Soy isoflavonoid effects on endogenous estrogen metabolism in postmenopausal female monkeys

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Endogenous estrogens are important determinants of breast cancer risk in postmenopausal women. In this study we evaluated the effects of dietary soy isoflavonoids on endogenous estrogen metabolism in a postmenopausal primate model. Ovariectomized female cynomolgus monkeys were randomized to receive one of three diets for 36 months: (i) isoflavonoid-depleted soy protein isolate (SPI−) (n = 29); (ii) soy protein isolate with 129 mg isoflavonoids/1800 kcal diet (8.6 mg isoflavonoids/kg body weight (BW), expressed in aglycone units) (SPI+) (n = 29) or (iii) isoflavonoid-depleted soy protein isolate with conjugated equine estrogens (CEE) at a dose of 0.625 mg/1800 kcal diet (0.042 mg CEE/kg BW) (n = 30). Mean plasma isoflavonoid concentrations in the SPI+ group were 946.9 ± 135.9 nmol/l, and equol was the primary circulating isoflavonoid (549.7 ± 61.6 nmol/l). The SPI− diet resulted in lower serum estrone (E1) after 29 (−26%, P = 0.03) and 34 months (−21%, P = 0.04) compared to the SPI+ diet, while urinary 2-hydroxyestrone (P = 0.005) and the 2 to 16α-hydroxyestrone ratio (P < 0.0001) were markedly higher in the SPI+ group compared to SPI−. Isoflavonoid treatment did not significantly alter gene markers of estrogen metabolism or estrogen receptor agonist activity in breast tissue. Within the SPI+ group, higher concentrations of serum equol (but not daidzein or genistein) corresponded to significantly lower serum E1, more benign 2-hydroxylated metabolites.

Introduction

Endogenous estrogens have a critical role in breast cancer development (1,2). Estrogens stimulate breast epithelial proliferation, promote expansion of responsive neoplastic cells and induce DNA mutations through genotoxic metabolites (1). Many well-established risk factors for breast cancer relate to lifetime estrogen exposure (2), and endogenous serum estrogens are important predictors of breast cancer risk in postmenopausal women (3). Clinical trials have also shown a strong protective effect of various antiestrogenic agents, which dramatically lower the incidence of hormone-dependent breast cancer (4,5). Strategies to reduce excessive estrogen exposure in postmenopausal women may thus have an important role in breast cancer prevention.

Estrogen effects within target tissues are largely determined by metabolic pathways of conjugation and catabolism (6–8). In postmenopausal women over 80% of endogenous estrogens circulate in an inactive form as either sulfate or glucuronide conjugates. The primary bioactive estrogens, estrone (E1) and estradiol (E2), are derived from deconjugation of E1 sulfate (E1S) by steroid sulfatase and, to a lesser extent, E1 glucuronide (E1G) by β-glucuronidase (8–10). E1 and E2 may then be converted to a variety of hydroxylated metabolites which are cleared from the body or further metabolized (6,7). Two key estrogen catabolites are 2-hydroxyestrone (2OH-E1) and 16α-hydroxyestrone (16αOH-E1), which are notable for their distinct biologic properties (11). While 2OH-E1 has limited estrogenic activity (and putative antiestrogenic effects), 16αOH-E1 is considered both estrogenic and genotoxic (11,12). The relative excretion of 2OH-E1 and 16αOH-E1 has thus been used as a marker of breast cancer risk (13), and preventive strategies have aimed at increasing this ratio.

Demographic evidence indicates that women in Asia have significantly lower serum estrogen concentrations (14) and about a 3-fold lower breast cancer risk (15) compared to US women, independent of body weight (BW). Asian women who have migrated to the US appear to lose this low-risk estrogen profile (16) and acquire comparable rates of breast cancer to US women (17), suggesting that specific lifestyle factors may strongly influence breast cancer risk related to estrogen exposure. One such factor is dietary soy, which is an important component in a variety of traditional Asian diets but scarce in typical Western diets (18). Soyfoods are rich in phytoestrogenic compounds called isoflavonoids, represented primarily by the glycosylated forms of genistein and daidzein. Daidzein may in turn be converted by intestinal bacteria to the related isoflavonoid equol (19). Regular intake of soy/soy isoflavonoids has been associated with reduced breast cancer risk (20–24), particularly in postmenopausal women with higher endogenous estrogens (25). Mechanisms driving any such effects have not been clearly identified, however, and variation across epidemiologic studies suggests that chemopreventive benefits of isoflavonoid intake may be limited to specific populations of women (24).

Soy isoflavonoids have structural similarities with mammalian estrogens and may interact with pathways of estrogen activity in the body. Once absorbed, isoflavonoids are extensively conjugated and catabolized by many of the same enzymes as estrogens (26,27), potentially altering physiologic systems of estrogen metabolism. Prior evidence supports this idea, indicating that isoflavonoids may modestly lower serum estrogen concentrations (28–30) and alter estrogen metabolite patterns (31,32) in some groups of women and potentially...
enhance clearance of exogenous estrogen (33). In the current study, we used a postmenopausal primate model to examine the long-term effects of soy isoflavonoids and conjugated equine estrogens (CEE), a widely used oral estrogen therapy, on markers of estrogen metabolism. Our hypothesis was that isoflavonoids would facilitate estrogen conjugation and catabolism.

