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Increased Urinary Excretion of 2-Hydroxyestrone but not 16α-Hydroxyestrone in Premenopausal Women during a Soya Diet Containing Isoflavones[1]

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ABSTRACT

Asian diets high in soy are associated with lower risk for breast cancer compared with Western diets. Moreover, higher levels of two putative carcinogenic metabolites of 17β-estradiol, 4- and 16α-hydroxyestrogen, and lower amounts of anticarcinogenic metabolites, 2-hydroxyestrogens, have been associated with greater breast cancer risk. In this study, we tested the hypothesis that consumption of a soya diet containing the weakly estrogenic isoflavones genistein and daidzein may alter the metabolism of 17β-estradiol to 2- and 16α-hydroxylated products. Eight premenopausal women were placed on a soya-containing, constant diet in a metabolic unit. The diet provided 400 kilocalories from soymilk and 113–202 mg/day (158 ± 26 mg/day, mean ± SD) isoflavones daily for a complete menstrual cycle. After a washout period of 4 months, the subjects consumed the same diet, but with soymilk that contained <4.5 mg/day isoflavones ("isoflavone-free"). Urine samples were collected for 24 h daily for the entire cycle during each soya diet period for the analysis of daidzein, genistein, and 2- and 16α-hydroxyestrone. Subjects excreted measurable amounts of daidzein (11.6–39.2 mg/day) and genistein (2.9–18.2 mg/day) during the isoflavone-rich soya diet but not during the isoflavone-free soya diet. The diet rich in isoflavones increased the cycle mean daily urinary excretion of 2-hydroxyestrone (averaged over the entire cycle) from 11.6 ± 2.06 to 17.0 ± 2.96 nmol/12-h ($P = 0.03$), a 47% increase. However, the mean daily excretion of 16α-hydroxyestrone did not change (7.0 ± 1.14 nmol/12-h during the isoflavone-free and 7.7 ± 1.25 nmol/12-h during the isoflavone-rich diet; $P = 0.36$). The ratio of 2-hydroxyestrone to 16α-hydroxyestrone was higher during the isoflavone-rich soya diet (2.6 ± 0.34) than during the isoflavone-free diet (2.0 ± 0.32; $P = 0.01$), a 27% increase. These results suggest that soya isoflavones increase the metabolism of endogenous estrogens to the protective 2-hydroxylated estrogens in women, and this may play an important role in lowering 17β-estradiol levels and the long-term risk for breast cancer.

INTRODUCTION

Breast cancer rates in Asian populations are significantly lower than in Western populations (1). Migration studies indicate that this may be attributable largely to differences in lifestyle, and diet in particular (2, 3). Traditional Asian diets may be protective because they are often rich in soya products (20–60% of daily protein provided by soy) and other components such as fiber, and lower in fat (4). There is epidemiological evidence to suggest that soya consumption in particular is associated with reduced rates of breast, prostate, and endometrial cancers (5–12). Isoflavone levels were lower in the urine of breast cancer cases compared with case-controls of population in Shanghai, China (13).

Soya contains a variety of potentially cancer-preventive components, including the weakly estrogenic isoflavones, daidzein and genistein (14). These isoflavones, which are present at levels of 1–2 mg/g protein, can act as estrogen agonists or antagonists; inhibit cell proliferation, angiogenesis, and tyrosine kinase; and induce cell differentiation (reviewed in Ref. 14). All of these biological effects may contribute substantially to cancer prevention. Populations consuming large amounts of soy excrete high levels of these isoflavones and their metabolites (15–17).

Breast cancer risk is associated with higher levels of certain endogenous hormones, and especially ovarian steroids (18–21). Many studies have led to recognition that higher levels of 17β-estradiol are risk indicators for breast cancer (19). 17β-Estradiol regulates breast cell proliferation and therefore can promote breast cancer cell growth. We and others have studied the effects of soya consumption on reproductive hormones and factors that are potential markers of breast cancer risk (11, 22–31).

We found previously that consumption of soya for 1 month significantly reduced serum levels of 17β-estradiol and progesterone in women (26). In a separate study, we further showed that these decreases occur through the entire menstrual cycle (32, 33). Soya-induced reduction of 17β-estradiol can be a consequence of reduced synthesis and/or increased metabolism of ovarian steroids. To elucidate further the mechanism by which soya reduces estrogen levels, we examined the effects of soya diets with and without isoflavones on estrogen metabolites in regularly cycling women.

17β-Estradiol is extensively metabolized, primarily in the liver, to 2-, 4-, and 16α-hydroxylated estrogens (Fig. 1), which have differential capacities to influence mammary tumorigenesis (34–39). 2-Hydroxylated estrogens are suggested to be anticarcinogens, whereas 4- and 16α-hydroxylated estrogens may enhance cancer development. These properties may relate to their differential mutagenic, genotoxic, carcinogenic, angiogenic, and cytotoxic effects, and differential affinity for estrogen receptors (35, 38–44).

