Past Oral Contraceptive Use and Current Dietary Soy Isoflavones Influence Estrogen Metabolism in Postmenopausal Monkeys (*Macaca fascicularis*)

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Abstract

Estrogen metabolism may play an important role in mammary carcinogenesis in postmenopausal women. We evaluated the effects of prior oral contraceptive (OC) treatment and current soy isoflavone consumption on endogenous estrogen metabolite concentration and biomarkers of tissue estrogen exposure in a monkey model. One hundred eighty-one female cynomolgus macaques were randomized to receive OC or placebo for 26 months premenopausally, then ovariectomized and randomized to one of three diets for 36 months: an isoflavone-depleted soy protein isolate (Soy−) diet, a diet containing soy protein isolate with a human equivalent of 129 mg isoflavone/d (Soy+), or a Soy− diet supplemented with conjugated equine estrogens (CEE+) at a human equivalent dose of 0.625 mg/d. Reverse-phase high-performance liquid chromatography directly coupled with tandem mass spectrometry was used to measure the concentrations of estrogen species in urine samples. Generally, prior OC treatment was associated with significantly reduced urinary estrogen metabolites (25–55% reduction; $P < 0.05$ for each versus OC−). Animals that consumed isoflavones postmenopausally had increased urinary 2-hydroxyestrone and 16α-hydroxyestrone (50% and 56% increases, respectively), but reduced levels of 2-hydroxyestradiol, 2-methoxyestradiol, and 17-epiestriol (92%, 63%, and 66%, respectively), compared with animals fed a Soy− diet. Isoflavones did not have widespread effects on uterine or mammary proliferation biomarkers, whereas prior OC significantly reduced two of three proliferation end points in the endometrium. Premenopausal OCs may have long-term systemic effects on response to estrogen and its metabolism whereas postmenopausal dietary isoflavones may alter endogenous estrogen metabolism in a modest but selective manner.

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Disclosure of Potential Conflicts of Interest

C.E. Wood and J.M. Cline were previous members of an advisory board for Wyeth-Ayerst Laboratories, Inc. J.M. Cline is a co-inventor of "Treatment of Endometriosis Using Soy Phytoestrogens." The other authors disclosed no potential conflicts of interest.

Introduction

According to the American Cancer Society, cancers originating in the breast and uterus are estimated to be the first and fourth most common sites of cancer incidence among women, respectively, accounting for a projected 32% of new cancer cases in 2007 (1). Given the functions of these tissues, estrogen responsiveness plays a critical role in their normal and pathologic character. Lifetime overexposure to estrogens has long been linked to the etiology of breast and endometrial cancer through various life characteristics such as age at menarche and menopause, parity status, exogenous hormone use, obesity, and diet (2,3).

Most hypotheses implicating estrogen in the cause or promotion of hormone-responsive cancers identify a mitogenic role possibly promoting carcinogenesis by stimulating growth of neoplastic cells. Over the past two decades, however, more investigations have focused on the role of certain estrogen metabolites [i.e., hydroxy estrogens (OHE) and their quinone species] as genotoxins and mitogens in promoting cancer (4). For example, 16α-hydroxyestrone is proposed to be an inducer of proliferation (5,6), and 4-hydroxyestradiol and 4-hydroxyestrone are postulated to be direct (through DNA adduct formation) and indirect (through generation of reactive oxygen species) producers of DNA damage in carcinogenesis (7). Estrogen metabolites modified at the C2 position, however, may act as inhibitors of carcinogenesis and angiogenesis (8). Based, in part, on these findings, a number of studies have sought to link an increased ratio of 2-hydroxyestrogen to 16-hydroxyestrogen formation to decreased risk for breast cancer, with mixed results (9).

The relationship between estrogen and breast cancer risk has driven many recent studies to investigate how dietary and lifestyle factors may interact with estrogen exposure. Several studies suggest that dietary soy may decrease serum levels of E_1 (10–12) and 4-hydroxyestrone (13) and increase the ratio of 2-hydroxyestrone/16-hydroxyestrone (13–16), although the latter effect may be dependent on gut flora conversion of the soy isoflavone daidzein to equol (17).

