Dietary Soy Isoflavones Inhibit Estrogen Effects in the Postmenopausal Breast

Charles E. Wood,¹ Thomas C. Register,¹ Adrian A. Franke,² Mary S. Anthony,² and J. Mark Cline¹

¹Department of Pathology/Section on Comparative Medicine, Wake Forest University School of Medicine, Winston-Salem, North Carolina and Cancer Research Center of Hawaii, Honolulu, Hawaii

Abstract

Soy isoflavones are promising dietary agents for prevention of breast cancer. Isoflavones bind estrogen receptors (ER) and may variably act as either estrogen agonists or antagonists depending on the estrogen environment. In this study, we used a postmenopausal primate model to evaluate interactive effects of dietary soy isoflavones and estrogen on risk markers for breast cancer. The experiment followed a randomized factorial design in which 31 ovariectomized adult female cynomolgus monkeys were divided into social groups of three to four animals each and rotated through eight different diets containing the human equivalent of 0, 60, 120, or 240 mg/d soy isoflavones with a dose of oral micronized 17β-estradiol (E₂) corresponding to either a low (0.09 mg/d) or a high (0.5 mg/d) postmenopausal estrogen environment. Treatment periods lasted 4 months with a 1-month washout period between diets. The highest isoflavone dose resulted in significantly lower breast proliferation and uterine size in the high-estrogen environment. These effects were accompanied by divergent changes in breast markers of ER activation in which pS2 expression was significantly lower and progesterone receptor expression was significantly higher following the 240 mg isoflavone dose. All isoflavone doses resulted in lower serum estrone and E₂ concentrations in the high-estrogen environment. In contrast, isoflavone treatment had no significant estrogen agonist effects and minimal antagonistic effects in the lower-estrogen environment. These findings show that in the presence of estrogen higher doses of dietary soy isoflavones may alter ER signaling and induce selective antagonistic effects in the breast. (Cancer Res 2006; 66(2): 1241-9)

Introduction

Many of the major risk factors for breast cancer relate to estrogen exposure (1). Estrogen-associated risk is most apparent in the postmenopausal period, when the majority of breast cancer cases occur. Numerous prospective studies indicate that postmenopausal women with high serum estrogens have at least a 2-fold increase in breast cancer risk (2, 3). Conversely, recent clinical trials indicate that treatment with antiestrogenic agents may prevent at least half of postmenopausal breast cancer cases (4, 5). Such agents include tamoxifen and raloxifene, which are classified as selective estrogen receptor modulators (SERM) for their ability to competitively inhibit ERs in the breast while providing selective estrogen agonist actions in other tissues.

Soy isoflavones are a class of estrogen-like compounds that have been characterized previously as natural SERMs (6). Recently, soy isoflavones have become widely used as a dietary supplement (7), particularly among postmenopausal women and breast cancer survivors seeking a safe natural alternative to estrogen therapy (8). Demographic and epidemiologic studies indicate that high dietary intake of soy isoflavones may lower breast cancer risk (9–12). Soy isoflavone effects in the breast are controversial, however, with evidence supporting both estrogen agonist and antagonist properties (13, 14). Isoflavones elicit clear estrogenic effects in low-estrogen rodent and cell culture models (15, 16) but may inhibit 17β-estradiol (E₂) effects in higher-estrogen systems (16–18), suggesting that relevant effects of dietary soy isoflavones may depend strongly on estrogen context.

To investigate this idea, we evaluated the interactive effects of dietary soy isoflavones and estrogen on risk markers for breast cancer in a postmenopausal primate model. The primary end point of this study was breast epithelial proliferation as determined by Ki-67 (MIB-1) immunostaining. Ki-67 expression is an important prognostic indicator in human breast cancer (19) and has been used extensively in our model to predict risk associated with hormonal agents (20, 21). Secondary end points included key markers of ER activity in the breast, serum estrogens, and measures of estrogen exposure in other reproductive tissues. We hypothesized that increasing doses of soy isoflavones would have estrogen agonist effects within a low-estrogen context and estrogen antagonist effects in a higher-estrogen postmenopausal environment.

Subjects and Methods

Animal subjects. The animal subjects in this project were surgically menopausal cynomolgus macaques (Macaca fascicularis). These animals have several distinct reproductive similarities to women, including a nonseasonal 28-day menstrual cycle, cyclic ovarian hormone profile, and natural menopause (22, 23). Unlike women, menopause occurs in macaques very late in their life span, generally within 5 years of natural death (24). Naturally menopausal animals are therefore relatively rare and short-lived, and ovariectomized macaques are more widely used as a primate model of menopause. An important feature of the macaque model is the unique degree of similarity to the human breast. Despite differences in gross appearance, human and macaque breasts have similar lobuloalveolar and ductal patterns, cytokeratin phenotype, sex steroid receptor expression, steroidogenic enzyme distribution and activity, and responses to endogenous and exogenous hormones (25–27).