Materials and methods

Animal subjects

Eighty-eight adult female cynomolgus monkeys (Macaca fascicularis) were obtained from the Institute Pertanian Bogor in Bogor, Indonesia. These animals were pair-fed a larger dose of the experimental diet (120 kcal/kg BW daily split into two feedings) to match the energy intake of the monkeys on the control diet. The monkeys were fed the premenopausal oral contraceptives (OC) and postmenopausal soy isoflavonoids and CEE (34). The animals in the present study comprised the subset of animals that did not receive OC in the premenopausal period. Average age at the beginning of this study was 9.1 ± 0.1 years. Animals were considered multiparous based on historical records and uterine histology.

Female cynomolgus macaques have numerous reproductive similarities to women, including a 28 days menstrual cycle, comparable ovarian hormone patterns and natural ovarian senescence (35,36). Macaques have >95% overall genetic coding sequence identity to humans (37), including key genes involved in breast cancer susceptibility (38), and analogous patterns of steroidogenic enzyme activity (39). Human and macaque mammary glands are similar in terms of microanatomy and development (40), sex steroid receptor expression (41), responses to exogenous estrogens (42), and the age-related development of hyperplastic and neoplastic lesions (43).

All procedures in this study were conducted in compliance with State and Federal laws, standards of the US Department of Health and Human Services and guidelines established by the Wake Forest University Animal Care and Use Committee. 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.

Study design and treatments

All animals were ovarioctomized at the end of the 2.5 years premenopausal period to make them surgically menopausal. The postmenopausal study then followed a three-group, parallel-arm design, lasting for 36 months. Monkeys were randomized by social group to receive one of three diets during this time: (i) isoflavonoid-depleted soy protein isolate (SPI) (n = 29); (ii) soy protein isolate with the human equivalent of 129 mg/day of isoflavonoids (SPI+) (n = 29) or (iii) isoflavonoid-depleted soy protein isolate with CEE at a dose metabolically scaled to >0.625 mg/day in women (n = 30). All animals remained in their original social groups for the entire experiment. The SPI– diet (SUPRO 670-IF) contained isolated soy protein that had been alcohol-washed to remove the isoflavonoids. The SPI+ treatment group received soy protein isolate (SUPRO 670-HG) containing isoflavonoids at a dose ~129 mg/day for women (91 mg genistein, 31 mg daidzein and 7 mg glycitein, in aglycone equivalents). The CEE group received alcohol-washed soy protein isolate (SPI–) plus CEE (Premarin, Wyeth-Ayerst Laboratories, Radnor, PA, USA) at a dose comparable to 0.625 mg/day for women. CEE and isoflavonoid doses were scaled to 1800 kcal of diet (the estimated daily intake for US women) so that monkeys fed 120 kcal of diet/kg of BW consumed ~8.6 mg isoflavonoids/kg BW or 0.042 mg CEE/kg BW. This caloric adjustment of dose adjusts for differences in metabolic rates between the monkeys and human subjects. The isolated soy proteins used for this study were generously provided by Solae, a division of Dupont (St Louis, MO, USA). Monkeys were fed 120 kcal/kg BW daily split into two feedings. Diets were formulated to be isocaloric and equivalent for macronutrients.

Serum isoflavonoids

Serum isoflavonoid concentrations (genistin, daidzein, equol, dehydrodaidzein and O-desmethylangolensin) were measured in serum collected 4 h after feeding at month 34 of the postmenopausal period. Isoflavonoid concentrations were determined by high performance liquid chromatography-mass spectrometry in the laboratory of Dr Stephen Barnes (University of Alabama, Birmingham, AL), as described previously (44).

Serum and urinary estrogens

E₂, E₁S and E₂G concentrations were measured in samples collected in months 29 (serum) and 34 (serum and urine) of the postmenopausal period. Blood was collected by femoral venipuncture following sedation with ketamine and stored at ~80°C. Urine was pan-collected after placing each animal in a single cage overnight. Urine samples were centrifuged at 7500g for 10 min to remove any sediment and stored at ~20°C. E₁ and E₂S concentrations were quantitated by radioimmunooassay using commercially available kits and protocols from Diagnostic Systems Laboratories (E₁, DSDL-8700; E₁S, DSDL-5400, Webster, TX, USA). E₂G was determined using a competitive enzyme immunoassay kit from Immunoetrics (London, UK) (45). Immunoassays were performed at either the Yerkes Primate Research Center Endocrine Core Laboratory (E₁, 29 months) or the Clinical Pathology Laboratory of the Comparative Medicine Clinical Research Center, Wake Forest University School of Medicine (all other assays). Intra- and interassay coefficients of variation were <10% for all assays.

Urinary 2 and 16α-hydroxysterogen metabolites were measured using a commercially available competitive immunoassay kit (Estramat, Immunocare, Blue Bell, PA). Details of the assay protocol have been described previously (46). Urine samples from animals treated with CEE were initially diluted 1:4, as recommended in the kit protocol. Absorbance was determined at 30 and 50 min with a GENios plate reader (Tecan Systems, San Jose, CA) at a wavelength of 405 nm. Data were analyzed with Magellan software (Tecan Systems) using a four-parameter model. Urinary creatinine was measured using a COBAS Fara Bioanalyzer (Roche, Indianapolis, IN).