The relationships of these metabolites to breast cancer risk have been studied in humans with varying results (34, 35, 45–52). Regular exercise increases the levels of 2-hydroxyestrone and decreases the risk of breast cancer in women (53, 54). Women with breast cancer had higher levels of 16α-hydroxyestrone compared with non-breast cancer controls (34, 35). Ratios of 2-hydroxyestrone to 16α-hydroxyestrone were found to be higher in the breast cancer cases compared with controls (45–49, 55). However, some studies failed to support this pattern of breast cancer-associated changes in oxidative estrogen metabolism (50–52).

Studies of the effects of dietary intervention on hydroxylated estrogen metabolites are few, but in general they support the idea that diet can substantially influence oxidative estrogen metabolism. For example, a high protein diet increased the 2-hydroxylation but not the 16α-hydroxylation of tracer estradiol (56). Indole-3-carbinol, a dietary component, increased ratios of 2-hydroxyestrone to estriol in healthy women (57–59). Diets supplemented with soya decreased urinary excretion of 4- and 16α-hydroxyestrone but had no effect on 2-hydroxyestrone in free-living women on unrestricted diets (30). The present study in women consuming defined diets tested the hypothesis

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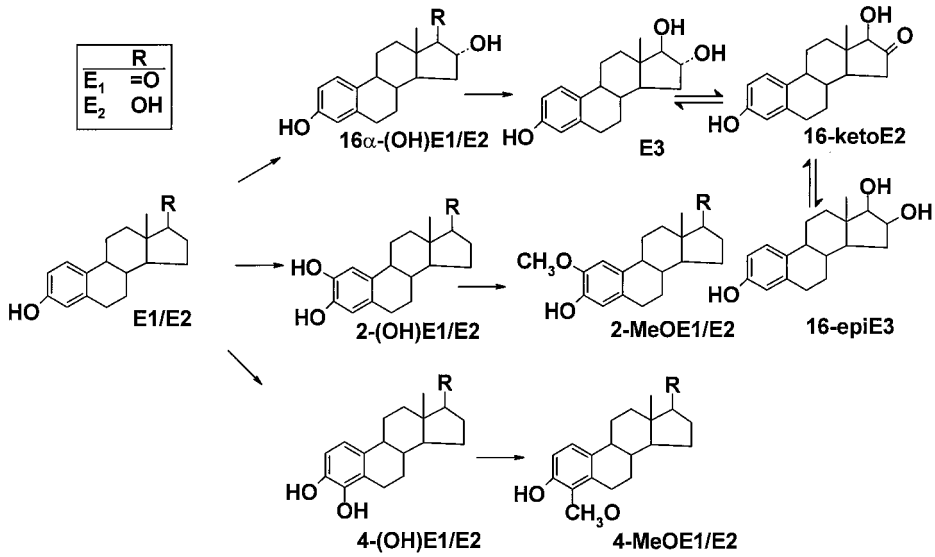

Fig. 1. The metabolism of estrogen. *E1*, estrone; *E2*, 17β-estradiol; *E3*, estriol.

that isoflavones in soya can modulate oxidative metabolism of endogenous estrogens.

MATERIALS AND METHODS

Study Design. This was a longitudinal crossover study to determine the effect of a soya diet rich in isoflavones on estrogen metabolism in premenopausal women. The study consisted of two controlled diet periods, each lasting for a full menstrual cycle, with an intervening 4-month washout period. In a previous study, we found that the effect of a soya diet on ovarian hormones did not persist for more than 2 months after termination of soya consumption (26). The study procedures were approved by the Institutional Review Board of the University of Texas Medical Branch. Written informed consent was obtained from each subject.

Subject Selection. Subjects were premenopausal women who were healthy as determined by history, physical examination, standard blood cell counts, clinical chemistry determinations, and serum ferritin levels. Vegetarians, smokers, and those consuming more than two alcohol-containing drinks per month were excluded. All subjects had a BMI[3] <36 kg/m^2, had no significant recent changes in weight or eating habits, had regular menstrual cycles, and had not taken contraceptive medications during the 6 months prior to the study. Contraceptive medications were not allowed during the study. Small doses of acetaminophen or aspirin were permitted during the study. One woman took replacement levothyroxin (0.1–0.25 mg/day) for hypothyroidism and was determined to be euthyroid, and another took sertraline (25 mg/day) for mild depression before and during both dietary intervention periods. Six subjects were Caucasians, and two were African Americans. All but one African-American woman were nulliparous. Table 1 summarizes the characteristics of the study subjects.

Soya Feeding and Sample Collections. Subjects were admitted twice to the GCRC at UTMB, each for a complete menstrual cycle, with an intervening four cycles between admissions. Subjects were admitted on cycle day 2 and discharged on cycle day 2 of the subsequent cycle. Day 1 was the first day of menstrual spotting, and was recorded for every cycle while enrolled in the study.

During both admissions to the GCRC, subjects consumed a constant diet calculated to maintain body weight, based on the Harris-Bennedict equation with adjustment for physical activity (60, 61). The subjects consumed their usual home diets during the 4-month washout period.