We investigated how the use of OCs premenopausally and/or dietary isoflavone consumption postmenopausally might alter the estrogen metabolite profile using a cynomolgus monkey model. This model allows a degree of control over dietary and hormonal treatments not possible in human primate subjects while also providing primate-specific reproductive physiology. We examined whether these two factors affected tissue biomarkers of estrogen exposure and proliferation and the relationship between those biomarkers and concentrations of selected urinary estrogen metabolites. We hypothesized that the isoflavone-rich diet would produce an estrogen metabolite profile with lower levels of genotoxic or mitogenic estrogen metabolites and higher amounts of benign or beneficial metabolites, whereas the premenopausal OCs would have no significant effects on postmenopausal estrogen metabolism.

Materials and Methods

Animals and Study Design

One hundred eighty-one premenopausal cynomolgus monkeys (*Macaca fascicularis*) were obtained from the Institut Bogor, Indonesia. These monkeys were used in a multifaceted, randomized, controlled study designed to investigate the effects of premenopausal OCs and postmenopausal soy isoflavones or CEE on cardiovascular, bone, neurobiology, and cancer-risk marker end points. Data on many of these end points as well as diet composition and study design have been reported previously (12,16,18–22).

In brief, half of the premenopausal monkeys were given a triphasic oral contraceptive (OC+) over a 26-mo period (Triphasil, Wyeth Pharmaceuticals). Initial randomization before the premenopausal treatment phase was based on body weight and plasma total cholesterol/high-

density lipoprotein cholesterol ratios and stratified by social group status. At the end of this premenopausal phase of the study, the animals were ovariectomized to make them surgically menopausal.

The postmenopausal phase of this study was a three-group, parallel-arm design, with treatments lasting 36 mo. An equivalent number of social groups were sampled from each premenopausal condition and randomized to the postmenopausal dietary interventions. The treatment diet included soy protein isolate containing isoflavone (Soy+) at a dose approximately equivalent to 129 mg/d for women, expressed as aglycone units. The control diet contained isolated soy protein that had been alcohol washed to remove the isoflavone (Soy−), so that the protein source remained consistent between the groups. The third diet group was fed isoflavone-depleted soy protein supplemented with CEE (Premarin, Wyeth Pharmaceuticals) at a human equivalent dose of 0.625 mg/d as a positive estrogen control. The isolated soy proteins used for this study were generously provided by The Solae Company. The diets were formulated to be isocaloric for protein, carbohydrate, and fat and comparable for cholesterol, calcium, and phosphorus. The monkeys were fed ∼ 120 kcal/kg body weight split into two feedings (one third in the morning, two thirds in the afternoon). Urine samples were collected from each monkey housed singly in cages overnight during month 35 of the 36-mo postmenopausal phase of the study and frozen for subsequent analysis of metabolite concentrations. Mammary and uterine tissues were collected at necropsy, with samples from each animal flash frozen in liquid nitrogen and stored at −80°C for mRNA analyses and formalin fixed and paraffin embedded for histomorphometric analysis. All procedures involving animals were conducted in compliance with state and federal laws, standards of the U.S. Department of Health and Human Services, and guidelines established by the Wake Forest University Animal Care and Use Committee.

Estrogen Profiling of Macaque Urine Using High-Performance Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry Analysis

Female cynomolgus monkey urine was centrifuged to remove debris, aliquoted, and frozen at −20°C until analysis. Urine samples for 166 animals and complete treatment information for 163 animals were obtained. Endogenous estrogens and estrogen metabolites were quantified using reverse-phase high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry in selected reaction monitoring mode as previously described (23). The estrogens quantified were estrone (E_1), estradiol (E_2), and estriol (E_3). The estrogen metabolites measured were 16-epiestriol (16epiE_3), 17-epiestriol (17epiE_3), 16-ketoestradiol (16KE_2), 16α-hydroxyestrone (16αOHE_1), 2-methoxyestrone (2MeOE_1), 4-methoxyestrone (4MeOE_1), 2-hydroxyestrone-3-methyl ether (3MeOE_1), 2-methoxyestradiol (2MeOE_2), 4-methoxyestradiol (4MeOE_2), 2-hydroxyestrone (2OHE_1), 4-hydroxyestrone (4OHE_1), and 2-hydroxyestradiol (2OHE_2).

