

Decreased Ovarian Hormones during a Soya Diet: Implications for Breast Cancer Prevention¹

Lee-Jane W. Lu,² Karl E. Anderson, James J. Grady, Fortune Kohen, and Manubai Nagamani

Departments of Preventive Medicine and Community Health [L.-J. W. L., K. E. A., J. J. G.] and Obstetrics and Gynecology [M. N.], The University of Texas Medical Branch, Galveston, Texas 77555, and Department of Biological Regulation, The Weizmann Institute of Science, Rehovot 76100, Israel [F. K.]

ABSTRACT

Ovarian hormones are biomarkers for breast cancer risk. Soybean consumption may be responsible in part for lower levels of ovarian hormones and decreased rates of breast cancer in women in Asia compared with Western populations. Soybeans contain a significant amount of the isoflavones daidzein and genistein, which are weak estrogens. The purpose of this study was to determine whether soya feeding decreases circulating levels of ovarian hormones and gonadotropins. Ten healthy, regularly cycling women consumed a constant soya-containing diet on a metabolic unit, starting on day 2 of a menstrual cycle until day 2 of the next cycle. Blood and urine samples were obtained daily for one menstrual cycle before and during soy feeding. The diet was calculated to maintain constant body weight, included 400 kilocalories from a 36-ounce portion of soymilk, and provided 113–207 mg/day (154.0 ± 8.4 mg/day, mean \pm SE) of total isoflavones. For the group, the soya diet provided more carbohydrate and less protein than the home diets. Daily consumption of the soya diet reduced circulating levels of 17β -estradiol by 25% ($P < 0.01$, Wilcoxon signed rank test, two-tailed) and of progesterone by 45% ($P < 0.0001$) compared with levels during the home diet period but had no effect on luteinizing hormone or follicle-stimulating hormone. Mean menstrual cycle length did not change during the soya diet; a slight decrease in mean luteal cycle length was marginally statistically significant ($P = 0.06$). Urinary excretion of isoflavones was 33.8 ± 5.3 mg/day (mean \pm SE) and when expressed as percentage of intake, varied substantially ($21.9 \pm 3.3\%$ of intake; range, 9.1–36.7%) among the subjects. Mean daily serum levels of daidzein and genistein (free and conjugated forms) 15 h after soymilk were 2.89 ± 0.53 μ g/ml and 0.85 ± 0.22 μ g/ml, respectively, indicating systemic bioavailability of these substances. Secondary analyses by multiple regression showed that decreases in follicular and luteal phase 17β -estradiol levels were positively associated with urinary isoflavone excretion, an association affected by age, and were inversely associated with decreases in protein intake. Decreases in progesterone levels during the soya diet were inversely associated with increases in intakes of genistein and were affected by the interaction of the intakes of daidzein with energy or with fiber. Consumption of an isoflavone-containing soya diet reduced levels of ovarian steroids in normal women over the entire menstrual cycle without affecting gonadotropins. This suggests that at least under the conditions of this study, soya-induced reductions of circulating ovarian steroids are not mediated by gonadotropins. Decreases in ovarian hormones are related to isoflavones contained in soy and also to energy intake and other components such as protein and fiber but not fat. Our results may explain decreased ovarian hormone levels and decreased risk of breast cancer in populations consuming soya diets and have implications for reducing breast cancer risk by dietary intervention.

INTRODUCTION

Breast cancer is a serious public health problem, especially in more affluent Western countries (1). Geographic and ethnic differences in breast cancer risk may be attributable in large part to environmental factors (2–6). Immigrants to Western countries who generally adopt the dietary habits of the host country are at increased risk for breast and other hormone-dependent cancers (2, 6–8). Epidemiological studies have associated dietary consumption of soy products with a reduced risk for breast and other cancers (9–15). For example, in premenopausal women in Singapore and pre- and postmenopausal Asian women in California and Hawaii, breast cancer risk was inversely related to soy protein intake (10, 11, 13, 16). Consumption of rice and tofu (a soy product) was inversely related to prostate cancer risk among men of Japanese ancestry in Hawaii (9). Urinary levels of phytoestrogens were lower in breast cancer cases compared with case-controls (14, 17). These epidemiological observations are supported by results of animal studies in which soya feeding was protective against experimentally induced mammary and other organ cancers (12, 18).

Ovarian hormone levels (19–22) and related reproductive factors (e.g., age of menarche, menopause, and parity; Refs. 4 and 5) are known to influence breast cancer development. Increased levels of estrogens in blood and urine correlate with increased risk for breast cancer (22–24). Serum levels of estrogens and androgens are in general lower in women who live in or have recently immigrated from low-risk areas for breast cancer, such as rural China and Japan, than in women from high-risk areas such as Britain and the United States (20, 23, 25, 26). Large cohort studies, one involving women in New York (27) and another the nationwide Nurse's Health Study (28), found a positive association of serum estrogens and androgens and breast cancer development. 17β -Estradiol stimulates breast and endometrium cell proliferation (21). Progesterone antagonizes the proliferative effect of 17β -estradiol on the endometrium. However, the fact that breast cell proliferation increases during the luteal phase of the menstrual cycle, when progesterone concentrations are the highest, suggests that progesterone may enhance breast cell proliferation (21, 29).

Soya consumption may in part account for geographical variation in breast cancer incidence. Soya contains many chemopreventive components, including Bowman-Birk protease inhibitors, inositol phosphates, phytosterols, saponins, and the isoflavones genistein and daidzein (12). Mechanisms underlying the oncoprotective effects of soya have been studied in cell cultures and laboratory animals. For example, isoflavones have been shown to have estrogen agonist and antagonist effects and can induce differentiation and inhibit cell proliferation, angiogenesis, tyrosine kinase, and topoisomerase II. Bowman-Birk protease inhibitor influences cell transformation. In a previous study, we found that circulating ovarian hormone levels were decreased by 1 month of soya consumption in six premenopausal women when measured on 3 different days during the menstrual cycle (30). Because of the cyclic nature of ovarian hormones, to better assess their changes induced by a soya diet, we have measured circulating levels of ovarian hormones and gonadotropins in 10

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² To whom requests for reprints should be addressed, at Department of Preventive Medicine and Community Health, The University of Texas Medical Branch, 700 Harbor-side Drive, Galveston, TX 77555-1110. Phone: (409) 772-1730; Fax: (409) 772-9108; E-mail: LLu@utmb.edu.

women over an entire cycle once during usual home diets and once during a soya diet.

MATERIALS AND METHODS

Study Design. This was a longitudinal study that compared circulating hormone concentrations in premenopausal women during a 3-month baseline observation period while subjects consumed their usual home diets to those during 1 month of a controlled diet that included soymilk. The study was 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. Those who were vegetarians or smokers, consumed more than two alcohol-containing drinks/month, had experienced recent significant changes in weight or eating habits, had taken antibiotics within the preceding 3 months, had irregular menstrual cycles, or had taken contraceptive medications during the preceding 6 months were excluded. Contraceptive medications were not allowed during the study. Small doses of acetaminophen or aspirin were permitted. One subject was taking replacement levothyroxine, 0.1–0.25 mg/day, for hypothyroidism and was determined to be euthyroid. Another was taking sertraline, 25 mg/day, for mild depression before and during the study.