Tissue collection

After 36 months of treatment, monkeys were sedated with ketamine and euthanized using sodium pentobarbital (100 mg/kg, iv) as recommended by the Panel on Euthanasia of the American Veterinary Medical Association. Mammary gland and uterine tissues were removed at necropsy. A portion of tissue was frozen immediately in liquid nitrogen and stored at ~80°C. Remaining tissue was fixed at 4°C in fresh 4% paraformaldehyde solution (Fisher Scientific, Ratele). Rats were transferred to 70% ethanol. Frozen mammary tissues were used for intramammary mRNA analyses and E₂ measurement. Fixed mammary and uterine tissues were processed and stained with hematoxylin and eosin (H&E) using standard histology procedures.

Intramammary estradiol

Intramammary E₂ was measured by radioimmunooassay following tissue homogenization and extraction. This method was adapted from previous studies (9). Mammary tissue (mean weight, 90.6 ± 4.2 mg) was thawed, weighed and homogenized for 20 s in 1.5 ml of phosphate buffered saline (pH 7.4). Homogenate was extracted by adding a 3:2 solution of ethyl acetate/hexane (10 ml) and vortexing for 2.5 min. Samples were placed in an alcohol/dry ice bath for 1 min after settling. The organic phase was drawn off and evaporated under a gentle stream of filtered air. Residue was reconstituted in 250 µl of E₂ zero standard, and E₂ was measured by commercial radioimmunooassay (DSL-4800 ultra-sensitive), as described for serum E₁. Recovery of extracted E₂ was determined using controls spiked with a known amount of [2,4,6,7-3H(N)]E₂ (Perkin Elmer, Boston, MA) and found to be >95%.

Intramammary gene expression

Transcript levels for estrogen receptor (ER) alpha and genes involved in ER activation (TFPI, PGR), proliferation (Ki67) and estrogen metabolism were measured in breast samples using qRT–PCR. Macaque-specific qRT–PCR primers and probe sets were generated through the Applied Biosystems (ABI, Foster City, CA) Taqman Assay-by-Design service (47) using cDNA sequences from published sequences or from PCR products generated using human primer sets. Genbank accession numbers and primer/probe sequences for these genes are provided in Supplementary Table I. Human Taqman assays were used for PGR (Assay ID: Hs00606901_m1) and β-glucuronidase (Assay ID: Hs99999908_m1). To eliminate genomic DNA contamination, all probes were placed across an exon junction with the exception of 17β-HSD type 1. The probe for this target was placed over a deletion in a highly similar pseudogene, and RNA was pretreated with DNeasy (Qiagen, Valencia, CA) to remove any genomic DNA. Also of note, the sulfotransferase gene (SULT1E1) corresponded to the enzyme form characterized previously in human breast cells (48), while mRNA for a second estrogen sulfotransferase (variably referred to as STE, SULT1A1 or hSULT2) was detected in macaque liver but not breast.

RNA was extracted from frozen mammary biopsies using Tri Reagent (Molecular Research Center, Cincinnati, OH) and quantitated at A260 nm using a Shimadzu DU650 spectrophotometer (Beckman Coulter, Fullerton, CA). An aliquot of RNA (5.0 µg/sample) was reverse transcribed using a High Capacity cDNA Archive Kit (ABI), and portions of this cDNA archive were then diluted 1:10 in a separate test plate. qRT–PCR reactions (20 µl volume) were performed using an ABI Prism 7000 Sequence Detection System and associated Taqman reagents. The thermocycling protocol
involved initial incubations of 2 min at 50°C and 10 min at 95°C followed by 40 PCR cycles of 95°C for 15 s and 60°C for 1 min. Relative expression was determined using the ΔΔCt method described in ABI User Bulletin #2 (available online at http://www.aml.uni-freiburg.de/core-facility/taqman/user_bulletin_2.pdf). GAPDH was used as the internal calibration gene, and stock breast tissue was used as an external calibrator for plate-to-plate adjustment. Calculations were performed utilizing ABI Relative Quantification SDS Software v1.1.

Histomorphometry

Breast and uterine morphology was quantified by histomorphometry, as described previously (34,42). Briefly, H&E-stained slides were digitized using a Hitachi VK-C370 camera and video capture board (SciOn LG-3; SciOn, Frederick, MD), and measurements were obtained using the SAS statistical package (version 8; SAS Institute, Cary, NC). A two-tailed significance level of 0.05 was chosen for all comparisons. All variables were evaluated for their distribution and equality of variances among groups, and log10 transformations were performed where appropriate to improve normality and homogeneity of variance. For log-transformed data, reported values were retransformed to the original scale using the inverse log. Data are reported either as mean ± standard error for untransformed data or mean (90% confidence interval) for retransformed data. Body mass index was screened initially as a covariate for serum estrogens but excluded in the final model due to negligible effects on model strength or intergroup comparisons. Three breast samples were excluded due to poor RNA intactness (2 CEE) or cDNA quality (1 SPI). Otherwise, sample size for a given endpoint was based on serum, urine or tissue availability.

