarify the relationship between soy protein products, isoflavones and bone health (16), the effect of SPI and isoflavone feeding at each life stage should be further evaluated (33).

Another new finding in the current report is that gene expression is affected differently in bone by E2 and SPI treatment. Consistent with serum bone turnover marker data, SPI significantly up-regulated ALP gene expression, but dramatically down-regulated RANKL gene expression, indicating dual effects of SPI diet on both osteoblasts and osteoclasts or their precursors. The data from Figure 6 fully support increases in serum bone formation markers. The significance and molecular basis of down-regulated RANKL gene expression by SPI are under further investigation in our laboratory.

In our current report, we found that E2 markedly up-regulated both osteocalcin and ALP mRNA expression in the bone tissue, despite lowering levels of protein and activity in serum. These data are consistent with previous publications (44, 45). The origin of these discrepancies between serum protein level and bone specific gene expression remains unclear. This discrepancy between serum bone formation markers and mRNA levels in bone at sacrifice may be due to the fact that serum markers represent the sum of effects on bone formation during the entire period of this short-term experiment, whereas gene expression in bone represents a snapshot of the dynamic effects of E2 and diet on bone formation at the time of sacrifice and may represent the rebound in bone formation rates which are known to occur after longer periods of E2 exposure (46). Alternatively, the difference in gene expression and serum proteins may reflect post-transcriptional effects or inhibition of osteoclacin and ALP secretion during the acute phase of E2 treatment (32).

The gene expression changes in the OVX E2+SPI group suggest that E2 and SPI have antagonistic effects in bone tissue. Feeding SPI down-regulated expression of both estrogen receptors alpha and beta. In contrast, E2 had small or negligible effects on estrogen receptor expression. The effects of E2+SPI on expression of the two estrogen receptor mRNAs also showed antagonism similar to other findings in this report. These findings are also consistent with previously observed antagonistic effects of E2 and SPI in reproductive tissues (47, 48). Clearly potential estrogen receptor mediated effects of SPI in bone and modulation of SPI-effects by endogenous estrogens require further investigation.

In summary, we have compared the effect of SPI, WPH and RPI on bone in both intact and ovariectomized rapidly growing female rats and further examined the bone effects of SPI in relation to the effects of E2 alone or in combination. We found that SPI had superior anabolic effects to enhance bone mineral density and bone mineral content in intact young female rats and in treatment of OVX-induced osteopenia in female rat models more so than other protein sources. Moreover, we provided strong evidence that SPI exerts different effects on bone than E2 in the absence of endogenous estrogens and has antiestrogenic effects on bone in the presence of endogenous estrogens. Estrogen and SPI effects on bone clearly differ and further studies are needed to assess bone effects of SPI feeding in males and females during different developmental windows where endogenous estrogen concentrations and ER expression profiles differ.

We would like to thank the following people for their technical assistance: Brandy Yarberry, Janelle Johnson, Mark Robinette, Pam Treadaway, Mat Ferguson and Tammi Delari.

1. National Osteoporosis Foundation. http://www.nof.org (accessed July 6, 2003).
2. Writing Group for the Women's Health Initiative Investigators. Risks and benefits of estrogen plus progestin in healthy postmenopausal women. J Am Med Assoc 288:321–333, 2002.
3. Ordovas JM, Corella D. Nutritional genomics. Annu Rev Genomics Hum Genet 5:71–118, 2004.
4. Sacks FM. Dietary phytoestrogens to prevent cardiovascular disease: early promise unfulfilled. Circulation 111(4):385–387, 2005.
5. Setchell KD. Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. Am J Clin Nutr 68: 1333S–1346S, 1998.
6. Massey LK. Dietary animal and plant protein and human bone health: a whole foods approach. J Nutr 133(3):862S–865S, 2003.