The soya diet consisted of three daily rotating menus and included a daily 36-oz portion of soymilk. The daily energy distribution of the soya diet was 35.5% fat, 14.0% protein, and 50.1% carbohydrate, which is similar to typical Western diets (62). Each 36-oz portion of soymilk provided 400 kilocalories, 37.9 g of protein, 20.3 g of fat, and 16.6 g of carbohydrates, and was ingested daily between 5 and 8 p.m. without other foods in place of an evening meal. During the first admission, subjects ingested a homogenized, pasteurized soymilk prepared from soybeans. This soymilk contained a significant quantity of isoflavones (113–202 mg/day) and no preservatives (Banyan Foods, Houston, TX). Lots selected for the study were frozen until the day of ingestion. Several different lots were used for this study, but each subject ingested soymilk from the same lot throughout the study. During the second admission, the subjects ingested a different soymilk preparation containing <4.5 mg/day isoflavones but similar amounts of energy and macronutrients (Protein Technologies Inc., St. Louis, MO) as the soymilk used for the first soy-feeding period. All meals and soymilk were consumed in the GCRC under direct supervision. Subjects continued their daily routines, including work, study, and exercise.

Fasting blood samples were obtained between 7 and 9 a.m. on cycle days 5 and 7, and then daily from day 9 until the second day of the subsequent cycle for measurement of LH and to determine the date of the LH surge. Two 12-h urine collections were obtained daily from cycle day 2 to cycle day 2 of the subsequent cycle. Urine samples were refrigerated during collection and then stored at -20°C until analyzed. One daily 12-h urine was started immediately after soymilk ingestion and was used for analysis of estrogen metabolites. Isoflavones were measured in both daily 12-h urine collections.

Analysis of Isoflavones in Soymilk and Urine. The isoflavone content in soymilk and in urine were analyzed by a gas chromatography-flame ionization detection method as described (63). Results were calculated from the internal standard added and were expressed as weight of the free forms of daidzein and genistein, respectively.

Table 1 *Demographic characteristics, nutrient intake, isoflavone intake, and urinary levels of isoflavones (mean ± SD) of study subjects*

	Isoflavone-rich	Isoflavone-free
Age (years)	33 ± 6.1	NC[a]
Weight (kg)	71.2 ± 15.4	69.2 ± 14.3
Height (m)	1.63 ± 0.05	NC
BMI (kg/m^2)	26.6 ± 5.2	25.8 ± 4.0
Energy intake (kcal)	2347 ± 175	2354 ± 163
Fat (%)	35.4 ± 0.9	35.6 ± 1.0
Protein (%)	14.0 ± 0.5	14.0 ± 0.5
Carbohydrate (%)	50.1 ± 0.8	50.1 ± 0.8
Daidzein intake (mg/day)	73.2 ± 16.9	~0.94[b]
Genistein intake (mg/day)	84.9 ± 13.3	~2.6[b]
Daidzein excretion (mg/day)	24.6 ± 10.1	~0[b]
Genistein excretion (mg/day)	9.2 ± 6.1	~0[b]
Cycle length (days)	26.6 ± 2.9	25.8 ± 1.9

[a] NC, no change.
[b] $P < 0.0001$.

[3] The abbreviations used are: BMI, body mass index; GCRC, General Clinical Research Center; UTMB, University of Texas Medical Branch; LH, luteinizing hormone; CYP, cytochrome P450; CI, confidence interval.

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Analysis of 2-Hydroxyestrone and 16α-Hydroxyestrone. 2-Hydroxyestrone and 16α-hydroxyestrone concentrations in urine were determined by ELISA using a commercially available kit (Immunacare, Bethlehem, PA) after enzymatic hydrolysis of urine with β-glucuronidase and sulfatase (provided in the kits). Upon receipt, the kits were stored immediately according to the supplier's instruction. The kit included a urine sample that served as a positive control, which was included with each assay. These estrogen metabolites were measured in a 12-h urine sample for each day of both dietary periods (24–30 daily samples per subject in each dietary period). A urine sample from one of the study subjects was included in each run and served as an additional control.

Daily excretion of 2- and 16α-hydroxyestrone was expressed as nmol/12-h, and daily ratios of 2-hydroxyestrone to 16α-hydroxyestrone were calculated. Plasma levels of LH were measured on days near ovulation by specific immunoassay using a commercial kit (Diagnostic Laboratory Inc., Webster, TX) to determine the day of LH surge, which was used as a reference point to separate the follicular and the luteal phases of the cycle. The luteal phase was defined as beginning 1 day after the LH surge.

Statistical Analysis. Daily excretion of 2- and 16α-hydroxyestrone (nmol/12-h) and the daily ratios of 2-hydroxyestrone to 16α-hydroxyestrone were averaged for the entire cycle and for the follicular and luteal phases. The cycle daily mean values during the two different soya diets were then compared by paired *t* test (two-tailed).