Statistics

Treatment group and tertile differences were evaluated as a one-way analysis of variance (ANOVA) using a general linear model. Analyses were performed using the SAS statistical package (version 8; SAS Institute, Cary, NC). A two-tailed significance level of 0.05 was chosen for all comparisons. All variables were evaluated for their distribution and equality of variances among groups, and log10 transformations were performed where appropriate to improve normality and homogeneity of variance. For log-transformed data, reported values were retransformed to the original scale using the inverse log. Data are reported either as mean ± standard error for untransformed data or mean (90% confidence interval) for retransformed data. Body mass index was screened initially as a covariate for serum estrogens but excluded in the final model due to negligible effects on model strength or intergroup comparisons. Three breast samples were excluded due to poor RNA intactness (2 CEE) or cDNA quality (1 SPI). Otherwise, sample size for a given endpoint was based on serum, urine or tissue availability.

Results

Body weight/body mass index

No significant differences were observed among treatment groups for BW. Final BW for SPI−, SPI+ and CEE groups was 3.14 ± 0.12, 3.29 ± 0.12 and 3.11 ± 0.12 kg (ANOVA P = 0.53). Mean change in BW during the study for SPI−, SPI+ and CEE groups was +0.22 ± 0.06, +0.26 ± 0.06 and +0.24 ± 0.06 kg (ANOVA P = 0.89).

Serum isoflavonoids

Total serum isoflavonoid concentrations in the SPI+ group were 946.9 ± 135.9 nmol/l (242.2 ± 34.8 ng/ml), similar to that reported previously in human soy intervention studies (26). However, the primary isoflavonoid in this study was equol, which generally accounts for <10% of total serum isoflavonoids in human subjects consuming soy (49). Individual isoflavonoid concentrations (nmol/l) were as follows: equol, 549.7 ± 61.6; genistein, 222.8 ± 61.9; daidzein, 137.1 ± 31.4; dihydrodaidzein, 24.3 ± 4.0 and o-desmethylangolensin, 12.9 ± 1.7. Total serum isoflavonoid concentrations from all SPI− animals were <80 nmol/l.

Serum estrogens

The SPI+ group had lower serum concentrations of E1 at 29 months (−26%, P = 0.03) and 34 months (−21%, P = 0.04) compared to the SPI− group, while E1S (+6%, P = 0.84 at 29 months; −6%, P = 0.73 at 34 months) and E1G (−13%, P = 0.16 at 34 months) were not significantly different (Table I). The percent of unsulfated E1 in the SPI+ group was 32% lower than the SPI− group at 29 months (P = 0.13) and 14% lower at 34 months (P = 0.09). CEE

### Table I. Serum concentrations of estrone and estrone conjugates

<table>
<thead>
<tr>
<th></th>
<th>SPI−</th>
<th>SPI+</th>
<th>CEE</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>29 months (4 h fast)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>E1 pg/ml</td>
<td>49.0</td>
<td>36.5</td>
<td>347.5</td>
<td>0.03</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>(45.5–54.0)</td>
<td>n = 29</td>
<td>n = 23</td>
<td>n = 30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1S ng/ml</td>
<td>0.32</td>
<td>0.34</td>
<td>10.08</td>
<td>0.84</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>(0.28–0.38)</td>
<td>n = 21</td>
<td>n = 23</td>
<td>n = 28</td>
<td></td>
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<tr>
<td>% Unsulfated E1</td>
<td>13.6</td>
<td>9.3</td>
<td>3.2</td>
<td>0.13</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>(11.5–16.0)</td>
<td></td>
<td></td>
<td>(2.8–3.7)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>34 months (18 h fast)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1 pg/ml</td>
<td>37.8</td>
<td>29.8</td>
<td>140.5</td>
<td>0.04</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>(34.8–40.9)</td>
<td>n = 29</td>
<td>n = 29</td>
<td>n = 30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1S ng/ml</td>
<td>0.34</td>
<td>0.32</td>
<td>2.96</td>
<td>0.73</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>(0.31–0.38)</td>
<td>n = 29</td>
<td>n = 29</td>
<td>n = 30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Unsulfated E1</td>
<td>10.3</td>
<td>8.9</td>
<td>4.9</td>
<td>0.09</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>(9.7–10.8)</td>
<td></td>
<td></td>
<td>(4.4–5.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1G pg/ml</td>
<td>53.8</td>
<td>46.6</td>
<td>69.9</td>
<td>0.16</td>
<td>0.01</td>
</tr>
<tr>
<td>(50.1–57.8)</td>
<td>n = 29</td>
<td>n = 29</td>
<td>n = 30</td>
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<td></td>
</tr>
<tr>
<td>% Unglucuronidated E1</td>
<td>41.3</td>
<td>39.3</td>
<td>65.8</td>
<td>0.49</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>E1(E1+E1G) (39.2–43.4)</td>
<td>(37.2–41.4)</td>
<td>(63.4–68.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Unconjugated E1</td>
<td>8.5</td>
<td>7.3</td>
<td>4.4</td>
<td>0.09</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>E1(E1+E1(E1+E1G)) (8.0–9.1)</td>
<td>(6.8–7.8)</td>
<td>(4.1–4.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Values represent means (90% CI). Data were analyzed using one-way analysis of variance. SPI−, soy protein without isoflavones; SPI+, soy protein with the human equivalent of 129 mg isoflavonoids per day; CEE, SPI− with conjugated equine estrogens at a dose equivalent to 0.625 mg per day in women. E1, estrone; E1S, estrone sulfate; E1G, estrone glucuronide. For conversion to SI units, multiply by the following conversion factors: 3.70 for E1 (pmol/L); 2.85 for E1S (nmol/l) and 2.24 for E1G (pmol/L).
treatment resulted in markedly higher concentrations of serum E\textsubscript{1} and E\textsubscript{1S} (P < 0.0001 at 29 and 34 months) and moderately higher E\textsubscript{1G} (P = 0.01 at 34 months) compared to SPI−. The greater relative increase in E\textsubscript{1S} compared to E\textsubscript{1} led to significantly higher percentage of conjugated E\textsubscript{1} in the CEE group (P < 0.0001 versus SPI−), >95% of which was sulfated. Within the SPI+ group, total isoflavonoid concentrations 4 h after feeding were >6000 times higher than serum E\textsubscript{1} and >500 times higher than serum E\textsubscript{1S}.