Study design. Thirty-one adult female cynomolgus monkeys with an average age of 14.3 ± 0.5 years were imported from the...
Institute Pertanian Bogor (Bogor, Indonesia). Animals were ovariectomized and divided into social groups of three to four animals each. Groups were then rotated through eight different diets in a randomized 2 × 4 factorial Latin-square experiment in which all animals received all diets. Two animals died of causes unrelated to the study procedures and were subsequently replaced, leaving 29 to 31 total animals per diet at the end of the study.

Diets contained the human equivalent of 0, 60, 120, or 240 mg/d soy isoflavones (in aglycone units) along with oral micronized E2 at doses equivalent to either 0.09 mg/d (low E2) or 0.5 mg/d (high E2) in women. Each of the eight dietary periods lasted for 4 months followed by a 1-month washout period. The low E2 dose was given to provide serum estrogen levels comparable with a nonobese naturally postmenopausal woman. The higher dose of E2 was designed to simulate the estrogen environment of a postmenopausal woman with increased estrogen concentrations either from obesity or from low-dose estrogen replacement. Isoflavone doses approximated a traditional Asian diet (60 mg), a high-dose soy diet (120 mg), and pharmacologic supplementation (240 mg) representing approximately twice the isoflavone dose attainable by dietary means. Monkeys were fed ~120 kcal/kg body weight once daily. Isoflavone and E2 doses were scaled to an 1,800 kcal diet (the estimated daily intake for a U.S. woman), so that monkeys were given ~0, 4, 8, or 16 mg isoflavones/kg body weight and either 0 or 33 μg E2/kg body weight. This caloric adjustment of dose accounts for differences in metabolic rates between the monkeys and the human subjects (28).

The protein source for all isoflavone-containing diets was a combination of casein-lactalbumin and soy protein isolate (standardized across all isoflavone diets), whereas casein-lactalbumin was the sole protein source for the control diets. To attain the 120 and 240 mg isoflavone doses, the base soy protein diet was supplemented with a purified isoflavone extract. The isoflavone profile of the diet components was determined by high-pressure liquid chromatography using established procedures. The soy protein isolate contained 53.3% genistein, 39.6% daidzein, and 7.1% glycitein, whereas the isoflavone extract contained 61.7% genistein, 32.6% daidzein, and 5.7% glycitein (expressed in aglycone units; data provided by the manufacturer). Quality-control measures included individual batch analysis for macronutrients, micromes, and isoflavones. Manufacturing protocols required <5% variation in isoflavone content between batches, and all study diets were specifically formulated to adjust for any changes in isoflavone content among batches. The isolated soy protein and isoflavone concentrate were provided by Solae, a division of Dupont (St. Louis, MO). Diets were formulated to be isocaloric and equivalent for macronutrients, cholesterol (0.20 mg/kcal), calcium (0.75 mg/kcal), and phosphorus (0.70 mg/kcal).

All procedures involving these animals were conducted in compliance with state and federal laws, standards of the U.S. Department of Health and Human Services, and guidelines established by the Wake Forest University Animal Care and Use Committee (ACUC). The facilities and laboratory animal program of Wake Forest University are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

Breast biopsies. At the end of each dietary period, the animals were anesthetized with ketamine and butorphanol for breast biopsy, blood collection, uterine ultrasound, vaginal cytology, and body weight measurement. For the breast biopsy, a 1.5-cm incision was made in a preselected breast quadrant and a small (~0.4 g) sample of mammary gland was removed. The incision was sutured, and the animals were monitored and given analgesia during recovery following ACUC-approved clinical procedures. The biopsy site was tattooed to prevent later resampling at the same site. Half of the biopsy sample was snap frozen in liquid nitrogen; the other half was fixed at 4°C in 4% paraformaldehyde for 24 hours and then processed for histology using standard procedures.

Serum isoflavonoids. Serum concentrations of isoflavones and isoflavone metabolites (genistein, daidzein, equol, glycitein, dihydrogenistin, dihydrodaidzein, and O-desmethylygonein) were determined by liquid chromatographic–photodiode array mass spectrometric analysis using techniques described previously (29). Isoflavonoid measurements were done on 24-hour fasted serum samples collected at the time of biopsy from all animals (n = 29–31 per diet) and 4-hour postfeeding samples from a subset of animals (n = 7–9 per diet). Serum isoflavonoid concentrations were also measured on a subset of fasted samples following the 1-month washout period (n = 4 per diet).