RESULTS

A crossover study design with controlled diets during both dietary periods was used to determine the role of isoflavones in regulating the oxidative metabolism of 17β-estradiol in women with regular menstrual cycles. Table 1 shows that the subjects consumed the same amounts of energy and macronutrients during both dietary periods. The energy distribution of the controlled diet was similar to that of typical Western diets (62), but included 400 kilocalories/day from soymilk. The subjects ingested large amounts of isoflavones (~158 mg/day) during the first dietary period, and very little (<4.5 mg/day) during the second. On average, the subjects' weight, BMI, and cycle length did not change during the study.

Intake and Excretion of Isoflavones. Isoflavone content in soybeans is known to vary naturally with season of harvesting, storage, and geographic location of growth (64, 65). Therefore, each lot of soymilk used for the first dietary period was analyzed for isoflavone content. However, the composition in terms of fat, carbohydrate, and protein in the soymilk lots varied no more than 5%. Each subject consumed the same lot of soymilk throughout the first dietary period, but because of variation in isoflavone contents between lots consumed by different subjects, the amount of daidzein ingested ranged from 49.6 to 106.4 mg daily and that of genistein ranged from 63.4 to 100.3 mg (Table 1). Approximately 85 mol % of these isoflavones were present as the glucoside conjugates daidzin and genistin. According to the manufacturer (Protein Technologies), the isoflavone content of this product was ~4.5 mg for the 36-oz daily portion; the portion also contained 37.9 g of protein and 16.6 g of carbohydrate. Soy oil (20.3 g) was added to equalize the fat content of this product to that of the isoflavone-rich soymilk used for the first dietary period.

Daidzein and genistein were measurable in all urine samples collected during the first dietary period, when subjects ingested isoflavone-rich soymilk. The mean daily urinary excretion of daidzein (excreted as daidzein glucuronides and sulfates and expressed as the free forms) was 24.6 ± 10.1 mg/day (mean ± SD; range, 11.6–39.2 mg/day; Table 1). The mean daily genistein excretion was 9.2 ± 6.1 mg/day (range, 2.9 to 18.2 mg/day). The isoflavone excretion expressed as percentage of intake was 33.3 ± 11.3% (range, 19.6–50.7%; not shown in Table 1) for daidzein and 11.2 ± 7.6% (range, 1.6–21.3%) for genistein. There was substantial interindividual variation in urinary excretion of both daidzein and genistein.

Isoflavones were measured in all urine samples from the entire isoflavone-free cycle from two of the eight study subjects, and as expected, isoflavones were not detected. These data suggested that the isoflavone-free soya diets were indeed low in isoflavones. Urinary levels of isoflavones in the remaining six subjects were not analyzed and were assumed to be isoflavone-free.

Intra- and Interassay Variability of 2-Hydroxyestrone and 16α-Hydroxyestrone. The intraassay variability (coefficient of variation) for measurement of 2- and 16α-hydroxyestrone was usually <15%, and if necessary, assays were repeated until variability of the duplicates was within 15%. These estrogen metabolites were measured in the first daily 12-h urine collected immediately following soymilk ingestion. The kits included a urine sample to serve as a positive control. The intra- and interassay variabilities of both hydroxylated estrogen standards were <10%. Although the intraassay variability for 2- and 16α-hydroxyestrone of the external positive control included in the kits was <10%, their interassay variability was 54 and 60%, respectively. However, we also included a urine sample from one of our study subjects in every batch of the immunoassay to determine the assay variability and to serve as an additional quality control. The interassay variability of the internal positive control was <9% for 2-hydroxyestrone and <15% for 16α-hydroxyestrone, which indicates that the assays were reproducible from day to day. It is not clear why the control with the same lot number supplied with the kit displayed more variability than the control sample from one of the study subjects.

Effect of Isoflavones on 2-Hydroxyestrone Levels in Urine. The daily urinary excretion of 2-hydroxyestrone averaged for all subjects throughout a menstrual cycle during the isoflavone-rich and the isoflavone-free soya diets is shown in Fig. 2*A*. Data in Fig. 2*A* were compiled using the day of serum LH surge as a reference point. Because interindividual variability in cycle length generally was associated with more variation in follicular phase length than the luteal phase length (66), using the LH surge as a reference point provided more consistent cyclical profiles. The cyclical profiles of 2-hydroxyestrone during both soya dietary periods resembled the typical cyclical profiles of 17β-estradiol (67, 68). Mean daily levels of 2-hydroxyestrone were higher over the entire cycle when the subjects were consuming the isoflavone-rich soya diet than when they were consuming the isoflavone-free soya diet. The increases were apparent in both the follicular and luteal phases.