**Urinary estrogens**

Creatinine-adjusted urinary E\textsubscript{1} concentrations tended to be lower in the SPI+ group (−54%, P = 0.06 versus SPI−), while urinary E\textsubscript{1S} (−12%, P = 0.68) and E\textsubscript{1G} (−27%, P = 0.19) concentrations were not significantly different between SPI+ and SPI− groups (Table II). The calculated percentage of unconjugated urinary E\textsubscript{1} was marginally lower in the SPI+ group (−36%, P = 0.09 versus SPI−), similar to serum. Urinary E\textsubscript{1} (+266%, P = 0.004), E\textsubscript{1S} (+1695%, P < 0.0001) and E\textsubscript{1G} (+463%, P < 0.0001) were all significantly higher in the CEE group compared to SPI−. A significant correlation was noted between urine and serum 34 months values for E\textsubscript{1} (r = 0.50, P < 0.0001), E\textsubscript{1S} (r = 0.70, P < 0.0001) and E\textsubscript{1G} (r = 0.33, P = 0.003).

Two primary catabolites of E\textsubscript{1} are 2OH-E\textsubscript{1} and 16αOH-E\textsubscript{1} (11). Concentration ranges of these metabolites in our model were similar to those reported previously in postmenopausal women (32). Urinary 2OH-E\textsubscript{1} concentrations were significantly lower in the SPI+ group (+72%, P = 0.005 versus SPI−) while 16αOH-E\textsubscript{1} concentrations were moderately lower (−26%, P = 0.05 versus SPI−), producing a ratio of 2OH-E\textsubscript{1} to 16αOH-E\textsubscript{1} more than twice that of SPI− (+132%, P < 0.0001) (Table II). CEE treatment increased both 2OH-E\textsubscript{1} (+383%) and 16αOH-E\textsubscript{1} (+174%) (P < 0.0001 for both versus SPI−), also producing a higher ratio of 2OH-E\textsubscript{1} to 16αOH-E\textsubscript{1} (+93%, P = 0.002 versus SPI−).

**Intramammary markers of estrogen metabolism**

To investigate related changes within breast tissue, we measured tissue E\textsubscript{2} and gene markers of estrogen metabolism. Intramammary E\textsubscript{2} concentrations were similar to those reported previously in postmenopausal women (9). Among diet groups, intratissue E\textsubscript{2} was significantly lower in the SPI+ group (P = 0.02) and higher in the CEE group (P = 0.009) compared to SPI− (Figure 1A). Key enzymes regulating E\textsubscript{2} formation include aromatase (Arom), reductive 17β-hydroxysteroid dehydrogenase (HSD) enzymes (types 1 and 5), sulfatase (STS) and β-glucuronidase (GLU), while enzymes favoring estrogen conjugation or deactivation include estrogen sulfotransferase (SULT), oxidative 17β-HSD enzymes (types 2 and 4) and various UDP-glucuronosyltransferase (UGT) enzymes (Supplementary Figure 1). Partial sequences of macaque genes corresponding to these enzymes exhibited high sequence identity to related human sequences (>94% for all) (Supplementary Table I), and mRNA for each enzyme was detected in mammary tissue. Mammary expression levels were greatest for the 17β-HSD enzymes, STS and β-GLU, and lowest for the UGT enzymes and aromatase.