Briefly, calibration standards were prepared in charcoal-stripped estrogen-free human urine by adding 20 µL of the deuterium-labeled endogenous estrogens and estrogen metabolites working internal standard solution (1.6 ng of deuterium-labeled estrogen metabolites) and various volumes of estrogen metabolite working standard solution, which typically contained between 0.02 and 19.2 ng of estrogen metabolites. Quality control samples were prepared in estrogen-free urine at three levels (0.12, 0.96, and 6.4 ng of estrogen metabolites/mL). Animal urine (0.5 mL) was used for each analysis and samples from each treatment groups were randomized in each run of the assay. Because endogenous estrogens and their metabolites in urine are mostly present as glucuronide and sulfate conjugates, a hydrolysis step was included using β-glucuronidase/sulfatase from *Helix pomatia* (type H-2). After hydrolysis, estrogen metabolites were extracted with dichloromethane. The organic phase was isolated, evaporated to dryness under nitrogen gas, and reconstituted with a sodium bicarbonate buffer containing dansyl chloride for subsequent derivatization of the estrogen metabolites. Calibration standards

and quality control samples were hydrolyzed, extracted, and derivatized following the same procedure as that of unknown urine samples. After derivatization, all samples were analyzed by reverse-phase high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry in selected reaction monitoring mode using a Finnigan TSQ Quantum-AM triple quadrupole mass spectrometer coupled to a Surveyor high-performance liquid chromatography system (ThermoFinnigan). Both the high-performance liquid chromatography and mass spectrometer were controlled by Xcalibur software (ThermoFinnigan). The lower limit of quantitation for each estrogen metabolite is 0.02 ng/0.5 mL urine sample and the intrabatch precision (percent coefficient of variation) for each estrogen metabolite is provided in Table 1. Two animals were excluded due to uncertainty about the urine samples based on their mass spectrometry profiles, leaving data from 161 animals for subsequent analyses. Animal urine creatinine was measured using an ACE ALERA automated analyzer (Alfa Wassermann, Inc.).

Histomorphometry

Breast and uterine epithelial area and thickness were quantified by histomorphometry, as described previously (24). Briefly, H&E-stained slides were digitized using a Hitachi VK-C370 camera and video capture board (Scion LG-3, Scion, Inc.). Area and thickness measurements were taken with public domain software (NIH Image version 1.60).[4] Three microscopic fields were randomly selected and examined at higher magnification. Percent glandular and epithelial areas were determined by manual tracing of total epithelial units within the endometrium and expressed as a percentage of the total area examined. Percent lobular area in the mammary tissue sections was determined in the same manner. Endometrial thickness was measured at the point of greatest perpendicular depth. H&E-stained mammary glands and uteri were also evaluated qualitatively for histologic changes by two board-certified veterinary pathologists (J.M.C. and N.D.K.). All measurements were made blinded to treatment group. Adjacent tissue sections from each animal were used for histomorphometry and immunostaining.

Mammary mRNA Expression Analysis by Reverse Transcription-PCR

To evaluate changes in estrogen exposure in the mammary gland, Ki67, pS2, and progesterone receptor mRNA levels were measured. Primer-probe set assays were used for human Ki67 and progesterone receptor from the premade inventory of gene expression assays from Applied Biosystems (ABI; assay ID nos. Hs00606991_m1 and Hs00172183_m1, respectively). Design of a macaque-specific primer-probe set for pS2 and reverse transcription-PCR protocol specifics have previously been reported (16). Relative expression amounts (R_0) of mRNA were calculated using a DART analysis worksheet (25) with the change in raw fluorescence (Δ_{Rn}) data generated by the ABI Prism software. The R_0 values were normalized to an internal control (glyceraldehyde-3-phosphate dehydrogenase) and an external plate control (mammary tumor sample) to account for interplate differences in reaction efficiency because the entire sample set could not be run on a single plate.