Fig. 2*B* shows the individual mean daily levels (expressed as amount excreted in 12 h), averaged over a cycle or a menstrual phase, and the group mean daily levels during the two soya diet periods. All eight subjects had higher mean daily levels of 2-hydroxyestrone averaged over the full cycle during the isoflavone-rich soya diet than during the isoflavone-free soya diet, and seven of eight subjects had higher mean daily levels over both the follicular and luteal phases ($P < 0.05$ for the group). The group mean total cycle level of 2-hydroxyestrone during the isoflavone-rich soya diet (17.0 ± 2.96 nmol/12-h, mean ± SE) was 1.5-fold higher than that during an isoflavone-free soya diet (11.6 ± 2.06 nmol/12-h; $P = 0.03$). The group mean level during the follicular phase was 1.5-fold higher during the isoflavone-rich diet (15.9 ± 2.85 nmol/12-h) than during the isoflavone-free diet (10.5 0 ± 0 1.97 nmol/12-h, $P = 0.03$). The group mean luteal phase level of 2-hydroxyestrone was 1.4-fold higher during the isoflavone-rich diet (18.4 ± 3.25 nmol/12-h) than during the isoflavone-free diet (12.9 ± 2.28 nmol/12-h; $P = 0.05$). Thus, a diet containing isoflavones increased 2-hydroxyestrone levels by >40% compared with the diet essentially free of isoflavones, and the increase was observed throughout the menstrual cycle.

Effect of Isoflavones on 16α-Hydroxyestrone Excretion. Fig. 3*A* shows the mean daily urinary excretion of 16α-hydroxyestrone averaged for all subjects in relation to the LH surge during the two

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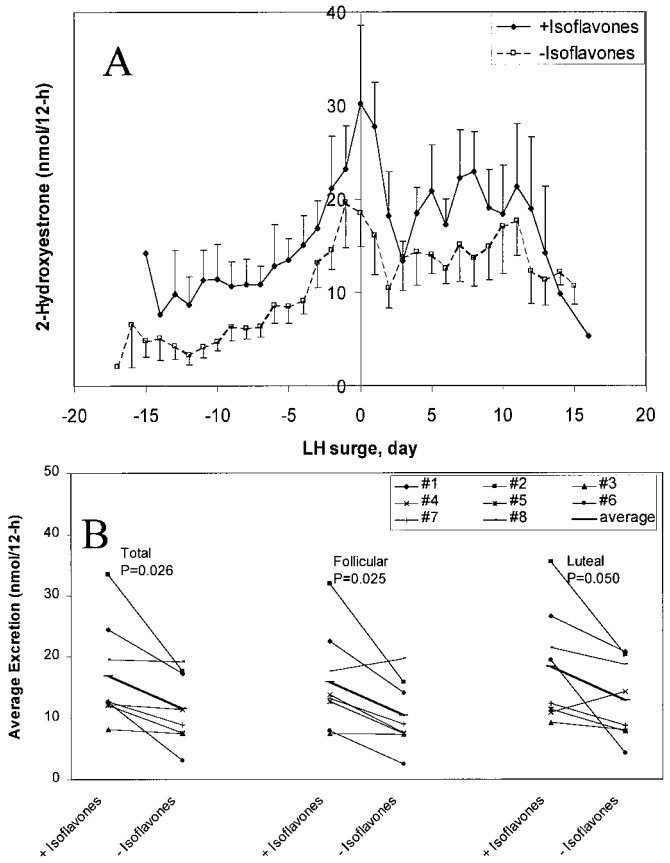

Fig. 2. Effects of soya isoflavone consumption on urinary excretion of 2-hydroxyestrone in premenopausal women. Subjects (n = 8) consumed a constant calculated soya diet for a full menstrual cycle containing ~150 mg/day isoflavones (*+isoflavones*), and 4 months later on an identical diet deficient in isoflavones (*−isoflavones*) for another full menstrual cycle. *A*, daily levels (expressed as amount/12-h) averaged for the group and plotted in relation to serum LH surge (day 0 in *X-axis*); *B*, individual and group daily means for the total cycle, the follicular phase, and the luteal phase. Differences related to diet were compared by paired t test (two-tailed). *Numbers* in legend for *B* indicate identification numbers of study subjects.

different soya diet periods. The mean daily levels of 16α-hydroxyestrone did not differ between the two soya dietary periods. This was confirmed by comparing the individual mean daily levels averaged over the cycle or each menstrual phase between the two dietary periods (Fig. 3*B*). The group mean daily level of 16α-hydroxyestrone averaged over the entire cycle during the isoflavone-rich soya diet (7.7 ± 1.25 nmol/12-h, mean ± SE) was not significantly different from that during the isoflavone-free soya diet (7.0 ± 1.14 nmol/12-h; P = 0.36). The group mean follicular phase level during the isoflavone-rich diet (7.1 ± 1.21 nmol/12-h) was not significantly different from that during the isoflavone-free diet (6.2 ± 1.09 nmol/12-h; P = 0.49). The group mean luteal phase level of 16α-hydroxyestrone during the isoflavone-rich diet (8.6 ± 1.59 nmol/12-h) was not significantly different from that during the isoflavone-free diet (7.9 ± 1.31 nmol/12-h; P = 0.19). Thus, 16α-hydroxyestrone levels were not affected by the presence of isoflavones in the soya diets during either phase of the cycle.