Among genes involved in estrogen formation or activation, we found significantly lower STS (P = 0.0002) and 17β-HSD type 1 (P = 0.005) expression in the CEE group compared to SPI− but no significant differences between SPI+ and SPI− (P > 0.1 for all). Neither CEE nor SPI+ significantly altered mRNA for Arom (ANOVA P = 0.80), 17β-HSD type 5 (ANOVA P = 0.09) or β-GLU (ANOVA P = 0.40) (Figure 1B). Among genes involved in estrogen conjugation or deactivation, 17β-HSD type 2 mRNA was higher in the CEE group (P = 0.04 versus SPI−), while no significant group differences were seen in 17β-HSD type 4 (ANOVA P = 0.37), sulfotransferase (ANOVA P = 0.15) or any of the UGT enzymes examined (ANOVA P = 0.92 for 1A9, P = 0.24 for 2B7, P = 0.10 for 2B15) (Figure 1C). UGT 1A1 values, while not significantly different among

### Table II. Urinary concentrations of estrone, estrone conjugates and estrone metabolites

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>SPI−</th>
<th>SPI+</th>
<th>CEE</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>E\textsubscript{1}</td>
<td>0.71</td>
<td>0.33</td>
<td>2.60</td>
<td>SPI− versus SPI+&lt;br&gt;0.06&lt;br&gt;0.004</td>
</tr>
<tr>
<td>ng/mg creatinine</td>
<td>(0.53–0.96)</td>
<td>(0.25–0.44)</td>
<td>(1.90–3.55)</td>
<td></td>
</tr>
<tr>
<td>E\textsubscript{1S}</td>
<td>2.84</td>
<td>2.49</td>
<td>50.98</td>
<td>&lt;0.0001&lt;br&gt;&lt;0.0001</td>
</tr>
<tr>
<td>ng/mg creatinine</td>
<td>(2.26–3.58)</td>
<td>(1.99–3.11)</td>
<td>(39.91–65.11)</td>
<td></td>
</tr>
<tr>
<td>% Unsulfated E\textsubscript{1}</td>
<td>17.6</td>
<td>11.2</td>
<td>4.7</td>
<td>&lt;0.0001&lt;br&gt;&lt;0.0001</td>
</tr>
<tr>
<td>E\textsubscript{1}/(E\textsubscript{1}+E\textsubscript{1S})</td>
<td>(14.6–21.2)</td>
<td>(9.4–13.4)</td>
<td>(3.9–5.7)</td>
<td></td>
</tr>
<tr>
<td>E\textsubscript{1G}</td>
<td>23.2</td>
<td>16.9</td>
<td>130.5</td>
<td>&lt;0.0001&lt;br&gt;&lt;0.0001</td>
</tr>
<tr>
<td>% Unsulfotransferase (SULT)</td>
<td>(19.5–27.6)</td>
<td>(14.3–19.9)</td>
<td>(108.7–156.7)</td>
<td></td>
</tr>
<tr>
<td>% Unconjugated E\textsubscript{1}</td>
<td>2.9</td>
<td>1.9</td>
<td>1.9</td>
<td>0.09&lt;br&gt;0.12</td>
</tr>
<tr>
<td>E\textsubscript{1}/(E\textsubscript{1}+E\textsubscript{1S}+E\textsubscript{1G})</td>
<td>(2.4–3.5)</td>
<td>(1.6–2.3)</td>
<td>(1.6–2.3)</td>
<td></td>
</tr>
<tr>
<td>% Unconjugated E\textsubscript{1}</td>
<td>2.5</td>
<td>1.6</td>
<td>1.3</td>
<td>0.08&lt;br&gt;0.02</td>
</tr>
<tr>
<td>2OH-E\textsubscript{1}</td>
<td>2.1–3.0</td>
<td>1.4–1.9</td>
<td>1.1–1.6</td>
<td></td>
</tr>
<tr>
<td>ng/mg creatinine</td>
<td>(5.71–7.48)</td>
<td>(9.87–12.82)</td>
<td>(27.30–36.37)</td>
<td></td>
</tr>
<tr>
<td>16OH-E\textsubscript{1}</td>
<td>6.53</td>
<td>11.25</td>
<td>31.51</td>
<td>0.005&lt;br&gt;&lt;0.0001</td>
</tr>
<tr>
<td>ng/mg creatinine</td>
<td>(3.13–3.88)</td>
<td>(2.34–2.88)</td>
<td>(8.54–10.72)</td>
<td></td>
</tr>
<tr>
<td>2:16OH-E\textsubscript{1} ratio</td>
<td>(1.87–4.34)</td>
<td>(3.86–4.87)</td>
<td>(2.90–3.74)</td>
<td>&lt;0.0001&lt;br&gt;0.002</td>
</tr>
</tbody>
</table>

*<sup>a</sup>Values represent means (90% CI). Data were analyzed using one-way analysis of variance. SPI&minus; soy protein without isoflavones; SPI+, soy protein with the human equivalent of 129 mg isoflavonoids per day; CEE, SPI&minus; with conjugated equine estrogens at a dose equivalent to 0.625 mg per day in women. SPI−, n = 27; SPI+, n = 29; CEE, n = 24. E\textsubscript{1}, estrone; E\textsubscript{1S}, estrone sulfate; E\textsubscript{1G}, estrone glucuronide.*
Isoflavonoids have previously been shown to induce estrogen agonist effects in cell culture and rodent models (50). To exclude any such effects in the breast, we measured intramammary gene markers of ER alpha expression, ER activation (TFF1, PGR) and cellular proliferation (Ki67). Transcript levels for ER (P = 0.005), TFF1 (P < 0.0001), PGR (P < 0.001) and Ki67 (P = 0.03) were all significantly higher in the CEE group, while no significant differences were found between the SPI+ and SPI− groups (P > 0.1 for all) (Figure 2).