Baseline Study Period. Day 1 of each cycle (the first day of menstrual bleeding) was recorded throughout the study. After enrollment, subjects underwent baseline studies as outpatients on the GCRC³ for at least 3 months but no more than 6 months while consuming their usual home diets prior to being placed on a soya diet for 1 month. Soy products were not part of the usual diet of any subject, and all were instructed to avoid soy products during the baseline observation period. The major purposes of the baseline observations were to assure that the subjects had regular cycles and to record cycle length. During the first month of the baseline period, blood was obtained on cycle days 5 (follicular phase), 12 (mid-cycle), and 22 (luteal phase) for measurement of 17 β -estradiol and progesterone. Subjects were retained in the study if luteal phase progesterone levels exceeded 4 ng/ml. During the second month of the baseline period, blood samples were collected on cycle days 5 and 7 and then daily from day 9 through the second day of the subsequent cycle. After a rest period of at least one menstrual cycle, subjects were studied as inpatients on the GCRC beginning on cycle day 2 and discharged on cycle day 2 of the next cycle. Intakes of energy, protein, carbohydrate, fat, and fiber during consumption of usual home diets were estimated using Block's Health Habit History Questionnaire (31), and the values were used for comparison with intakes during the soya diet.

Soya Diet Period. Subjects consumed a soya-containing diet for one menstrual cycle on the GCRC. Meals and soymilk were consumed under direct supervision. Soymilk used for this study was an homogenized, pasteurized preparation containing no preservatives, purchased in lots (Banyang Food Co., Houston, TX), stored frozen, and thawed on the day of consumption by the subjects. The soya diet, including both soymilk and non-soya foods, for each subject was calculated to match that needed to maintain constant body weight, based on the Harris-Benedict equation, with adjustment for physical activity (32, 33). The soya diet consisted of three rotating daily menus and included a 36-ounce portion of soymilk daily that provided 400 kilocalories, 37.9 g of protein, 20.3 g of fat, and 16.6 g of carbohydrate (analysis by Protein Technology Inc., St. Louis, MO). The content of protein, carbohydrate, and fat did not vary significantly between different lots of soymilk. Soymilk was ingested between 5 and 8 p.m. without other foods and in place of the evening meal. The energy distribution of the soya diet was 35.5% fat, 14.0% protein, and 50.1% from carbohydrate daily, which is similar in macronutrient distribution to that consumed by many residents of Western countries (34).

Subjects continued their usual daily activities, including work, study, and exercise. Basal body temperatures were recorded daily to determine the time of ovulation. Fasting blood samples were obtained between 7 and 9 a.m. on cycle days 5, 7, and 9 and then daily until the second day of the next cycle for

measurement of 17 β -estradiol, progesterone, gonadotropins, daidzein, and genistein. Two 12-h urine samples were collected daily starting on cycle day 2 and continuing until cycle day 2 of the next cycle for analysis of urinary excretion of daidzein and genistein. Sera were separated and stored immediately at -80°C until analysis. Urine samples were refrigerated during collection and then stored at -20°C until analyzed.

Blood cell counts, liver function tests, serum calcium, phosphorous, creatinine, cholesterol, HDL, LDL, triglycerides, and ferritin were determined on cycle days 3 and 15, as well as on day 2 of the next cycle during the baseline and the soya diet periods. If the serum ferritin fell below 10 ng/ml during the study, an oral iron supplement was provided.

Hormone Analysis. 17 β -Estradiol concentrations in the serum, after extraction with hexane and ethyl acetate (v/v, 3:2), were measured by a specific RIA as described previously (35). Progesterone levels were measured by direct RIA using commercial kits (Diagnostic Laboratory Inc., Webster, TX; Ref. 30). Blank and control sera were run with each assay. Assays were performed in duplicate. Levels of LH and FSH were measured by immunoradiometric assay using commercial kits (Diagnostic Laboratory, Inc.). The intra-assay coefficients of variation were 4–8%, and inter-assay variation was 5–9%. All of the samples from each subject, *i.e.*, baseline and treatment samples, were analyzed together in a single batch.

Analysis of Soya Isoflavones Genistein and Daidzein in Soymilk and in Urine. Isoflavone content in soymilk and urine was analyzed by a gas chromatography flame ionization detection method as described previously (36). Amounts of isoflavones in soymilk or urine were expressed as amounts of the aglycone forms.

Serum Levels of Daidzein and Genistein. Serum levels of daidzein and genistein were analyzed by competitive enzyme-linked immunoassays, using monoclonal antibodies generated against daidzein and genistein, and horseradish peroxidase conjugates of daidzein and genistein as tracers, as described previously (37, 38). The detection limit of the assay is 0.5 ng/well. Sera were obtained ~ 15 h after soymilk ingestion, and daily blood samples of each study cycle were individually measured for daidzein and genistein. Mean values for the entire cycle were calculated.

Data Analysis. The outcome measures were serum concentrations of 17 β -estradiol, progesterone, LH, FSH, and menstrual cycle length. Because of the cyclic nature of these hormones, summary measures of the data were used in the data analysis (39). The hormone data were expressed as AUCs, mean daily levels, peak levels, and rates, as appropriate for assessing changes in hormones over two entire cycles under the two different dietary conditions.

Serum levels of hormones, obtained once every other day initially and daily after day 9 until day 2 of the next cycle, were used to calculate area under the serum concentration *versus* time curves (AUC, concentration \times time) using WinNonlin software (Scientific Consulting, Inc., Cary, NC). AUC represents the integrated or cumulative exposure to these cyclic hormones during each dietary period. AUC was divided by the number of days to obtain the mean daily level. Peak levels represent the maximum levels recorded during the cycle and during the follicular and luteal phases. The rates of increase in 17 β -estradiol level leading up to the follicular (or mid-cycle) peak were calculated for each subject by solving for the parameter *b* in the second order polynomial regression equation: $Y = \text{intercept} + bX + cX^2$, where *Y* is the serum 17 β -estradiol level at day *X* of the cycle, using the day of the LH surge as the reference point. The statistical analysis compared the *b* estimates from before and during the soya diet. AUCs and mean daily levels were calculated for the entire cycle and for the follicular and luteal phases using the day of the serum LH surge as a reference point. The LH surge represents the end of the follicular phase and the beginning of the luteal phase. For graphical analysis of the time courses of hormone levels, values were plotted using the day of serum LH surge as a reference point.

Each outcome summary measure, *i.e.*, cycle length, AUC, mean daily levels, peak levels, and rates of increase, was analyzed across the entire cycle and then also within the follicular and the luteal phases. Most comparisons were for within-subject changes and used paired *t* tests or Wilcoxon signed rank tests.

Secondary analyses by multiple regression were carried out to further explore the possible mechanisms of the hormone changes between home diet and soya diet periods as related to study-associated variables. The equation for the regression analysis for changes in hormone, *Y*, is

³ The abbreviations used are: GCRC, General Clinical Research Center; LH, luteinizing hormone; FSH, follicle-stimulating hormone; AUC, area under the concentration time curve; BMI, body mass index; CV, coefficient of variation; LDL, low density lipoprotein; HDL, high-density lipoprotein.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 (X_1 \times X_2) + \epsilon$$

where β_0 is the intercept and β_1 is the parameter for one of the predictor variables of isoflavone levels, *i.e.*, intakes, plasma levels, or urinary levels of daidzein, genistein, or the sum of daidzein and genistein. The second variable in the model associated with parameter β_2 can be viewed as a covariate adjustment using one of the five possible variables for nutrients (*i.e.*, home – soya values for: energy, protein, fat, carbohydrate, or fiber), age, or BMI. The final term is the interaction of the two main effects, X_1 and X_2 . The residual error is denoted as ϵ . Each isoflavone level predictor variable was fitted separately with changes in hormone levels expressed as AUCs, mean daily levels, and peak values. Each of these models was then adjusted for age, BMI (kg/m²), or nutrient intake variables. Because of the small sample size, it was necessary to make each adjustment separately. To identify highly correlated variables for the regression models ($R > 0.5$), pair-wise correlations were computed. Because hormonal profiles are different in the follicular and luteal phases, the models were obtained separately for the total cycle and for the follicular and luteal phases. Not surprisingly, AUC, mean daily, and peak level were influenced in the same manner by all factors examined, and therefore, model results only for mean daily levels are presented. Likewise, because results for the total cycle were generally consistent with the results of separate analyses for the follicular and luteal phase, only the latter two are presented. For brevity of presentation, groups of related regression models are reported as a range of R^2 values plus the maximum P (*e.g.*, all $P \leq 0.05$).