Immunohistochemistry. Immunostaining procedures were done on fixed, paraffin-embedded mammary gland tissues using commercially available primary monoclonal antibodies for Ki-67 (Ki-67/MIB-1, DAKO, Carpinteria, CA), ERα (NCL-ER-6F11, Novoceastra, Newcastle upon Tyne, United Kingdom), progesterone receptor (PR; NCL-PGR, Novoceastra), and cleaved caspase-3 (Cell Signaling Technologies, Beverly, MA). Staining methods included antigen retrieval with citrate buffer (pH 6.0), biotinylated rabbit anti-mouse Fc antibody as a linking reagent, alkaline phosphatase–conjugated streptavidin as the label, and Vector Red as the chromogen (Vector Laboratories, Burlingame, CA). Cell staining was quantified by a computer-assisted counting technique, using a grid filter to select cells for counting and our modified procedure of cell selection, described previously (30). Numbers of positively stained cells were measured as a percentage of the total number examined (100 cells), and positive cells were scored for staining intensity (1+, 2+, or 3+) to obtain a H-score (intensity × % positive cells; ref. 31). All measurements were made blinded to treatment.

pS2 gene expression. Mammary gland expression of the estrogen-responsive epithelial cell marker pS2 (trefoil factor 1) was determined using quantitative real-time PCR (qRT-PCR). Macaque cDNA sequences for pS2 and the internal control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined from PCR products generated using human primer sets. Products were run on an agarose gel, purified, quantitated, and sequenced by the Wake Forest University Core Sequencing Laboratory. Macaque-specific qRT-PCR primer-probe sets were then generated through the ABI Taqman Assay-by-Design service. Assay specificities have been described previously (32). The following primer-probe sets were used: pS2-forward 5'-GTGCCCTCCATCTAATACCTACA-3', pS2-reverse 5'-TTATAGACACTTCTGACTGGATCTGC-3', and pS2 probe 5'-TCCCTCAGAAAGGAGTGTG-3' (NM003225.1, bp 241–315) and GAPDH-forward 5'-TGCCCTCATTGACCACCTTGTG-3', GAPDH-reverse 5'-ATTTGCTACAGCAACAGGCT-3', and GAPDH-probe 5'-ATTCCCTGTATAGACAAACAGA-3' (BC0140852, bp 1,773–1,841). To eliminate false signals from possible genomic DNA contamination, the probe for each target was placed across an exon boundary (indicated by base pairs in bold). Macaque and human target sequence identity was 98.7% for pS2 and 98.6% for GAPDH. RNA was extracted from frozen mammary biopsies using Tri-Reagent (Molecular Research Center, Cincinnati, OH) and quantitated using a Beckman DU650 spectrophotometer (Beckman Coulter, Fullerton, CA). An aliquot of RNA (2.5 μg/sample) was reverse transcribed using a High-Capacity
cDNA Archive kit (Applied Biosystems, Foster City CA). qRT-PCR was done on an Applied Biosystems ABI PRISM 7000 Sequence Detection System. qRT-PCR reactions (20 μL volume) were done in separate wells for each target using Taqman Universal Master Mix and associated reagents. The thermocycling protocol involved initial incubations of 2 minutes at 50°C and 10 minutes at 95°C followed by 40 PCR cycles of 95°C for 15 seconds and 60°C for 1 minute. Relative expression was determined using the ΔΔCt method described in the Applied Biosystems User Bulletin 2 (available online at http://www.ukl.uni-freiburg.de/core-facility/taqman/user_bulletin_2.pdf). Stock breast tissue was run in triplicate on each plate as an external calibrator. Calculations were done using ABI Relative Quantification SDS Software version 1.1.

Uterine area. Uterine area was determined by transabdominal ultrasound using a Sonosite 180 portable ultrasound machine with a 5.0-MHz linear transducer (Sonosite, Bothell, WA). Images were recorded before and after each treatment phase. Uterine thickness and maximal transverse cross-sectional area were measured on a static representative digital image using public domain software (NIH Image 1.33; available at http://rsbweb.nih.gov/ij/download.html).