Equol tertile analysis
Animals in the SPI+ group exhibited considerable interindividual variation in serum equol, which on average comprised 58% of total serum isoflavonoids. In order to evaluate correlative effects of this variability, SPI+ animals were stratified into tertiles based on serum equol concentrations. The high-equol tertile had ~25–50% lower serum E₁ and E₁S compared to the low-equol tertile (P = 0.004 for E₁ at 29 months; P < 0.1 for other comparisons) (Table III). Estrogen-sensitive measures in reproductive tissues followed a similar pattern, as mammary gland area (−74%, P = 0.0001), uterine weight (−43%, P = 0.009) and endometrial thickness (−35%, P = 0.05) were all lower in the high-equol tertile. Gene markers of estrogen exposure (TFF1) and proliferation (Ki67) in the breast, while not significantly different by tertile, also tended to be lower in the high-equol subgroup. Based on simple regression analysis, serum equol correlated negatively with serum E₁ (r = 0.62, P = 0.002 at 29 months; r = 0.33, P = 0.08 at 34 months), mammary epithelial area (r = 0.59, P = 0.0007) and uterine weight (r = 0.40, P = 0.03), while serum genistein and daidzein did not correlate significantly with any of these measures (r < 0.4, P > 0.05 for all).

Discussion
In this study we investigated the long-term effects of dietary soy isoflavonoids on endogenous estrogen metabolism. Total serum isoflavonoid concentrations were comparable to those reported in human trials, while equol rather than genistein was the predominant serum isoflavonoid detected. The diet containing isoflavonoids resulted in lower concentrations of serum E₁ and increased excretion of 2-hydroxyestrone, producing an estrogen profile consistent with lower breast cancer risk in human populations. Within breast tissue, isoflavonoid intake resulted in lower intramammary E₂ but minimal effects on mRNA expression of key enzymes involved in local estrogen formation and conjugation. Among isoflavonoid-fed animals, serum equol concentrations were inversely associated with several estrogen-responsive risk markers in the breast and uterus. These findings suggest that exposure to certain soy isoflavonoids, equol in particular, may alter endogenous estrogen exposure by modulating pathways of estrogen catabolism.

Estrogens circulate predominantly as inactive sulfates which are deconjugated within peripheral tissues, selectively catabolized and excreted. As estrogen-like phytochemicals, soy isoflavonoids are metabolized using many of these same enzymatic pathways (26,27). In this study, long-term daily treatment with isoflavonoids (for the human equivalent of 9 years) resulted in 21–26% lower serum E₁, a finding consistent with prior epidemiologic evidence in postmenopausal women (28). We investigated two potential mechanisms for this finding, altered E₁ conjugation and increased E₁ catabolism/excretion. Our data suggest that dietary isoflavonoids have minimal effects on E₁ conjugation but potentially important effects on E₁ catabolism. While an aromatase-inhibiting effect cannot be excluded, in this study isoflavonoids did not alter aromatase mRNA in the breast or increase the ratio of androstenedione to E₁ (P > 0.1, data not shown). Moreover, structure-function studies have consistently
found a lack of inhibitory effect for soy isoflavonoids on aromatase activity (51,52).

In this study soy isoflavonoid intake resulted in 54% lower urinary E1 and 72% higher 2OH-E1. This pattern of low E1 and high 2OH-E1 excretion is similar to that reported in populations of high soy-consuming Asian women compared to US women (53). Several short-term intervention studies in women have reported similar (though less robust) findings, with isoflavonoid treatment generally increasing 2OH-E1 excretion and/or the \(2:16alpha\)OH-E1 ratio by 25–50% (31,32). In our model, we have also found that dietary isoflavonoids may enhance clearance of exogenous oral estrogen (33), supporting the notion of a pro-catabolic isoflavonoid effect. These findings may relate specifically to equol, a daidzein metabolite found in high concentrations in the serum of soy-fed monkeys and rodents but only 43–47% of people (19). Recently, a trial in postmenopausal women reported greater 2OH-E1 excretion after soy intake but only in the subset of women producing equol (54). Similar results were found in a separate trial specifically comparing equol producers and non-producers (55). Potential mechanisms underlying this increase in 2OH-E1 excretion include induction of specific cytochrome P450 (CYP) enzymes for 2-hydroxylation (e.g. CYP 1A1 and 1A2) and inhibition of downstream enzymes for 2OH-E1 metabolism (e.g. peroxidases), which may lead to a variety of reactive quinone species (6). Further studies are needed to identify any such molecular targets and characterize isoflavonoid/equol effects on a wider spectrum of estrogen catabolites.