All statistical analyses were performed using SAS (SAS Institute, Inc., Cary, NC). All results were expressed as mean \pm SE, and all P s are from two-tailed tests.

RESULTS

Subject Enrollment and Dietary Intakes. Ten of the 26 women who were enrolled completed the study (Table 1). They were (137 \pm 7)% (mean \pm SE) of ideal body weight and had regular cycles (CV in cycle length during the 3-month baseline of 5.7–11%). Seven were Caucasians, three were African-Americans, and nine were nulliparous. (Table 1). The demographic variables of the dropouts were in general very similar to those who completed the study regarding age (29.7 \pm 7.7 years; $P = 0.31$, Student’s t test), body weight (69.9 \pm 13.9 kg; $P = 0.77$), height (1.7 \pm 0.7 m; $P = 0.55$), and BMI (25.1 \pm 5.1 kg/m²; $P = 0.40$) except race (3 African-Americans, 1 Asian, 3 Hispanics, and 9 Caucasians). The dropouts all occurred during the baseline observation period. The main reason for the dropouts was frequency of blood drawing.

Body weight in all subjects who completed the study was not significantly different during home and soya diet periods (Table 1). For the group, average energy and fat provided by home diets and the soya diet did not differ significantly. The soya diet on average provided more energy from carbohydrate than did the home diets (50.1 *versus* 45.3% of calories; $P = 0.02$), less energy from protein (14 *versus* 17%; $P = 0.01$), and less fiber (6 *versus* 18.3%; $P = 0.001$; Table 1). There was individual variation in these dietary differences.

Table 2 Intakes and urinary and serum levels of daidzein and genistein (expressed as free forms) during the soya diet

	Mean	SE	Range
Intakes			
Daidzein (mg/day)	68.8	3.7	49.6–106.4
Genistein (mg/day)	85.2	5.4	63.4–120.0
Isoflavones (mg/day)	154.0	8.4	113–207
Daidzein (mg/cycle)	1844.1	143.0	1190–2784
Genistein (mg/cycle)	2285.4	201.4	1522–3840
Isoflavones (mg/cycle) ^a	4129.5	329.6	2712–6624
Urinary excretion			
Daidzein (mg/day)	24.6	3.5	11.15–39.16
Genistein (mg/day)	9.2	1.9	1.35–18.20
Isoflavones (mg/day)	33.8	5.3	13.0–57.4
Daidzein (mg/cycle)	644.1	99.2	279–1131
Genistein (mg/cycle)	240.5	49.7	36.5–437
Isoflavones (mg/cycle)	884.6	146.5	350–1553
% intake excreted in urine (amount excreted/amount ingested)			
Daidzein	35.1	4.2	19.6–58.1
Genistein	11.1	2.3	1.6–21.3
Isoflavones	22.0	3.3	9.1–36.7
Serum levels (sum of free and conjugated forms)			
Daidzein (μg/ml)	2.95	0.53	0.45–6.17
Genistein (μg/ml)	0.85	0.22	0.14–2.16

^a Isoflavone refers to both daidzein and genistein.

Intakes of Soya Isoflavones. The soymilk used in this and previous studies (36) contained the isoflavones daidzein and genistein, mostly as conjugates (~85 mol-% glucoside conjugates and ~15 mol-% free). Because soybean isoflavone content varies with season of harvesting and storage (40), there was variation in isoflavone content between lots. Because different subjects ingested soymilk from different lots, isoflavone intake varied among the subjects. However, each subject ingested the same lot throughout the soya-feeding period.

Table 2 shows that subjects ingested 68.8 \pm 3.7 mg/day ($n = 10$) of daidzein and 85.2 \pm 5.4 mg/day of genistein. The daily total isoflavone intakes varied from 113 to 207 mg/day (154.0 \pm 8.4 mg/day). Because of interindividual variation in cycle length as well as isoflavone content of the soymilk lots, isoflavone intake over entire cycles varied from 2712 to 6624 mg/cycle (4129.5 \pm 329.6 mg/cycle). The individual intakes were used to determine the association of isoflavones and observed changes in various hormones (see below).

Urinary and Serum Levels of Isoflavones. Isoflavones were detected in all daily urine samples of the 10 subjects during the entire soya-feeding period. There were large interindividual variations in urinary excretion of daidzein and genistein, when expressed as total cycle excretion, mean daily excretion (total cycle excretion/cycle length), and percentage of intake excreted (CV, 46% for daidzein and 63% for genistein; Table 2). There was also considerable intraindividual variation in isoflavone excretion (CV, 26% for daidzein and 28% for genistein). Subjects excreted more daidzein (35.1 \pm 4.2%; range, 19.6–58.0%) than genistein (11.3 \pm 2.3%; range, 1.6 to 21.3%;

Table 1 Characteristics of study subjects

	Home diet			Soya diet			<i>P</i>
	Mean	SD	Range	Mean	SD	Range	
Age, yr	32.7	6.6	23–42	32.7	6.6	23–42	
Body weight, kg	71.5	13.7	52.9–97.9	71.5	13.7	52.9–97.9	
Height, m	1.6	0.1	1.56–1.74	1.6	0.1	1.56–1.74	
BMI, kg/m ²	26.4	4.7	19.43–36.85	26.4	4.7	19.43–36.85	
Cycle lengths, mean day	26.6	1.6	25–29	26.0	2.0	23–29	
Variation in cycle lengths, CV, %	7.39	1.78	5.7–11.0				
Total calories	2277	785	1332–3440	2341.1	156.9	2069–2576	0.79
% fat	36.2	6.6	24.2–45.6	35.5	0.9	34.3–37.0	0.74
% carbohydrate	45.3	5.6	38.1–57.6	50.1	0.7	49.0–51.0	0.02
% protein	17.0	3.2	12.7–24.6	14.0	0.5	13.3–14.7	0.01
Fiber, g	18.3	7.8	7.1–29.5	6.0	0.8	5.2–7.2	0.001

Table 2 and Fig. 1) as reported in previous studies (41, 42). Average daily levels of daidzein and genistein (sum of free and conjugated forms) over a cycle in serum were 2.95 $\mu\text{g/ml}$ (range, 0.45–6.17 $\mu\text{g/ml}$) and 0.85 $\mu\text{g/ml}$ (range, 0.14–2.16 $\mu\text{g/ml}$), respectively, with substantial interindividual variation (Table 2).