Vaginal cytology. To evaluate vaginal keratinocyte maturation, animals were sedated and vaginal swabs were taken before and after each treatment phase. Vaginal epithelial cells were collected from the anterior vagina with a cotton swab, rolled onto a glass slide, and fixed using a commercial fixative (Spray-cyte, Surgipath Medical Industries, Richmond, IL). Slides were stained using a modified Papanicolaou method. Maturation value was calculated using the formula: maturation value = (0.2 × % parabasal cells) + (0.6 × % intermediate cells) + (% of superficial cells).

Serum estrogens. Serum concentrations of E2, estrone (E1), and E1 sulfate (E1S) were measured on 24-hour fasted serum samples collected at the time of biopsy from all animals (n = 29-31 per diet). Estrogens were quantitated by RIA using commercially available kits and protocols from Diagnostic Systems Laboratories (Webster, TX; E2, DSL-4800 ultrasensitive; E1, DSL-8700; E1S, DSL-5400). For E2 assays, serum (0.5 mL) was extracted by adding ethyl ether (4 mL) and vortexing for 5 minutes. The aqueous layer was frozen in a dry ice/isopropanol bath, and the organic phase was decanted. Extracts were dried and reconstituted with zero-standard serum from the RIA kit. E2 samples with values falling below the lowest standard (<5.0 pg/mL) were set to 4.0 pg/mL for statistical analysis. Assays were done in the Clinical Pathology Laboratory at the Comparative Medicine Clinical Research Center, Wake Forest University School of Medicine. Normal ranges of sensitivity are as follows: E2 (5-750 pg/dL), E1 (7.5-2,000 pg/mL), and E1S (0.05-90 ng/mL). Intra-assay and interassay coefficients of variation were <10% for E2 and E1S. For E2, intra-assay and interassay coefficients of variation were 5.8% and 8.9% at 20 pg/mL and 6.0% and 9.3% at 250 pg/mL, respectively.

Statistics. A mixed general linear model with repeated measures was used to determine means and to test for main and interactive effects of E2 and isoflavone treatment. E2 (low, high) and isoflavone (0, 60, 120, 240) interactions were excluded from the final model. Uterine area was the only variable in which a trend for a carryover effect of high E2 treatment was noted (P = 0.06). Change in body weight across each dietary period was also used in our initial model as a random effect covariate. Body weight change significantly correlated with serum E2 (P = 0.02) and was thus kept as a covariate for serum estrogens. Adjustment for either body weight change or post-treatment body weight had negligible effects on other outcome measures. Contrasts were run between the control (0 mg isoflavone) and the 60, 120, and 240 mg isoflavone diets within each E2 category. Tests for P for trend were determined by multivariate regression analysis adjusting for repeated measures among subjects, dietary phase, and E2 treatment in the previous phase. All variables were evaluated for their distribution and equality of variances among diets, and log10 transformations were done where appropriate to improve homogeneity of variance. Data were analyzed using the SAS Statistical Package version 8 (SAS Institute, Cary, NC). A two-tailed significance level of 0.05 was chosen for all comparisons. Unless otherwise stated, all data are reported as mean ± SE.

Results

Serum isoflavonoid concentrations in the human physiologic range. Serum isoflavonoid concentrations were measured at 4 and 24 hours after feeding to evaluate dose effects and isoflavone metabolism in relation to human studies. Dietary isoflavone dose was strongly associated with serum concentrations in both low and high E2 environments (P for trend < 0.0001; Fig. 1). Mean serum isoflavonoid concentrations 4 hours after feeding increased from <15 nmol/L in the 0 mg isoflavone diets to 1,435.2 nmol/L in the 240 mg isoflavone diets, similar to the reported range of human serum concentrations following a high-dose soy meal (33). The higher E2 dose had no effect on serum isoflavonoid concentrations (P = 0.60). Comparison of 4- and 24-hour samples indicated 70% to 80% clearance from serum over a 20-hour postprandial period. The predominant circulating isoflavone metabolite in all monkeys was equol, which comprised 45% of total isoflavonoids at 4 hours after feeding and 77% at 24 hours after feeding. Total isoflavonoid concentrations following the 1-month washout period were 39.6 ± 2.9 nmol/L at 24 hours after feeding, similar to the 0 mg isoflavone control diets.

Body weight was also measured as an indicator of individual dietary intake. Overall average body weight increased from 3.10 to 3.44 kg (10.7%) over the study duration. Post-treatment body weights, by increasing isoflavone dose, were 3.25, 3.30, 3.38, and 3.29 ± 0.09 kg among low E2 diets and 3.14, 3.21, 3.24, and 3.21 ± 0.09 kg among high E2 diets. The high E2 diets resulted in slight but significant weight loss per dietary cycle (−0.05 ± 0.02 kg), whereas the low E2 diets resulted in mild weight gains (0.07 ± 0.02 kg; P < 0.0001). Among the high E2-treated animals, the highest isoflavone dose resulted in significantly less weight loss compared with the control diet (P = 0.01).