7. Yan L, Zhou B, Prentice A, Wang X, Golden MH. Epidemiological study of hip fracture in Shenyang, People's Republic of China. Bone 24(2):151–155, 1999.
8. Lacey JM, Anderson JJ, Fujita T, Yoshimoto Y, Fukase M, Tsuchie S, Koch GG. Correlates of cortical bone mass among premenopausal and postmenopausal Japanese women. J Bone Miner Res 6(7):651–659, 1991.
9. Ginty F, Prentice A. Can osteoporosis be prevented with dietary strategies during adolescence? Br J Nutr 92(1):5–6, 2004.
10. Anderson JJ, Garner SC. Phytoestrogens and bone. Baillieres Clin Endocrinol Metab 12(4):543–557, 1998.
11. Gao YH, Yamaguchi M. Anabolic effect of daidzein on cortical bone in tissue culture: comparison with genistein effect. Mol Cell Biochem 194(1–2):93–97, 1999.
12. Picherit C, Coxam V, Bennetau-Pelissero C, Kati-Coulibaly S, Davicco MJ, Lebecque P, Barlet JP. Daidzein is more efficient than genistein in preventing ovariectomy-induced bone loss in rats. J Nutr 130(7):1675–1681, 2000.
13. Setchell KD. Soy isoflavones—benefits and risks from nature's selective estrogen receptor modulators (SERMs). J Am Coll Nutr 20(5 Suppl):354S–362S, 2001.
14. Kronenberg F, Fugh-Berman A. Complementary and alternative medicine for menopausal symptoms: a review of randomized, controlled trials. Ann Intern Med 137(10):805–813, 2002.
15. Barnes S. Soy isoflavones—phytoestrogens and what else? J Nutr 134(5):1225S–1228S, 2004.
16. Weaver CM, Cheong JM. Soy isoflavones and bone health: the relationship is still unclear. J Nutr 135(5):1243–1247, 2005.
17. Kennedy AR. The evidence for soybean products as cancer preventive agents. J Nutr 125(3 Suppl):733S–743S, 1995.
18. Toba Y, Takada Y, Yamamura J, Tanaka M, Matsuoka Y, Kawakami H, Itabashi A, Aoe S, Kumegawa M. Milk basic protein: a novel protective function of milk against osteoporosis. Bone 27(3):403–408, 2000.
19. Uenishi K, Ishida H, Toba Y, Aoe S, Itabashi A, Takada Y. Milk basic protein increases bone mineral density and improves bone metabolism in healthy young women. Osteoporos Int 18(3):385–390, 2007.
20. Spence LA, Lipscomb ER, Cadogan J, Martin B, Wastney ME, Peacock M, Weaver CM. The effect of soy protein and soy isoflavones on calcium metabolism in postmenopausal women: a randomized crossover study. Am J Clin Nutr 81(4):916–922, 2005.
21. Zhao Y, Martin BR, Wastney ME, Schollum L, Weaver CM. Acute versus chronic effects of whey proteins on calcium absorption in growing rats. Exp Biol Med (Maywood) 230(8):536–542, 2005.
22. Black RE, Williams SM, Jones IE, Goulding A. Children who avoid drinking cow milk have low dietary calcium intakes and poor bone health. Am J Clin Nutr 76(3):675–680, 2002.
23. Gao X, Wilde PE, Lichtenstein AH, Tucker KL. Meeting adequate intake for dietary calcium without dairy foods in adolescents aged 9 to 18 years (National Health and Nutrition Examination Survey 2001–2002). J Am Diet Assoc 106(11):1759–1765, 2006.
24. Yu S, Fang N, Li Q, Zhang J, Luo H, Ronis M, Badger TM. In vitro actions on human cancer cells and the liquid chromatography-mass spectrometry/mass spectrometry fingerprint of phytochemicals in rice protein isolate. J Agric Food Chem 54(12):4482–4492, 2006.
25. Akihisa T, Yasukawa K, Yamaura M, Ukiya M, Kimura Y, Shimizu N, Arai K. Triterpene alcohol and sterol ferulates from rice bran and their anti-inflammatory effects. J Agric Food Chem 48(6):2313–2319, 2000.
26. Sugano M, Tsuji E. Rice bran oil and cholesterol metabolism. J Nutr 127(3):521S–524S, 1997.
27. Reeves PG, Nielsen FH, Fahey GC Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 123(11):1939–1951, 1993.
28. Badger TM, Ronis MJ, Hakkak R. Developmental effects and health aspects of soy protein isolate, casein, and whey in male and female rats. Int J Toxicol 20(3):165–174, 2001.