In previous studies in which the initiation of soya ingestion was not timed with the menstrual cycle, urinary excretion of isoflavones decreased during 1 month of soymilk ingestion (42). In this study, soymilk ingestion was timed to begin on cycle day 2. Because follicular phase length tends to vary more than luteal phase length, urinary excretion of isoflavones was plotted using the LH surge as a reference point in time that separates the follicular and luteal phases of the cycle. As shown in Fig. 1A, urinary excretion of ingested isoflavones decreased progressively during the menstrual cycle such that isoflavone excretion was greater during the follicular phase than the luteal phase.

This was examined further by calculating average daily excretion during the follicular and luteal phases for each subject. This confirmed that the excretion of daidzein (in all 10 subjects) and genistein (in 8 of 10 subjects) were both greater during the follicular phase than the luteal phase (daidzein excretion, 25.72 \pm 3.58 mg/day and 23.71 \pm 3.35 mg/day during the follicular and luteal phases, respec-

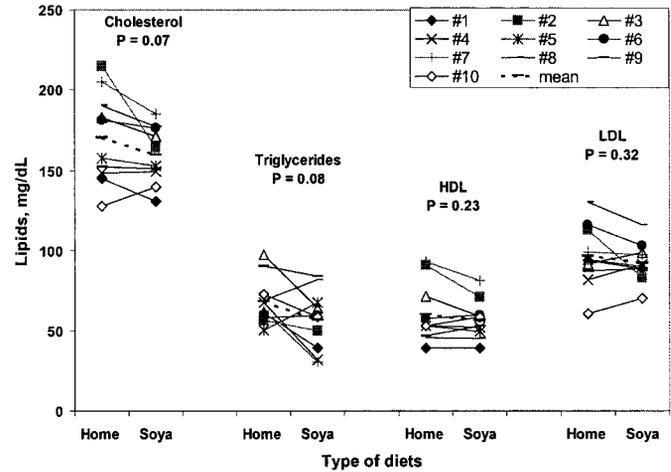


Fig. 2. Effects of an isoflavone- and soya-containing diet on serum lipid levels in 10 premenopausal women. Women provided three blood samples at the beginning, middle, and the end of a study cycle for one cycle before (*Home*) and for one cycle during soya feeding (*Soya*) for the analysis of cholesterol, triglycerides, LDL, and HDL. For study design, see the legend of Fig. 1. For statistical analysis, the average of all three measurements during the baseline period and the average of the last two measurements during the soya diet period were compared using two-tailed, paired *t* tests.

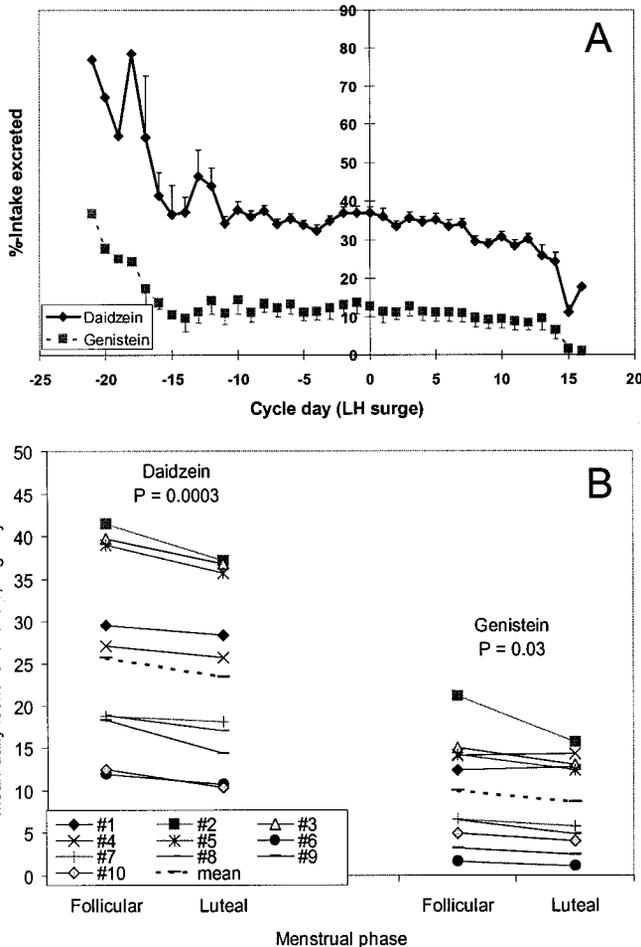


Fig. 1. Urinary excretion of soya isoflavones during 1 month of soymilk ingestion by 10 premenopausal women. Women with regular menstrual cycles on a metabolic unit ingested one 36-ounce portion of soymilk containing 70.7 \pm 5.0 mg of daidzein and 82.7 \pm 4.0 mg of genistein (85 mol-% as glucosides in soymilk) daily from cycle day 2 until day 2 of the next cycle with 24 h urine collection for the analysis of daidzein and genistein. A, the mean percentage of intakes recovered (bars, SE of amount excreted/dose of daidzein or genistein ingested) in relation to the day of LH surge in sera. B, the individual mean daily urinary excretion of daidzein and genistein (mg/day) averaged for each menstrual phase and the group mean. *P*s refer to two-tailed, paired *t* tests.

tively; *P* = 0.006; genistein excretion, 9.97 \pm 1.99 mg/day and 8.62 \pm 1.73 mg/day during follicular and luteal phases, respectively; *P* = 0.03; *n* = 10; Fig. 1B).

Effects on Serum Lipids. The effects of 1 month of soya consumption on serum lipid levels in this group of premenopausal women are shown in Fig. 2. Cholesterol, triglycerides, LDL, HDL, and the ratios of cholesterol to HDL were measured three times per cycle during the baseline and during soya diet periods, generally at cycle onset, mid-cycle, and at the end of the cycle. Averages during both dietary periods were calculated, with exclusion of the first measurement in the soya diet period, because this was taken after only one day of this diet. As shown in Fig. 2, during the soya diet period, the average serum cholesterol decreased by 6% (*P* = 0.07, paired *t* test), triglyceride by 17% (*P* = 0.08), HDL by 6% (*P* = 0.23), and LDL by 4% (*P* = 0.32). The ratios of cholesterol to HDL did not change significantly (not shown).

Effects on Menstrual Cycle Length. As shown in Fig. 3, consumption of soymilk containing 113–207 mg of isoflavones/day for 1 month did not significantly alter menstrual cycle length (1.2% decrease; *P* = 0.51) or follicular phase length (2.7% increase; *P* = 0.67) in this group of women but was associated with a decrease in average length of the luteal phase that approached statistical significance (6%; *P* = 0.07). By multiple regression analysis, decreases in luteal cycle lengths among individuals were positively and significantly associated with the amounts of daidzein and genistein ingested or excreted and with the decrease in protein intake during soya feeding, and there was some evidence of an interaction between these two variables (*R*², 0.51–0.54; all *P* < 0.05).