Inhibition of E2-induced effects by high-dose soy isoflavones. To determine whether dietary soy isoflavones alter estrogen-mediated targets in the breast, we first measured breast proliferation using the Ki-67 immunohistochemical marker. As shown in Fig. 2A, the high E2 diet resulted in 64% greater lobular proliferation overall (P < 0.0001 across all diets) and 87% higher proliferation between 0 mg isoflavone control diets (P < 0.0003). In the high E2 environment, the 240 mg isoflavone dose resulted in
51% lower E2-induced lobular Ki-67 expression (P = 0.04 compared with the 0 mg isoflavone/high E2 diet). As a result, lobular proliferation following the 240 mg isoflavone/high E2 diet did not differ significantly from the low E2 control diet (P = 0.06) in contrast to the highly significant difference between high and low E2 control diets. A trend for an inverse association between isoflavone dose and lobular proliferation was seen in the high E2 environment (P for trend = 0.09) but not in the low E2 environment (P for trend = 0.97). Proliferation in large ducts was increased 47% overall by the high E2 diet (P = 0.01) and 41% between 0 mg isoflavone control diets (P = 0.23). The 240 mg isoflavone dose completely abolished E2-induced ductal proliferation, although this effect was not significant (P = 0.22). In the low E2 environment, the 240 mg isoflavone dose had no significant effect on lobular proliferation.

**Figure 1.** Serum isoflavonoid concentrations at 4 and 24 hours after feeding by isoflavone dose. Isoflavonoids were determined by liquid chromatographic-photodiode array mass spectrometric analysis. A. ranges of serum isoflavonoid (IF) concentrations were dose dependent and comparable with those reported in human soy intervention studies. Values for each isoflavone dose represent a combination of low and high E2 diets. B. serum isoflavonoid concentrations at 24 hours after feeding were consistent between low and high E2 diets. Equol was the predominant isoflavonoid (n = 29-31 per diet). EQ, equol; DA, daidzein; GE, genistein; GLY, glycitein; DHDA, dihydrodaidzein; DHGE, dihydrogenistein; oDMA, O-desmethylangolensin. *, P < 0.0001 compared with the respective 0 mg control diet. Bars, SE.

**Figure 2.** Antagonistic effects of soy isoflavones on breast proliferation (A), uterine size (B), and vaginal maturation (C) in either low or high E2 environment. Proliferation was measured by immunohistochemical expression of the proliferation marker Ki-67 in breast lobular and ductal epithelium; uterine area was determined by transabdominal ultrasound; vaginal maturation was evaluated by cytologic assessment. The 240 mg isoflavone dose resulted in significantly lower lobular Ki-67 expression (A) and uterine area (B) in the high-estrogen (E2) environment and lower vaginal maturation (C) in the low E2 environment (n = 29-31 per diet). Horizontal lines, washout values measured 1 month after each diet (B and C). a, P < 0.05 compared with low E2 control; b, P < 0.05 compared with high E2 control. Bars, SE.
(P = 0.61) or ductal (P = 0.99) proliferation compared with the 0 mg isoflavone control group. No significant interaction between E2 and isoflavone treatment was seen for lobular or ductal proliferation due primarily to the lack of any estrogen-like isoflavone effects in the low E2 environment.

A similar pattern of effects was seen for uterine size, which was used as a surrogate marker of endometrial proliferation. The high E2 diet had a significant stimulatory effect on the uterus, increasing uterine area by 42% overall (P < 0.0001) and by 51% between low and high E2 control diets (P < 0.0001; Fig. 2B). In contrast, the low E2 diet did not significantly increase uterine area (P = 0.25 for low E2 diet versus washout diet). The highest isoflavone dose resulted in lower uterine area among the high E2 diets, blocking 33% of the high E2 effect (P = 0.01), whereas no isoflavone effects were detected among low E2 diets (P = 0.37 for 0 versus 240 mg isoflavone dose). Although no significant isoflavone dose trends were noted, a significant interactive effect for uterine area was noted (P for E2 × isoflavone = 0.01).