Effect of Isoflavones on the Ratio of 2-Hydroxyestrone to 16α-Hydroxyestrone. Because 2- and 16α-hydroxyestrone are metabolic products of estrone, many studies have examined the relationship between the ratios of 2-hydroxyestrone to 16α-hydroxyestrone and the relative risk of developing breast cancer, as discussed earlier. Fig. 4*A* shows that the cyclical profiles of this ratio closely resemble that of 2-hydroxyestrone. The ratios were higher during the isoflavone-rich soya diet than during the isoflavone-free soya diet. Fig. 4*B* shows that the mean daily ratios were higher during the isoflavone-rich soya diet than during the isoflavone-free soya diet in seven of the eight subjects when averaged over the entire cycle, over the follicular phase, or over the luteal phase. The group means during the isoflavone-rich and isoflavone-free soya diets were 2.6 ± 0.34 and 2.0 ± 0.32, respectively, for the entire cycle (P = 0.01); 2.5 ± 0.38 and 2.0 ± 0.32, respectively, for the follicular phase (P = 0.028); and 2.4 ± 0.34 and 2.0 ± 0.32, respectively, for the luteal phase (P = 0.037). The ratios were higher by 24–30% during the isoflavone-rich soya diet than during the isoflavone-free soya diet. The increase in the ratio is attributable primarily to an increase in the amount of 2-hydroxyestrone.

DISCUSSION

We showed previously that soya consumption consistently reduces circulating levels of 17β-estradiol in women (26). Because an alteration in estrogen metabolism is a potential mechanism for this effect of soya on levels of 17β-estradiol, excretion of the major estrogen metabolites was examined in this study during the consumption of two soya diets, one of which contained high levels of isoflavones (~158 mg/day) whereas the other was essentially isoflavone free (<4.5 mg/day). Isoflavones were measurable in urine during the isoflavone-rich soya dietary period. Urinary excretion of 2-hydroxyestrone was significantly greater over the entire cycle during the isoflavone-rich soya diet than during the isoflavone-free soya diet, but the excretion

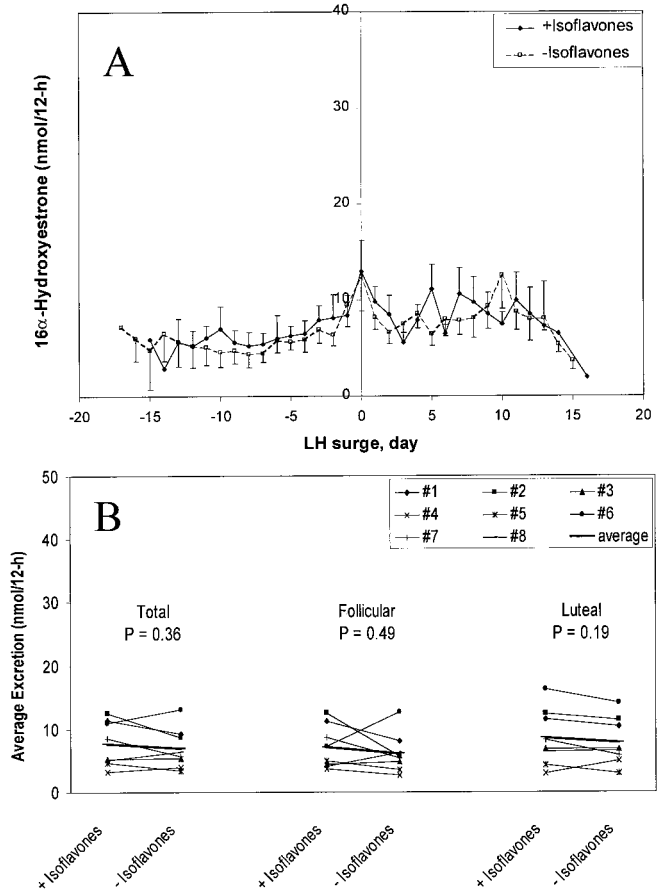

Fig. 3. Effects of soya isoflavone consumption on urinary excretion of 16α-hydroxyestrone in premenopausal women. *A*, cyclical mean daily levels for the group; *B*, individual and group daily means for the total cycle, the follicular phase, and the luteal phase. For study details, see legend of Fig. 2.