Effects on 17 β -Estradiol Levels. Differences in 17 β -estradiol levels were studied over the entire cycle as well as during the follicular and luteal phases. Because interindividual variation in cycle length is largely attributable to variation in follicular phase length (43, 44), the day of the LH surge was used as a reference point for separation of the follicular and luteal phases. The levels of 17 β -estradiol averaged for each day were decreased over the full cycle during soymilk ingestion (Fig. 4A), as were AUC (Fig. 4B), mean daily level (AUC/cycle length), and peak levels (Fig. 4C) of 17 β -estradiol in most subjects. These decreases in circulating 17 β -estradiol levels were evident when analyzed over the entire cycle, over the

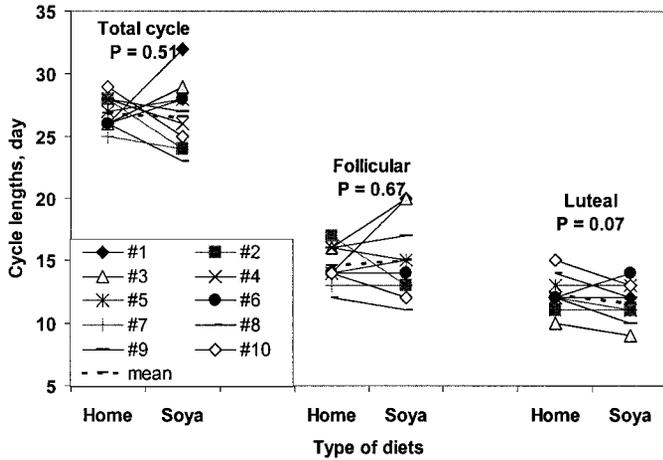


Fig. 3. Effects of soya feedings on menstrual cycle lengths of 10 premenopausal women. For study design, see the legend of Fig. 1. Subjects recorded day 1 of menstrual bleeding during the baseline and the soya diet periods. Follicular phase lengths are days between onset of menstrual bleeding and the day before LH surge. Luteal phase lengths are days from LH surge day to the day before subsequent menstrual bleeding. *P*s are from two-tailed, paired *t* tests.

follicular phase, and over the luteal phase. For the total cycle, follicular phase, and luteal phase AUCs (pg/ml × day), 17β -estradiol decreased by ~24% during soya diet feeding (all $P < 0.04$; Fig. 4B). Average daily levels (pg/ml) of 17β -estradiol were decreased by 19–23% during the follicular and the luteal phases of the cycle ($P \leq 0.05$; Fig. 4C). Maximal levels of 17β -estradiol during the follicular phase were suppressed to a similar degree during the soya diet (~28%; $P = 0.004$; Fig. 4C).

17β -Estradiol levels before the ovulatory peaks were fitted into the regression model: $Y = \text{intercept} + bX + cX^2$, where Y is the serum 17β -estradiol level on day X of the cycle. The mean for regression coefficient b was 96.0 ± 15.2 (SE) for all 10 subjects while consuming their own home diets and 45.5 ± 5.6 during the soya diet. The mean difference in bs during the two dietary periods was 50.5 ± 12.7 ($P = 0.003$). If one ignores the quadratic term, the soya diet decreased the average rate of increase of 17β -estradiol before the LH surge and the total amount of 17β -estradiol in serum over the full cycle.

The effects of intakes and levels of isoflavones, macronutrient intakes, age, and BMI on soy-induced decreases in 17β -estradiol were examined by multiple regression analysis as described in “Materials and Methods.” The results showed that during the soya diet, the decreases of both follicular and luteal phase 17β -estradiol levels were positively associated with subject’s age and with the urinary levels of daidzein, genistein, or the sum of daidzein and genistein, and with plasma levels of genistein, but were inversely related to the interaction of age and isoflavone levels ($R^2 = 0.60$ – 0.80 ; $P < 0.05$ for follicular phase levels; and $R^2 = 0.61$ – 0.68 ; $P < 0.05$ for luteal phase levels). The decrease in 17β -estradiol was also inversely associated with change in protein intake ($R^2 = 0.53$ – 0.64 ; $P < 0.05$ for follicular phase levels; and $R^2 = 0.81$ – 0.83 ; $P < 0.001$ for luteal phase levels), either with or without adjustment for age or urinary isoflavone levels. This suggests that a greater decrease in energy intake from protein during the soya diet may blunt the inhibitory effect of isoflavones on 17β -estradiol levels. This influence of protein intake on 17β -estradiol levels was greater during the luteal phase than during the follicular phase. Changes in 17β -estradiol were not predicted by variables such as BMI, fat intake, carbohydrate intake, or fiber intake. None of these independent variables singularly explained the outliers in Fig. 4. Group mean changes did not differ between Caucasians and African-Americans.

Effects on Progesterone Levels. Average daily levels of progesterone for the group of subjects during the luteal phase decreased during the soya diet (Fig. 5A). The luteal phase AUC (Fig. 5B; $P = 0.002$), mean daily level, and mean peak level (Fig. 5C; $P \leq 0.004$) of progesterone were lower in all 10 subjects during the soya diet than during the home diets. Progesterone levels were ~45% lower over the total cycle during the soya diet period than during the home diet period. Progesterone levels during the follicular phase were

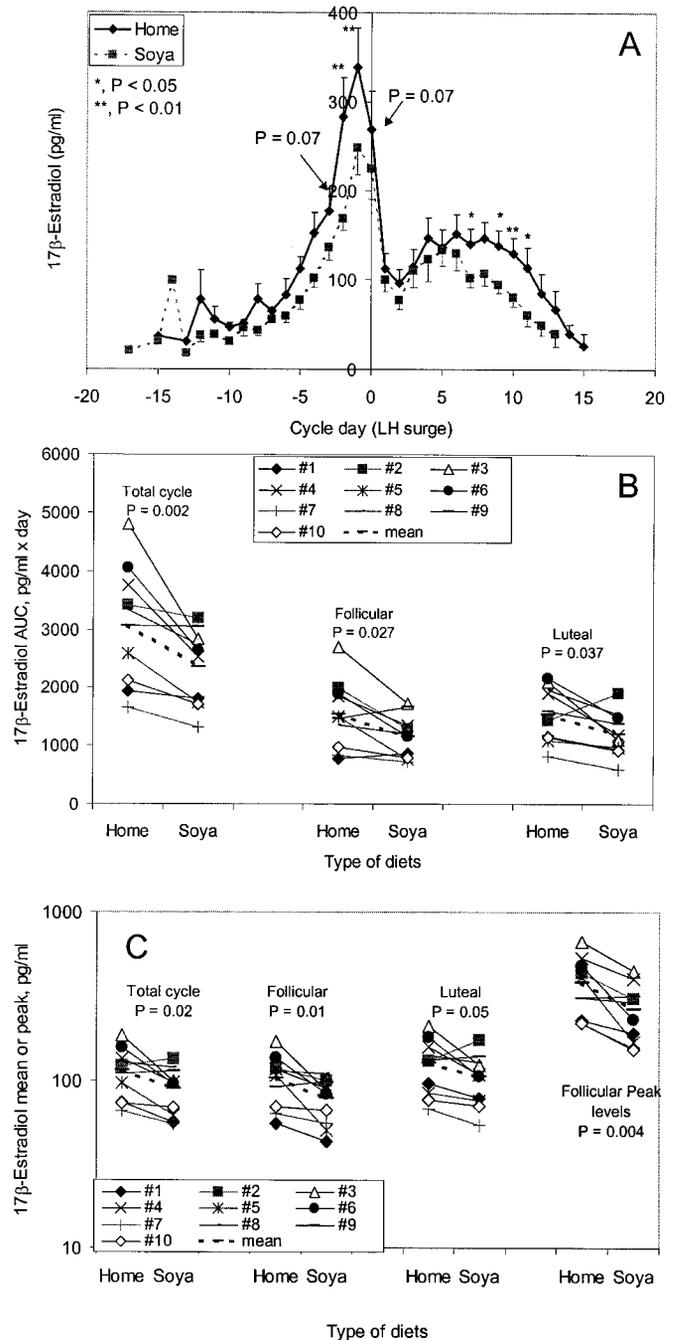


Fig. 4. Effects of 1 month of soya consumption on 17β -estradiol levels in 10 premenopausal women. Women provided one blood sample on cycle days 5, 7, and then daily from day 9 until day 2 of the subsequent cycle for one menstrual cycle while consuming their home diet (Home) and for one cycle during the soya feeding (Soya). For study design, see the legend of Fig. 1. A, the mean daily levels (means; bars, SE) of the group in relation to the day of serum LH surge over the full cycle. B, the AUCs; C (log Y-scale), individual mean daily levels of a cycle or a menstrual phase and individual peak levels and their group average. *P*s refer to a Wilcoxon signed rank test for differences of ovarian hormones between the home diet and the soya diet.