Statistical Analysis

All data were log-transformed before statistical analysis to establish homoscedasticity. Statistical significance was determined using factorial two-way ANOVA by treatment for log-transformed variables in which estrogen metabolite concentrations could be detected. Due to a large proportion (range, 25–70%) of measurements for several estrogen metabolite concentrations being undetectable (urinary levels of $17epiE_3$, $2MeOE_1$, $2MeOE_2$, $3MeOE_1$, $4MeOE_1$, $4MeOE_2$, $2OHE_2$, and $4OHE_1$), a two-part regression model was used to analyze

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[4]Available from: http://rsb.info.nih.gov/nih-image/.

differences in probability of detection by treatment group and levels of amount present in those monkeys with detectable levels (26). The number of animals in each treatment group with detectable levels of each metabolite and the total percentage of detectable measurements of the 161 urine samples are provided in Table 2. The overall mean amount of the hormone present in the sample was estimated by multiplying the probability of detection by the amount detected in those with detectable hormone amounts, and the variance was estimated using both components of the model as well (27). Data represented in all figures are from analyses including all treatment groups unless otherwise indicated. We did not expect any significant effects of past OCs on any end points examined, so to rule out potential confounding effects of OCs on the more recent postmenopausal treatment, we tested for interactions. Full factorial (OC treatment × postmenopausal diet) interaction effects were examined for all estrogen metabolites, as is common practice when evaluating main effects with ANOVA/general linear models. The interaction was only statistically significant for urinary $2MeOE_1$ levels, and therefore only significant main effects are presented unless otherwise stated. OC treatment effect compares OC treated (OC+; $n = 78$) versus not treated (OC−; $n = 83$) regardless of postmenopausal treatment, and postmenopausal diet effect examines CEE+ ($n = 54$) or Soy+ ($n = 55$) diet versus Soy− diet ($n = 52$) regardless of prior OC treatment. Analyses were done using the SAS statistical analysis software (version 9.1.3, SAS Institute).

Results

Premenopausal Usage of OC Results in Significantly Lower Levels of Most Estrogen Metabolites 3 Years after Surgical Menopause

As shown in Fig. 1 and Fig. 2, OC treatment (regardless of postmenopausal diet) resulted in significant decreases in the levels of all metabolites measured except for E_1 and $4MeOE_2$. Among the most abundant metabolites (Fig. 1A and Fig. 2A), overall percent changes ranged from a 25% reduction in E_1 to a 50% reduction in $2OHE_1$ with OC administration. Percent reductions were comparable among the less abundant metabolites (Fig. 1B and Fig. 2B), ranging from 25% to 57%.

Postmenopausal Dietary Isoflavones Induced Few Significant Effects on Postmenopausal Estrogen Metabolite Concentrations

Monkeys fed an isoflavone-rich diet (Soy+) postmenopausally had increased levels of urinary $2OHE_1$ and $16\alpha OHE_1$ excretion (50% and 56% increases, respectively) and decreased levels of $2OHE_2$, $2MeOE_2$, and $17epiE_3$ (92%, 72%, and 76% reductions, respectively) compared with those monkeys fed the Soy− diet (Fig. 1A and B). These changes in urinary estrogen metabolite excretion were significant regardless of premenopausal OC administration status. Urinary excretion of $2MeOE_1$ was differentially affected by the interaction of isoflavone and OC treatment. There was a 57% decrease in $2MeOE_1$ levels associated with the OC treatment among the monkeys not fed an isoflavone-rich diet. Alternatively, the addition of Soy+ treatment resulted in no significant difference in $2MeOE_1$ excretion between the control and OC-treated monkeys. The visually apparent difference in mean urinary $4OHE_1$ levels between Soy− and Soy+ treated monkeys was not statistically significant because the difference in frequency of detectable $4OHE_1$ between Soy+ and Soy− fed animals did not reach statistical significance ($P = 0.052$, two-tailed Fisher's exact test). Although none of the Soy+ monkeys had detectable levels of $4OHE_1$ in their urine, only four of the Soy− monkeys had detectable urinary concentrations of $4OHE_1$.