We also evaluated vaginal keratinocyte maturation, a sensitive indicator of estrogen exposure. The high E2 diet significantly increased vaginal maturation by 7% overall (P < 0.0001) and by 6% between low and high E2 control diets (P = 0.0004; Fig. 2C). Low E2 treatment also had a strong stimulatory effect on vaginal maturation (+31%; P < 0.0001 for the low E2 diet versus washout diet) in contrast to uterine area (Fig. 2B and C). Compared with the respective 0 mg isoflavone control diets, the 240 mg isoflavone dose resulted in significantly lower vaginal maturation values in the low E2 environment (P = 0.02) but not in the high E2 environment (P = 0.38). Isoflavone dose trends followed a similar pattern (P for trend = 0.03 and 0.14, respectively, for low and high E2 diets).

**Divergent effects of soy isoflavones on ER-mediated transcription.** To determine if these antagonistic effects were mediated by the ER, we next measured markers of ER expression and activity. As shown in Fig. 3A, isoflavone treatment did not affect ERα protein expression at any of the doses. A similar lack of treatment effect was seen for ERβ mRNA expression in breast tissue (data not shown). To evaluate ERα activity, we next measured mRNA content of pS2 within the breast using qRT-PCR. The promoter region of the pS2 gene contains a near-consensus estrogen response element to which activated ERα directly binds to induce transcription, and pS2 is thus a sensitive marker of ligand-dependent ERα activation (34). The high E2 diet strongly induced pS2 expression, increasing mRNA by 374% between low E2 and high E2 control diets (P < 0.0001; Fig. 3B). The 240 mg isoflavone dose in turn resulted in 62% less E2-induced pS2 expression (P = 0.006 versus 0 mg isoflavone control diet). In the low E2 environment, the 240 mg dose resulted in a nonsignificant 22% decrease in pS2 expression. Inhibitory effects of isoflavones on pS2 were dose dependent within the high E2 environment (P for trend = 0.03). Similar results were also obtained using β-actin in place of GAPDH as the internal calibrator.

We next evaluated immunohistochemical expression of PR, another ER-mediated target. Unlike pS2, PR lacks a complete estrogen response element and is more indirectly regulated by estrogen agonists (35). High E2 treatment increased lobular PR expression by 55% overall (P < 0.0001) and by 34% between control diets (P = 0.07). However, in contrast to pS2, the 240 mg isoflavone dose resulted in significantly higher PR expression in lobular breast epithelium in the high E2 environment (P = 0.04 versus 0 mg isoflavone control diet; Fig. 3C).

**Figure 3.** Divergent effects of soy isoflavones on markers of ERα activity. ERα expression was measured by immunohistochemical expression in breast lobular and ductal epithelium. ERα activity was determined by quantitation of pS2 gene expression and immunohistochemical expression of PR in breast tissue. Isoflavone treatment did not significantly alter ERα expression (A). However, in the high-estrogen (E2) environment, the 240 mg isoflavone dose resulted in significantly reduced pS2 expression (B) and increased PR expression (C; n = 29-31 per diet). pS2 expression is expressed relative to GAPDH. a, P < 0.05 compared with low E2 control; b, P < 0.05 compared with high E2 control. Bars, SE.

High doses of soy isoflavones induce a variety of proapoptotic effects in cell culture (36). To rule out the possibility that proapoptotic effects were driving the reduced proliferation and pS2 expression in the breast, breast biopsies were immunostained...
for cleaved caspase-3, a central downstream product of apoptosis. Relatively low levels of cleaved caspase-3 expression were detected in the breast epithelium (<1% positive cells overall), and no significant isoflavone effects were detected (data not shown).

**Decreased serum estrogens with isoflavone treatment.**

Finally, we evaluated isoflavone effects on serum estrogen concentrations. All isoflavone doses resulted in significantly lower E2 and E1 24 hours after feeding in the high E2 environment (P < 0.05 for all isoflavone doses versus 0 mg isoflavone control group; Table 1), whereas no significant differences were seen in the low E2 environment. Similarly, isoflavone dose was inversely associated with serum E2 and E1 in the high E2 environment only (P for trend = 0.04 and 0.06, respectively). E1S was also lower after isoflavone treatment, although no significant treatment differences were noted. The increase in serum estrogens from the high-estrogen diet was 42% less for E2, 18% less for E1, and 10% less for E1S following the 240 mg isoflavone diet compared with the 0 mg isoflavone diet (Fig. 4A). Body weight data, isoflavone concentrations, and 4-hour postfeeding serum E2 concentrations from a subset of high E2 animals all indicated that lower serum estrogens