Data from the CEE group suggest that exogenous oral conjugated estrogens may negatively regulate estrogen activation within tissues. Over 95% of serum and urinary \(E_1\) from CEE was conjugated, with \(E_1\) predominating in serum and \(E_1\)G in urine. Within the breast, CEE resulted in decreased mRNA for two key enzymes responsible for \(E_2\) formation (sulfatase and 17\(beta\)-HSD type 1) and increased transcripts of the \(E_2\)-buffering 17\(beta\)-HSD type 2 enzyme. CEE also significantly decreased the estrogen precursors

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### Table III. Tertile analysis of SPI+ group based on serum equol concentrations

<table>
<thead>
<tr>
<th>Equol tertiles</th>
<th>P values</th>
<th>Trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Equol</td>
<td>Low</td>
<td>Mid</td>
</tr>
<tr>
<td>ng/ml</td>
<td>47.5</td>
<td>117.4</td>
</tr>
<tr>
<td>(E_1): 29 months</td>
<td>(43.4–52.1)</td>
<td>(107.6–128.2)</td>
</tr>
<tr>
<td>(E_1)S: 29 months</td>
<td>n = 6</td>
<td>n = 10</td>
</tr>
<tr>
<td>ng/ml</td>
<td>0.52</td>
<td>0.25</td>
</tr>
<tr>
<td>(E_1): 34 months</td>
<td>(0.40–0.68)</td>
<td>(0.19–0.33)</td>
</tr>
<tr>
<td>(E_1)S: 34 months</td>
<td>n = 9</td>
<td>n = 7</td>
</tr>
<tr>
<td>ng/ml</td>
<td>34.8</td>
<td>34.1</td>
</tr>
<tr>
<td>Mammary gland</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mammary epithelial area</td>
<td>(0.91–1.41)</td>
<td>(0.47–0.72)</td>
</tr>
<tr>
<td>Mammary Ki67 mRNA</td>
<td>6.2</td>
<td>2.9</td>
</tr>
<tr>
<td>Mammary 2\textsuperscript{\textasciitilde}\textsuperscript{AMY} mRNA</td>
<td>(4.3–8.8)</td>
<td>(2.1–4.1)</td>
</tr>
<tr>
<td>Mammary TFF1 mRNA</td>
<td>129.8</td>
<td>43.1</td>
</tr>
<tr>
<td>Mammary 2\textsuperscript{\textasciitilde}\textsuperscript{AMY} mRNA</td>
<td>(75.3–223.6)</td>
<td>(24.3–76.4)</td>
</tr>
<tr>
<td>Uterus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uterine weight</td>
<td>(0.69)</td>
<td>0.42</td>
</tr>
<tr>
<td>g/kg BW</td>
<td>(0.59–0.80)</td>
<td>(0.37–0.49)</td>
</tr>
<tr>
<td>Endometrial thickness</td>
<td>1.16</td>
<td>0.99</td>
</tr>
<tr>
<td>mm</td>
<td>(1.00–1.34)</td>
<td>(0.86–1.14)</td>
</tr>
</tbody>
</table>

*Values represent means (90% CI). Data were analyzed using one-way analysis of variance. \(n = 9–10\) per group unless otherwise indicated. SPI+ = soy protein with the human equivalent of 129 mg isoflavonoids per day. For conversion to SI units (nmol/l), multiply equol values by 4.13. \(E_1\), estrone; \(E_1\)S, estrone sulfate.
androstenedione and testosterone compared to SPI− (P < 0.05 for both), reported previously (56). This apparent autoregulation of local estrogen formation, which is supported by prior in vitro work (57), may help explain the greater breast cancer risk associated with small changes in endogenous post-menopausal E1 or E2 (produced locally within tissues) (3) compared to exogenous estrogen administration (58).

Evidence from this study suggests that chronic exogenous hormone treatment may in turn alter dietary isoflavonoid metabolism. Total serum isoflavonoid concentrations at 34 months were 65% higher in the SPI+ subset of animals in the present study (OC−) compared with the other half of SPI+ animals in the larger trial (34) that received 2.5 years of oral contraceptives (OC+) premenopausally (P = 0.02). (At the start of the premenopausal phase, animals were randomly assigned by social group to OC− and OC+ treatments and then treated in parallel with otherwise identical casein/lactalbumin-based diets over the same time frame.) The difference in isoflavonoids was most pronounced for equol, which was 75% higher in OC− versus OC+ animals (P = 0.008). Genistein (+62%) and daidzein (+47%) were also higher in OC− versus OC+ animals, although these differences were not significant (P > 0.1 for both). Potential mechanisms for such an effect include persistent alteration of hepatic isoflavonoid metabolism, changes in enterohepatic circulation and/or shifts in gut flora leading to altered isoflavonoid bioavailability. This observation suggests that prior hormone use may be a contributing factor for any potential differences in isoflavonoid metabolism between women in the US and women in Asia (e.g. 59), who are less likely to use OC (60).

Results of this study identify soy isoflavonoids as potential dietary determinants of endogenous estrogen metabolism. We found a high degree of similarity between monkey and human serum and urinary estrogen patterns, intramammary estrogen concentrations and sequence identity for estrogen-metabolizing enzymes. Our data indicate that long-term isoflavonoid intake may facilitate specific pathways of estrogen 2-hydroxylation, which could in turn increase estrogen excretion and lower estrogen exposure in reproductive tissues. We found no estrogen agonist effects of isoflavonoids, consistent with previous data in this model (33,34,61). Future studies should focus on more detailed analysis of estrogen hydroxylation pathways, potential isoflavonoid effects on estrogen-related DNA damage in the breast and the specific role of equol in estrogen carcinobolism.

Supplementary data

Supplementary data are available at Carcinogenesis online

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Conflict of Interest Statement: None declared.

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