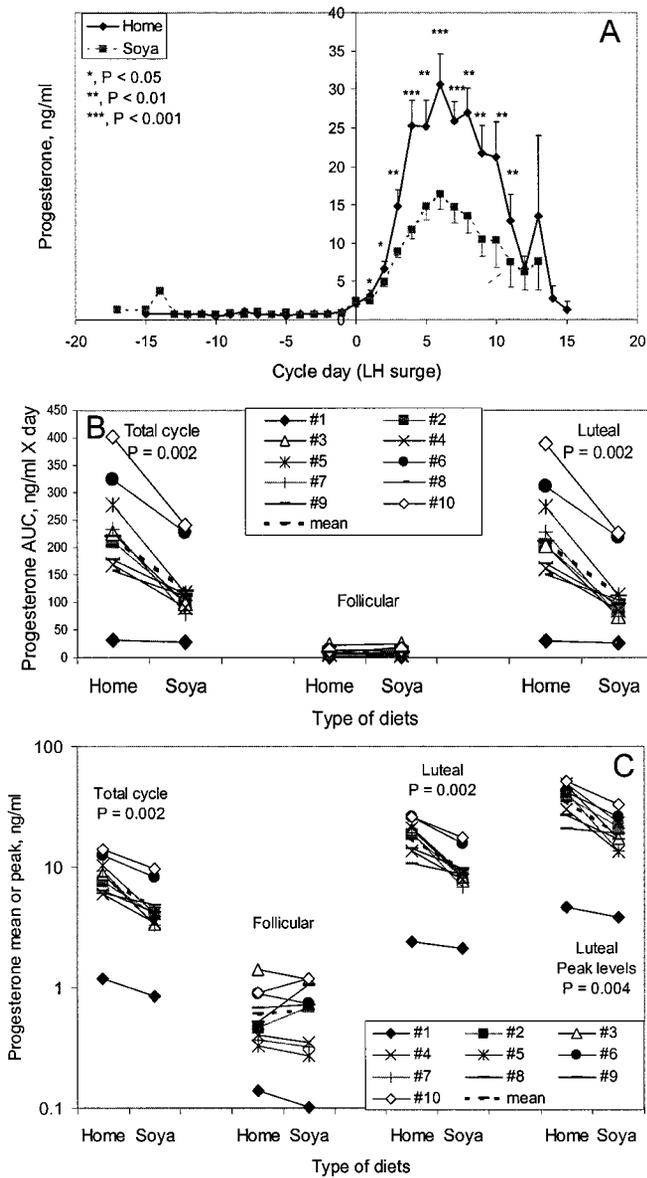


Fig. 5. Effects of 1 month of soya consumption on progesterone levels in 10 premenopausal women. A, mean daily levels for the group in relation to LH surge day; bars, SE. B, individual AUCs; C, individual mean daily levels of a cycle or a menstrual phase and individual peak levels and the group average. For study design, see legends of Figs. 1 and 4.

generally very low, as expected, and the difference during the two dietary periods was not statistically significant ($P = 0.84$).

Multiple regression analysis showed that the higher the intake of total isoflavone, in particular genistein, and the greater the decrease in energy and fiber intake during soy diet, the less was the decrease in progesterone level ($R^2 = 0.87-0.90$; all $P \leq 0.002$), with or without adjustment for age and intakes of protein, carbohydrate, or fat. Intakes of energy and fiber modified the influence of isoflavone and daidzein dose ($R^2 = 0.87-0.94$; all $P \leq 0.05$), but not genistein dose, on progesterone level change induced by the soya diet. Urinary or serum levels of daidzein and genistein and the sum of these isoflavones were not significantly related to changes in progesterone. When urinary or serum isoflavone levels were included in the regression analysis, energy, protein, carbohydrate, fat, and fiber intakes became significant predictors for changes in serum progesterone levels ($R^2 = 0.46-0.87$; $P < 0.05$). By this analysis, providing more energy during the soya diet compared with the home diet, for example, led to a greater

soya-induced decrease in progesterone level. Other study-associated variables, such as fat intake, carbohydrate intake, and age, were not predictors for progesterone changes. Qualitatively, group mean changes for African-Americans ($n = 3$) were greater than those of Caucasians ($n = 7$) but without statistical significance.

Effects on Gonadotropin Levels. Circulating levels of gonadotropins LH and FSH were measured during both dietary periods to determine whether the soya diet might influence 17β -estradiol and progesterone levels (Figs. 4 and 5) by decreasing the levels of these gonadotropins, perhaps by the interaction of the weakly estrogenic isoflavones genistein and daidzein with receptors in the pituitary and hypothalamus. As shown in Fig. 6, consumption of the soya diet for 1 month did not influence the total cycle, mean daily, or peak levels of LH and FSH.

Autocorrelation Analysis. The cycle profiles of ovarian steroids and gonadotropins, as shown in Figs. 4 and 6, of our study subjects are typical of those found in premenopausal women (44-46). These figures indicate that intracycle variation is substantial for all individuals. To assess the within-subject correlation during one cycle of home diet measurements, autocorrelations for each subject were plotted for progesterone and 17β -estradiol (Fig. 7). These correlations give information about how much a single measurement correlates with previous measurements. For 17β -estradiol (Fig. 7A), measurements within a few days (*e.g.*, <2 days) are highly correlated, but for >4 days apart the correlations diminish. For progesterone (Fig. 7B),

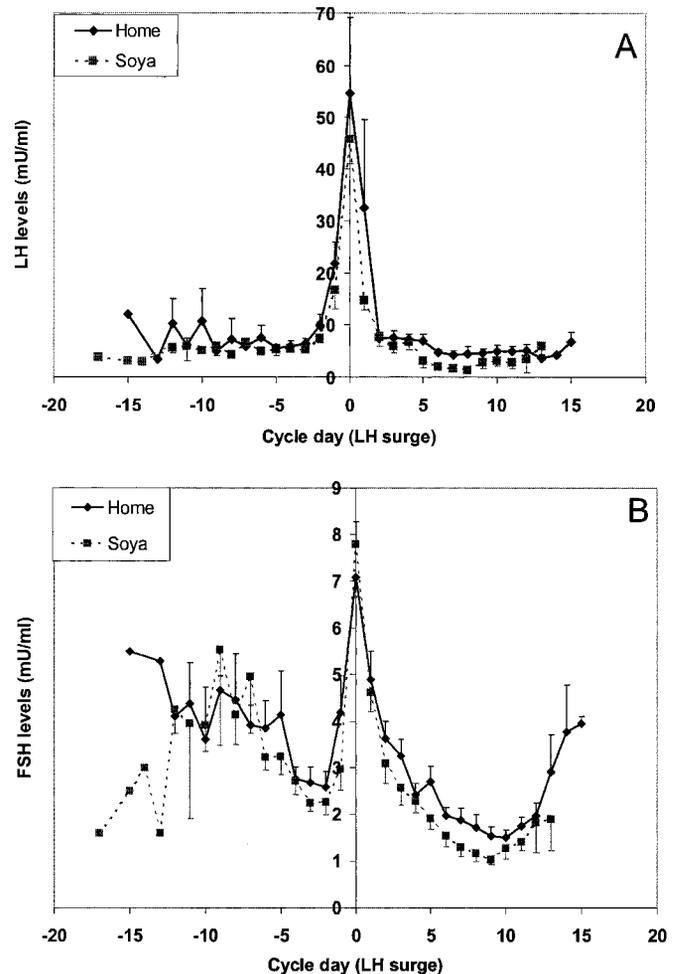


Fig. 6. Effects of 1 month of consumption of a soymilk-containing diet on mean (bars, SE) cyclical levels of LH (A) and FSH (B) in premenopausal women. For study design, see the legends of Figs. 1 and 4.