### Table 1. Effects of soy isoflavones on serum estrogen concentrations

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NOTE: Mean ± SE. Each treatment condition included the same 29 to 31 animals. Data were analyzed using a mixed general linear model adjusting for repeated measures among subjects and change in body weight across each dietary period. Serum estrogens were measured by RIA in serum collected 24 hours after feeding. Isoflavone doses are expressed in human equivalents. All isoflavone doses resulted in significantly lower E2 and E1 in the high-estrogen postmenopausal environment. Note also that E2 values in the low- and high-estrogen environments approximate upper and lower serum E2 quintiles in naturally postmenopausal women. For conversion to SI units, multiply by the following conversion factors: 3.7 for E2 and E1 (pmol/L) and 2.8 for E1S (nmol/L).

*P < 0.05 compared with high-estrogen control group.

Figure 4. Effects of high-dose soy isoflavones on serum estrogen concentrations. A, the 240 mg isoflavone dose significantly blunted the effect of the high E2 diet on serum E2 and E1 (n = 29-31 per diet). *P < 0.05 compared with the 0 mg isoflavone diet. B, time course analysis of serum E2 and E1 following the high E2 diet alone (E2) or with 240 mg isoflavone (E2 + isoflavone) showed significantly greater clearance of E2 and E1 by 24 hours after feeding in the E2 + isoflavone group (n = 3-4 per diet). *P < 0.05 compared with the 4-hour time point. Bars, SE.
in isoflavone-treated animals were not due to decreased dietary intake. To further explore this observation, we measured serum E2 and E1 at 4, 8, 12, and 24 hours after feeding in high E2 animals receiving either 0 or 240 mg isoflavone (n = 3-4 per diet). Serum E2 and E1 concentrations were similar at 4 hours after feeding between the two diets (P = 0.78 for E2 and P = 0.84 for E1) but declined more rapidly in the isoflavone-fed animals (Fig. 4B). Compared with 4 hours after feeding, serum E2 and E1 were significantly lower at 24 hours in the 240 mg isoflavone group (P = 0.008 for E2 and P = 0.001 for E1) but not in the 0 mg isoflavone group (P = 0.09 for E2 and P = 0.08 for E1). Area under the curve measurements also indicated 36% lower E2 and 25% lower E1 for the isoflavone-treated group in the 12- to 24-hour period.

Discussion

Estrogen exposure is an important determinant of breast cancer risk, particularly in postmenopausal women (1–3). In this article, we showed in an established primate model that dietary soy isoflavones alter estrogen effects in the postmenopausal breast. Higher doses of soy isoflavones decreased breast proliferation, modified ER activity, and lowered serum estrogen concentrations. Antagonistic isoflavone effects were largely dose dependent and tissue specific. These findings establish a relationship between estrogen context and isoflavone effects in the breast and suggest that high soy intake may contribute to breast cancer prevention in specific populations of at-risk women.

The estrogenic activity of isoflavones was first reported >50 years ago (37). Since this time, numerous in vitro studies have confirmed that isoflavones bind ERs (13), induce distinct changes in ER conformation (38), and elicit a variety of ER-mediated effects (14, 15). Soy isoflavones and their metabolites may also inhibit binding of E2 to ERα (13, 16) and block estrogen effects in vivo (17, 18), leading to speculation that isoflavones may function as natural SERM-like compounds (6). Although isoflavones have far less affinity for ERα than E2 (13), serum isoflavonoid concentrations after a high-dose soy meal may exceed postmenopausal E2 concentrations by at least 10,000-fold. In this study, high physiologic isoflavone doses inhibited estrogen-induced proliferation in the breast and induced divergent effects on ER-mediated targets as seen with synthetic SERMs. Tamoxifen, for example, has been shown to decrease proliferation (38), increase PR expression (20, 39), and decrease pS2 expression (40) in the breast, providing a similar profile to that seen with high-dose isoflavone treatment. These data indicate that dietary soy isoflavones, rather than simply blocking ER transactivation, may induce unique ER-driven expression patterns in breast tissue. Contrary to our initial hypothesis, higher doses of soy isoflavones did not have estrogen agonist effects in a low-estrogen setting. This result, however, is consistent with recent human trials (e.g., ref. 41) as well as a previous long-term study in our model (42), all of which used doses comparable with or lower than the 120 mg isoflavone dose given in this study. Discrepancies between these results and previous rodent studies (43), including our own (18, 29), may relate to estrogen context, isoflavone dose and formulation, and various species differences. Rodents, unlike primates, have complete loss of estrogens with ovarietomy, and this total sex steroid deficiency may sensitize tissues and obscure potential hormone interactions. Still, isoflavone doses required to induce uterotropic effects in rodent models are at least five times higher (on a caloric scale) than those found in a soy-based diet, and based on these data, our lack of estrogenic effects is not surprising. On a provisional note, we should also point out that these findings do not exclude potential agonist effects of isoflavones on occult breast tumors or endometrial proliferation and that the estrogenic properties of soy formulations may vary considerably depending on specific isoflavone composition or route of administration.