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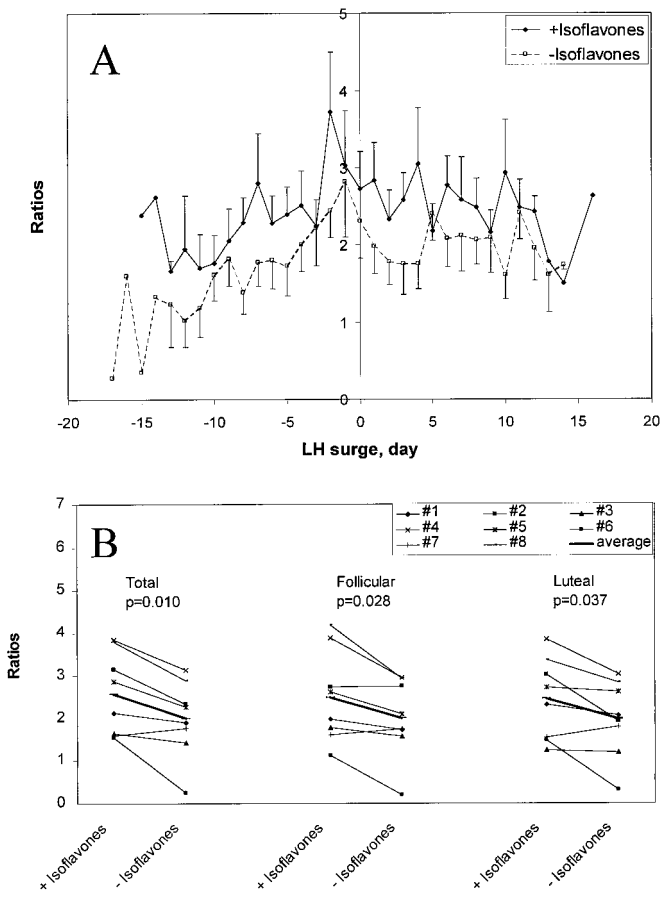

Fig. 4. Urinary ratios of 2-hydroxyestrone to 16α-hydroxyestrone in relation to LH surge day (*A*), and individual and group daily mean ratios during an isoflavone-containing soya diet (*+isoflavones*) and an isoflavone-free (*−isoflavones*) soya diet averaged over the entire menstrual cycle, the follicular phase, and the luteal phase (*B*). For study design, see legend of Fig. 2. Urinary ratio of 2-hydroxyestrone to 16α-hydroxyestrone was calculated for each day of the cycle for each subject.

of 16α-hydroxyestrone did not differ. Likewise, the ratios of 2-hydroxyestrone to 16α-hydroxyestrone were higher during the isoflavone-rich diet than during the isoflavone-free diet.

The two diets were isocaloric, and as expected, the body weights and BMIs of the subjects did not change. The two diets also had similar types and amounts of fat, protein, and carbohydrate. Thus, intake of energy and macronutrients cannot account for the difference in 2-hydroxyestrone excretion. Because the two soya diets were virtually identical except for differences in isoflavone content, the results indicate that isoflavones in the soya diet were responsible for the increase in 2-hydroxyestrone excretion. Although these results suggest that isoflavones can mediate a potentially important metabolic effect of soya feeding on estrogen metabolism, it should be noted that isoflavones were removed by alcohol extraction in preparing the isoflavone-free soya product, and it is possible that this process might remove one or more other components, presently not identified, that could alter estrogen metabolism.

The increase in 2-hydroxyestrone excretion presumably reflects an increase in 2-hydroxylation of endogenous estrogen, and suggests that isoflavones affect the enzyme(s) involved in the formation of 2-hydroxyestrone. Genistein and daidzein have been shown to inhibit 17β-hydroxysteroid oxidoreductase, the enzyme that catalyzes the conversion of estrone to 17β-estradiol (69). Isoflavones are weak competitive inhibitors of aromatase, the enzyme that converts testosterone to 17β-estradiol (70). However, these two enzymes are not involved in estrogen 2-hydroxylation, which has been shown to involve CYP1A1 (71), CYP1A2, and CYP3A4 (72). Genistein is a poor inhibitor of CYP1A1 *in vitro* (73) and a poor inducer of CYP1A1 in mice (74). The differential effects of isoflavones, and perhaps other alcohol-extractable components of soya, on cytochrome P450 enzymes involved in estrogen 2-, 4-, and 16α-hydroxylation deserves further study because the different pathways of estrogen hydroxylation lead to products that have differential influence on carcinogenesis as discussed below (42).

16α-Hydroxyestrone is reactive and can bind covalently to protein, damage DNA, and increase cell transformation rates (43, 75). Both 2- and 4-hydroxyestrogens can undergo redox cycling, leading to the formation of free radicals (76) that can directly damage DNA (38, 41). 4-Hydroxyestrone is a carcinogen in the hamster kidney. However, 2-hydroxyestrone has not been found to induce tumors, possibly because of its shorter half-life than that of 4-hydroxyestrone (40, 41). Moreover, 2-hydroxyestrone inhibits cell growth in cultures (77, 78), and after further metabolism, 2-methoxyestrogens are formed (39). 2-Methoxyestradiol exhibits strong antiangiogenic effects, inhibits tubulin formation, and is cytotoxic to tumor cells in culture (39, 79). The 2-, 4-, and 16α-hydroxyestrogens bind to estrogen receptors with differential affinity, and subsequent receptor-mediated responses vary accordingly (38). Compared with the parent compound 17β-estradiol, 16α-hydroxyestrone binds more strongly and covalently to estrogen receptors and induces more persistent biological effects (75). 4-Hydroxyestrone binds with similar affinity and 2-hydroxyestrone with lower affinity compared with 17β-estradiol. Therefore, 2-hydroxylation presumably is a more oncoprotective pathway for estrogen metabolism than is 4- or 16α-hydroxylation. The ability of soya isoflavones to increase estrogen 2-hydroxylation, as indicated by the effects on 2-hydroxyestrone excretion reported here, have important implications for breast cancer prevention.