29. Fang N, Yu S, Badger TM. Comprehensive phytochemical profile of soy protein isolate. J Agric Food Chem 52(12):4012–4020, 2004.
30. Morta T, Kiriyama S. Mass production method for rice protein isolate and nutritional evaluation. J Food Sci 58:1393–1397, 1993.
31. Lecka-Czernik B, Moerman EJ, Grant DF, Lehmann JM, Manolagas SC, Jilka RL. Divergent effects of selective peroxisome proliferator-activated receptor-gamma 2 ligands on adipocyte versus osteoblast differentiation. Endocrinology 143(6):2376–2384, 2002.
32. Chen JR, Haley RL, Hidestrand M, Shankar K, Liu X, Lumpkin CK, Simpson PM, Badger TM, Ronis MJ. Estradiol protects against ethanol-induced bone loss by inhibiting up-regulation of receptor activator of nuclear factor-kappaB ligand in osteoblasts. J Pharmacol Exp Ther 319(3):1182–1190, 2006.
33. Reinwald S, Weaver CM. Soy isoflavones and bone health: a double-edged sword? J Nat Prod 69(3):450–459, 2006.
34. Setchell KD, Clerici C, Lephart ED, Cole SJ, Heenan C, Castellani D, Wolfe BE, Nechemias-Zimmer L, Brown NM, Lund TD, Handa RJ, Heubi JE. S-equol, a potent ligand for estrogen receptor beta, is the exclusive enantiomeric form of the soy isoflavone metabolite produced by human intestinal bacterial flora. Am J Clin Nutr 81(5):1072–1079, 2005.
35. Gu L, House SE, Prior RL, Fang N, Ronis MJ, Clarkson TB, Wilson ME, Badger TM. Metabolic phenotype of isoflavones differ among female rats, pigs, monkeys, and women. J Nutr 136(5):1215–1221, 2006.
36. Kuipers BJ, Bakx EJ, Gruppen H. Functional region identification in proteins by accumulative-quantitative peptide mapping using RP-HPLC-MS. J Agric Food Chem 55(23):9337–9344, 2007.
37. Takada Y, Aoe S, Kumegawa M. Whey protein stimulated the proliferation and differentiation of osteoblastic MC3T3-E1 cells. Biochem Biophys Res Commun 223(2):445–449, 1996.
38. Matsuoka Y, Serizawa A, Yoshioka T, Yamamura J, Morita Y, Kawakami H, Toba Y, Takada Y, Kumegawa M. Cystatin C in milk basic protein (MBP) and its inhibitory effect on bone resorption in vitro. Biosci Biotechnol Biochem 66(12):2531–2536, 2002.
39. Balasch J. Sex steroids and bone: current perspectives. Hum Reprod Update 9(3):207–222, 2003.
40. Marcus R. Post-menopausal osteoporosis. Best Pract Res Clin Obstet Gynaecol 6(3):309–327, 2002.
41. Knight DC, Eden JA. A review of the clinical effects of phytoestrogens. Obstet Gynecol 87(5 Pt 2):897–904, 1996.
42. Mäkelä SI, Pylkkänen LH, Santti RS, Adlercreutz H. Dietary soybean may be antiestrogenic in male mice. J Nutr 125(3):437–445, 1995.
43. Tivesten A, Movérare-Skrtic S, Chagin A, Venken K, Salmon P, Vanderschueren D, Sävendahl L, Holmäng A, Ohlsson C. Additive protective effects of estrogen and androgen treatment on trabecular bone in ovariectomized rats. J Bone Miner Res 19(11):1833–1839, 2004.
44. Plant A, Tobias JH. Characterisation of the temporal sequence of osteoblast gene expression during estrogen-induced osteogenesis in female mice. J Cell Biochem 82(4):683–691, 2001.
45. Cai DJ, Zhao Y, Glasier J, Cullen D, Barnes S, Turner CH, Wastney M, Weaver CM. Comparative effect of soy protein, soy isoflavones, and 17 beta-estradiol on bone metabolism in adult ovariectomized rats. J Bone Miner Res 20(5):828–839, 2005.
46. Chow JW, Lean JM, Abe T, Chambers TJ. The anabolic effect of 17 beta-oestradiol on the trabecular bone of adult rats is suppressed by indomethacin. J Endocrinol 133(2):189–195, 1992.
47. Diel P, Hertrampf T, Seibel J, Laudenbach-Leschowsky U, Kolba S, Vollmer G. Combinatorial effects of the phytoestrogen genistein and of estradiol in uterus and liver of female Wistar rats. J Steroid Biochem Mol Biol 102(1–5):60–70, 2006.
48. Gallo D, Zannoni GF, Martinelli E, Ferlini C, Fabrizi M, Riva A, Morazzoni P, Bombardelli E, Scambia G. Estradiol and phytoestrogens differently influence the rodent postmenopausal mammary gland. Menopause 13(1):72–79, 2006.