The diets used in this study produced serum isoflavonoid concentrations relevant to human levels of exposure. Isoflavone doses equivalent to 60, 120, and 240 mg/d yielded mean 4-hour serum isoflavonoid concentrations of 298, 674, and 1,327 nmol/L, respectively, similar to those reported in human trials (34). Relative dose effects suggest that serum isoflavonoid concentrations above 1,000 nmol/L are required for significant antagonistic effects. This finding is consistent with a previous 3-year study in our model in which we reported significantly lower breast epithelial area specifically in animals with serum isoflavonoid concentrations above 1,000 nmol/L (42). Although the 240 mg dose is beyond that typically consumed in Asian diets, it is plausible that lower dietary doses taken over many years may provide similar effects. Another experimental consideration relates to equol, which was the predominant circulating isoflavone metabolite in all animals in this study. Equol is formed through enzymatic reduction of daidzein by gut bacteria in most mammalian species, although only ~30% of people produce equol after consuming soy (34). The antagonistic effects of dietary isoflavones (and resultant equol) seen in this experiment are consistent with previous sporadic data relating equol production with such characteristics as lower breast cancer risk (9), mammographic density (44), and serum estrogens (45). All these findings remain associative, however, and the relative importance of the equol-producing phenotype remains unclear.

Serum E2 concentrations in this study were comparable with those in postmenopausal women, which generally range from 6 to 20 pg/mL (2, 3). The low and high E2 control diets resulted in mean serum E2 of 8.8 and 18.2 pg/mL, providing a reasonable estimation for each end of the postmenopausal E2 spectrum. In the high E2 environment, all doses of soy isoflavones resulted in significantly lower serum E1 and E2. This finding supports previous studies in women showing that high soy intake may lower endogenous serum estrogens and possibly alter estrogen catabolism (46–48). However, evidence from this study is the first to indicate that soy or soy isoflavones may facilitate clearance of estrogens. Several mechanisms could be responsible for this effect, including decreased enteric estrogen reabsorption, increased hepatic or renal estrogen catabolism, and inhibition of estrogen deconjugation. The lack of a significant lowering of E1S is also noteworthy, providing indirect support for the latter mechanism. Isoflavone effects in the breast were found primarily at high dietary to pharmacologic doses within the high E2 environment. These data suggest that any relevant SERM-like isoflavone effects may be operable primarily in postmenopausal women with high estrogens and high daily serum isoflavone concentrations. The most important known factor underlying high postmenopausal estrogens is obesity (49), and women with high body mass index (and high estrogens) should thus benefit the most from higher doses of soy isoflavones. Recent studies from the Shanghai Breast Cancer Study support this idea, indicating significantly lower breast (50) and endometrial (51) cancer risk specifically in more obese women in the highest category of soy intake. A similar pattern was also reported in the Multiple Outcomes of Raloxifene Evaluation trial for raloxifene, which dramatically reduced breast cancer risk.
in postmenopausal women with higher estrogens while having no detectable effect in low-estrogen women (52). Findings of this study may also relate to the use of estrogen therapy for menopausal symptoms. The higher dose of E2 approximated a standard estrogen regimen for postmenopausal women, and soy isoflavones could potentially serve as a complementary supplement to limit exogenous estrogen exposure in the breast. Our findings, however, do not address potential interactions between soy isoflavones and combined hormone therapies (containing an estrogen and a progestin). Recent evidence has shown greater breast cancer risk associated with combined therapy than with estrogen alone (53), and it is not known whether isoflavones may alter progesterin-mediated effects in the breast.

We show in this study that dietary soy isoflavones modify estrogen effects on risk markers for breast cancer. Isoflavones antagonized E2 effects on breast proliferation in a dose-dependent manner, altered ER activity in the breast, and resulted in lower serum E2 concentrations. No significant estrogen-like isoflavone effects were identified even at doses several times that of a soy-based diet. This evidence suggests that a diet rich in soy isoflavones may have an estrogen-sparing effect in the postmenopausal breast, potentially limiting cancer risk associated with higher estrogen concentrations.

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References

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