Indole-3-carbinol, another potential dietary anticarcinogen, increases urinary ratios of 2-hydroxyestrone to estriol when administered to both obese and nonobese women (57, 59). This increase is due mostly to an increase in 2-hydroxyestrone, whereas 16α-hydroxyestrogen is less affected. This result is consistent with the pattern of changes induced by the administration of soya isoflavones reported here. The extent of increase in 2-hydroxyestrone excretion induced by 400 mg/day of indole-3-carbinol for 1 month was ~30% (57), and we observed a ~50% increase after 150 mg/day of isoflavones for 1 month. Thus, indole-3-carbinol and isoflavones appear to have similar potencies in affecting 2-hydroxyestrone levels. An increase in the protein/carbohydrate of the diet increased radiolabeled estradiol 2-hydroxylation but had no effect on estradiol 16α-hydroxylation (56). Collectively, these previous reports and the present observations indicate that 2- and 16-hydroxylation of endogenous estrogens can be differentially regulated by diet, and that specific macro- and micronutrients can modulate these pathways.

Xu *et al.* (30) in a study of women consuming self-selected diets found a decrease in 16α-hydroxyestrone excretion but no change in 2-hydroxyestrone during consumption of soya containing isoflavones. These results differ from ours perhaps because of differences in study design. Our study subjects consumed controlled and constant diets provided in a metabolic unit, whereas those of Xu *et al.* (30) consumed their usual home diets with and without soya supplementation. As discussed above, micro- and macronutrients can alter the oxidative metabolism of estrogen, and an effect of soya isoflavones may be influenced or obscured by other dietary changes. Because of cyclical changes in ovarian hormones, urine samples were obtained frequently and for one complete menstrual cycle for both dietary periods in our study, whereas a single 3-day collection was obtained in the study of

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Xu *et al.* (30). Washout periods also differed, and were four cycles in the present study and 3 weeks in the study of Xu *et al.* (30).

Studies in Caucasians, African Americans, and Asians have shown that ratios of 2-hydroxyestrone to 16α-hydroxyestrone are lower in breast cancer cases than controls by ~30% (45, 46, 48, 49). Thus, the 30% change in the ratio of 2-hydroxyestrone to 16α-hydroxyestrone induced by isoflavone exposure reported here is a degree of change that is likely to have biological significance.

In this study, our subjects ingested ~75 g (37.9 g of protein, 20.3 g of fat, and 16.6 g of carbohydrate) of soy food per day, which is within the range of soy food intake (geometric mean, 89.1 g/day; 95% CI, 67.0–118.5 g/day) but higher in terms of soy protein intake (geometric mean, 8.5 g/day; 95% CI, 6.8–10.8 g/day; highest, 39 g/day) consumed by women in Shanghai, China (80), who are at relatively low risk for breast cancer. Because of natural variability of isoflavones in soybeans, our subjects ingested 113–202 mg (based on actual measurement) of isoflavones per day, which is higher than the mean daily intake (geometric mean, 33.4 mg/day; 95% CI, 26.8–42.5 mg/day, estimated using database) but still within the reported intake range (highest, 150 mg/day) consumed by women in Shanghai (80) and in Japan (81). Our subjects excreted variable amounts of isoflavones with a mean of 33.8 mg/day (~88 nmol/mg creatinine; range, 33–149 nmol/mg creatinine, based on analysis of 24-h urine samples), which is 10-fold higher than the average excretion rate of isoflavones (geometric mean, 8.4 nmol/mg creatinine; 95% CI, 5.6–12.6 nmol/mg creatinine, based on analysis of morning spot urine samples) but within the excretion range (the highest being 120 nmol/mg creatinine) reported for Shanghai women (80). These differences between the isoflavone intake and excretion but not in the soy intake of our subjects and those of women in Shanghai are not unexpected and probably can be accounted for by differences in sample collection and data analysis. A detailed comparison of the isoflavone exposure levels between this intervention study and those observed in population-based studies, therefore, may not be possible. Nonetheless, the amounts of soy isoflavones consumed by our study subjects were within the upper portion of the ranges of consumption and excretion rates reported for oriental populations. Therefore, the change in 2-hydroxyestrone observed after soymilk consumption reported here may be biologically relevant for breast cancer prevention.

Epidemiological studies have linked soya consumption to a reduced risk for breast cancer. We previously demonstrated that 1 month of soya consumption reduced circulating levels of ovarian steroids in premenopausal women (26). The present study showed for the first time that 2-hydroxyestrone and the ratio of 2-hydroxyestrone to 16α-hydroxyestrone are increased in women by the consumption of a soya diet containing isoflavones. Thus, decreased circulating levels of ovarian hormones and an increase in the formation of potentially anticarcinogenic 2-hydroxyestrone may be dual mechanisms for breast cancer prevention by soya. In summary, our results provide data in healthy women that add support for the hypothesis that a soya diet can be effective in breast cancer prevention.

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