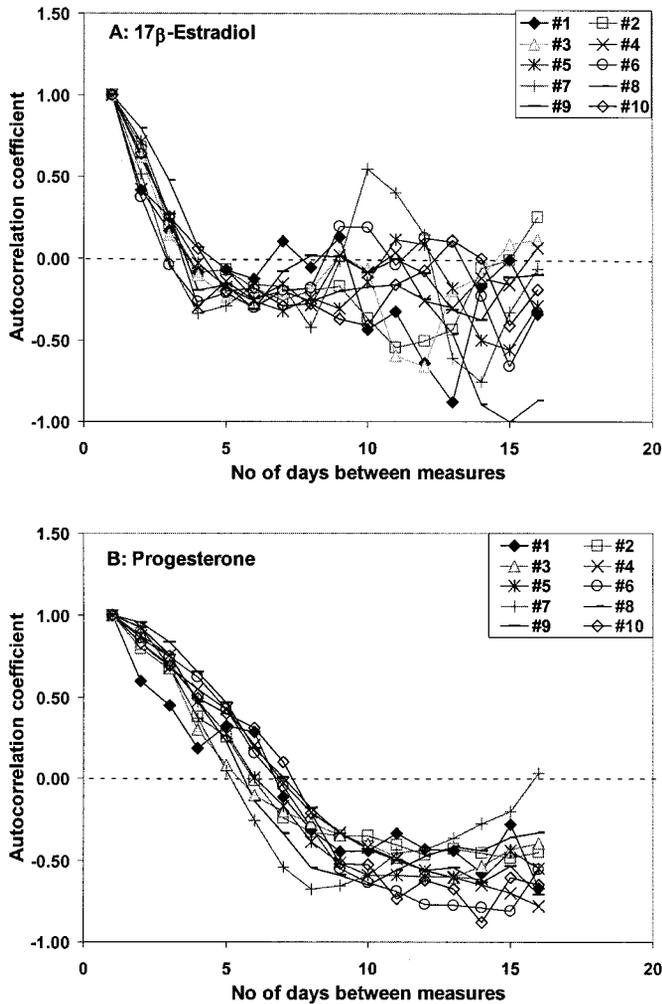


Fig. 7. Plot of autocorrelation for increasing lags between days, for each subject. The correlations were performed for observations between 2 and 15 days apart, because autocorrelation estimates for lags >15 days become unstable.

measurements within 3 days or >10 days apart are highly correlated, whereas the correlations drop off for measurements 3–9 days apart. The negative correlations for measurements >5 days apart reflect the cyclic nature of these two ovarian hormones. The similarity of the shapes and closeness of the lines for progesterone show that subjects follow a similar cyclical pattern, whereas the more scattered pattern for 17β-estradiol shows higher between-subject variability.

DISCUSSION

By controlling daily energy and nutrient intakes and obtaining blood samples for a full cycle, we have shown that soya feeding effectively reduces circulating levels of 17β-estradiol and progesterone (Figs. 4 and 5) and that this hormonal effect occurs throughout the menstrual cycle. This expands the result of a prior study during which subjects ate unrestricted while consuming soymilk as a supplement (30). Furthermore, we have shown that soya feeding has no effects on gonadotropins (Fig. 6). The soya diet also marginally shortened luteal phase duration (Fig. 3) and reduced serum lipids (Fig. 2). Decreases in serum 17β-estradiol were observed during both the follicular and the luteal phases of the menstrual cycle (Fig. 4), whereas decreases in progesterone were observed only during the luteal phase, when progesterone synthesis in ovaries is more active (Fig. 5). The results

suggest that the synthesis and/or metabolism of these two ovarian steroids may be affected by a soymilk-containing diet.

Secondary analysis by multiple regression, the use of which was made possible by individual differences among subjects in intakes of isoflavones (ranging from 113 to 207 mg/day; Table 2) and other nutrients and in metabolism and disposition of ingested isoflavones (Fig. 1) provided insight into other factors in the soya diet in addition to isoflavones that may influence these ovarian hormones. After adjustment for these variations, isoflavone intake and plasma and urinary levels of isoflavones were found to be associated with soya-induced changes in ovarian hormone levels.

The isoflavones daidzein and genistein in soybeans are weak estrogens and may bind to estrogen receptors (47) in the pituitary and hypothalamus and regulate ovarian hormone synthesis by influencing production of gonadotropins. However, despite the high daily intakes of isoflavones in this study (113–207 mg/day; Table 2), the levels of LH and FSH (Fig. 6) were not significantly different between the soya and the home dietary periods, even after controlling for individual differences in intakes and urinary levels of isoflavones. These data suggest that the inhibitory effects of the soya diet on ovarian steroid hormone levels may not be mediated by gonadotropins. Two other studies in which women ingested 45–60 mg of isoflavones/day found a suppressive effect on serum LH and FSH concentrations (48, 49). In the present study providing isoflavone doses >100 mg/day, no effects on serum LH and FSH were observed compared with basal non-soya diet (Fig. 6). This is consistent with the results of Duncan *et al.* (49). In fact, in the present study peak LH levels were higher during soya feeding in 8 of the 10 women. Comparisons of results from these different studies suggest the possibility that low doses of isoflavones may act as estrogen agonists in the hypothalamus and pituitary, whereas high doses do not. Additional studies in humans over a wide range of isoflavone dosages are needed to fully characterize whether gonadotropins mediate the effects of soya isoflavones on ovarian steroids. Alternatively, soya isoflavones may directly inhibit steroid synthesis enzymes in ovaries, as has been suggested by *in vitro* studies (50–53).

Multiple regression analysis has shown that individual decreases in 17β-estradiol level are not associated with individual differences in isoflavone intake but are positively associated with individual plasma and urinary levels of daidzein and genistein, and this association is influenced by age. This result suggests that isoflavones play a role in influencing 17β-estradiol levels. The lack of correlation between isoflavone intakes and changes in 17β-estradiol is attributable to large interindividual differences in plasma and urinary levels of isoflavones (Fig. 1 and Refs. 41, 42, and 54). We showed previously that urinary recoveries of ingested isoflavones varied from 3 to 80% (41, 42, 54). Isoflavones in soy exist mostly as glucosides (glycones), and the hydrolysis of these glycones to aglycones (free forms) by intestinal flora is thought to be necessary prior to systemic absorption (55). The large interindividual variability in urinary recovery of ingested isoflavones is attributable possibly to interindividual differences in composition of intestinal flora as discussed previously (41). Thus, plasma or urinary levels of isoflavones may be better biomarkers of soya exposure than isoflavone intake *per se*.

In contrast to the positive association between individual levels of isoflavones and individual decreases of 17β-estradiol, the inhibitory effect of soya on progesterone levels is inversely related to individual intakes of genistein with or without controlling for age and intake changes in macronutrients: protein, fat, and carbohydrate. The influence of daidzein intake on progesterone changes is affected by intakes of energy and fiber. It was surprising that despite this strong inverse relationship between isoflavone doses and decreases in progesterone, there was no correlation between urinary and serum levels of genistein

and daidzein and progesterone changes. When serum and urinary levels of both isoflavones were included in the regression analysis, all macronutrient intakes became significant predictors of progesterone level changes. These data suggest that the macronutrient components of the soya diet also influence progesterone levels. Dosage of genistein provided in soymilk in this study ranged from 63–100 mg/day. It remains to be determined whether genistein intakes lower than 63 mg/day might have a different dose-response relationship with progesterone level changes. Dose-response relationships for genistein are frequently biphasic or U-shaped in many biological systems. For example, the effects of genistein on progesterone synthesis (50), cell proliferation (56, 57), pituitary responsiveness to the stimulation of gonadotropin-releasing hormone (58), and bone loss (59) can be stimulatory or inhibitory, depending upon genistein dose. Additional studies with lower doses of soya isoflavones are needed to fully characterize human responses to soya isoflavone effects.

BMI and fat intake, each of which may influence hormone levels and cancer risk (60, 61), do not explain the observed soya-induced changes in ovarian steroid hormone levels, because none of our study subjects gained weight during the month of soya feeding. This is not surprising because the soya diet was eucaloric compared with the home diets for the group. Total fat intakes for the group were similar during both dietary periods (Table 1) and approximated the amounts of fat (*e.g.*, 35%) commonly consumed by populations in the United States (34). Despite the high fat content, fat intake change is not a predictor for hormonal effects. The study subjects consumed more energy from carbohydrate by the soya diet than by the home diets (Table 1), but this change in carbohydrate intake was not a predictor of soya-induced inhibition of ovarian hormone levels. Protein intake was somewhat lower during the soya diet than during the home diets of this group of study subjects (14% *versus* 17% of total energy, respectively; Table 1). This change in energy intake from protein appears to have modified soya-induced changes in 17β -estradiol but not progesterone. The regression data suggest that increased energy intake from protein during the soya diet enhanced soya-induced inhibition of 17β -estradiol levels and modified the influence of isoflavone levels on luteal phase lengths. Altered fat:carbohydrate (60) and protein:carbohydrate ratios (62), where energy intake remains constant, have been shown to affect sex hormone levels. In this study, a decrease in dietary protein appears to have lessened the isoflavone-related reduction in 17β -estradiol. More dietary intervention studies are needed to determine the role of protein and other specific nutrients on sex hormone levels in humans.

In the present study, in which the lengths of the follicular and luteal phases were estimated based on daily measurement of serum LH, a significant decrease in luteal phase length was observed after controlling for the isoflavone levels and change in protein intake. Effects of soya feeding on menstrual cycle lengths have been examined in five other studies (30, 48, 49, 63, 64), four of which showed an increase or a trend toward an increase in the lengths of the follicular phase or total cycle during soya ingestion (30, 48, 63, 64). By accurately measuring the occurrence of serum LH surge day, we showed that soya feeding significantly reduced luteal phase lengths after controlling for isoflavone levels. Because breast cells are more proliferative during the luteal phase than the follicular phase, a shortening of the luteal phase may reduce the length of time of proliferation of breast cells, and thereby reduce the probability of neoplastic transformation and breast cancer development.

In a prior study, in which women were provided the same amount of soymilk as in this study for 1 month but began soymilk ingestion 3–6 days after the onset of menstrual bleeding rather than on cycle day 2 as in this study, and consumed self-selected hospital diets rather than a controlled diet as in this study, soya feeding was found to

reduce ovarian hormone levels when measured on cycle days 5, 12, and 22 (30). Therefore, two different study designs involving two different groups of premenopausal women both have shown that consumption of a soya diet can lower circulating ovarian steroid hormone levels (30). The sample size of our study is small, and this makes inference to a larger population difficult. However, as shown in Fig. 5, all 10 subjects had decreases in progesterone levels during the soy diet. On the basis of a binomial event, the lower bound of the 95% confidence limits for the probability of success for 10 successes in 10 trials is 0.69. This implies that our results can be generalized to at least 70% of the population randomly sampled. Despite a small sample size, our results are consistent with the observation in Japanese women of a 25% reduction of 17β -estradiol level after consumption of soymilk containing 109 mg isoflavones daily and also with the result of a cross sectional study showing an inverse relationship between soya intake and 17β -estradiol levels (64, 65).

The following additional studies showed effects of soya feedings on ovarian steroids that differ from data presented here. With lower doses of isoflavones (<65 mg/day) an increase (48, 66) or no effect (49, 63) of soya feedings on serum 17β -estradiol levels were found. Our present and previous studies (30) have noted an effect of soya with isoflavones on progesterone levels. Others have not noted such an effect. The studies of Duncan *et al.* (49) and Petrakis *et al.* (66), which did not control for energy intake, as was done in the present study, and provided soya as a supplement, found no effects on 17β -estradiol levels. Our regression analysis indicates that endocrine effects of soya feeding are complex and may relate to multiple dietary components, including doses of isoflavones as discussed above and characteristics of the subjects such as age, which may explain the differing endocrine effects of studies of soya feeding.

As shown in Fig. 7, single measurements of blood ovarian hormone levels have limited ability to project the actual cycle levels of ovarian hormones in cycling women. Daily blood samples may have enhanced the capacity of this study to detect changes in circulating ovarian hormones. Differences in results among reported studies might also be attributable in part to differences in soya preparations. The soya preparation used in this study was a homogenate prepared from whole soybeans and contained compounds other than isoflavones and might differ from the preparations used by other investigators. The other chemopreventive components of soya are Bowman-Birk protease inhibitor, which was detected in our soya preparation, inositol phosphates, phytosterols, and saponins, and these have established biological effects in laboratory animals and in cell cultures (reviewed in Refs. 12, 67). Whether the latter compounds can influence ovarian steroids remains to be determined.

Serum concentrations of cholesterol and other lipids tended to decrease during soya feeding (Fig. 2). These effects approached statistical significance ($P = 0.07$ – 0.08 , in the two tailed *t* test; Fig. 2). Small decreases in serum lipids have also been observed in other studies in subjects with normal lipid levels (48, 68). Soya has been reported to be quite effective in lowering serum lipids in hypercholesterolemic patients (69, 70). Given the role of fat in cancer risk (61), a small decrease in serum lipids if it occurred over an extended period might be significant for cancer risk reduction.

In summary, a diet maintaining energy intake and containing soya and weakly estrogenic isoflavones is effective in reducing circulating levels of ovarian hormones in premenopausal women without apparent effect on the levels of gonadotropins. Analysis of data suggests that multiple components of the soya diet including isoflavones may modulate ovarian hormone levels directly and the effect is not mediated by gonadotropins. Because decreased levels of ovarian hormones may reduce breast cell proliferation and breast cancer risk, the results

of this study have implications for breast cancer prevention by dietary intervention.

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