Mammary and Endometrial Biomarkers of Estrogen Exposure and Proliferation Were Most Significantly Changed in CEE-Treated Monkeys, with Some Modest Changes Associated with Isoflavone or OC Treatments

CEE treatment resulted in at least a doubling of average endometrial thickness, percent glandular and epithelial areas in the endometrium, and percent lobular area in the mammary tissues compared with monkeys fed the control diet alone (Table 3). Compared with monkeys on the control Soy− diet, those treated with CEE also experienced a 2-fold increase in Ki67, a 6-fold increase in progesterone receptor, and a 12-fold induction in pS2 mRNA expression within mammary tissues (Table 3). Overall, premenopausal OC administration (regardless of postmenopausal treatment) modestly reduced average thickness and percent epithelial area of the endometrium by 23% and 17% respectively (Table 3). Its effect on mammary Ki67 mRNA expression was much more robust: increasing levels by 110% compared with placebo-treated control animals (Table 3). Postmenopausal isoflavones were associated with a 48% reduction in mammary expression of Ki67mRNA compared with Soy− animals (Table 3), whereas it had no significant effect on any other tissue biomarker of estrogen exposure or proliferation. All of these effects, however, are in stark contrast to the wide-ranging and large increases in all proliferation and estrogen exposure end points stimulated by CEE treatment.

Premenopausal and Postmenopausal Treatment Effects on Mammary Gland and Endometrial Proliferation End Points May Occur through Changes in Estrogen Metabolism

To further explore significant treatment effects on biomarkers of estrogen-induced proliferation, we used a simple mediation analysis based on the concepts of Baron and Kenny (28). Using linear regression modeling, we explored whether changes in estrogen and estrogen metabolite levels mediated significant premenopausal or postmenopausal treatment effects on mammary and endometrial measures of proliferation. Table 3 illustrates the significant relationships between the dietary and hormonal treatments and proliferation biomarkers. Table 4 summarizes those relationships and lists the estrogens and/or estrogen metabolites that cause the treatment-outcome effect to drop to nonsignificance once added to the regression model. Inclusion of each mediation variable reduced the significant percent change in treatment group means by at least 9%, and each of these variables alone was significantly correlated with the indicated proliferation end point (data not shown). E_1 and E_2 levels seemed to be the primary mediators for CEE-associated increases in endometrial percent epithelial area. The percent change was reduced from a 120% increase in percent epithelial area without any mediating variables to nonsignificant 26% and 56% increases after inclusion of E_1 or E_2, respectively, in the regression model. Similarly, percent increase in mammary percent lobular area was reduced by 18% (from 154% to 136%) with E_2 incorporation. In contrast, $2OHE_1$ and $16\alpha OHE_1$ variability most strongly attenuated OC-associated reductions in endometrial thickness (23% decrease without mediating variables reduced to a 3% or 4% decrease with $2OHE_1$ or $16\alpha OHE_1$ included, respectively) and percent epithelial area (percent decrease also reduced to 3% or 4% with $2OHE_1$ or $16\alpha OHE_1$). Mediation by $16\alpha OHE_1$ also produced a 13% reduction of percent decrease in mammary Ki67 mRNA expression associated with Soy+ diet (45% decrease in mammary Ki67 expression without mediators lessened to a nonsignificant 31% decrease after $16\alpha OHE_1$ inclusion).

Discussion

In this study, we investigated the long-term effects of prior OCs and/or dietary soy isoflavones on markers of endogenous estrogen metabolism and proliferation in the breast and uterus. Premenopausal OC administration resulted in a significant reduction of most endogenous urinary estrogen metabolites measured during postmenopausal years almost 3 years after OC treatment. The results suggest that premenopausal OC use may alter subsequent exposure to estrogen even after OC use is ceased. This effect could be through a reduction of endogenous

biosynthesis or an increase in catabolism or excretion of estrogens, but elucidation of the exact mechanism is still necessary. Recently, Chan and colleagues (29) also reported a reduction in urinary estrogen concentrations in postmenopausal women who were previous users of OCs, supporting our current findings.

Varied long-term effects of past OC use have also been noted in other studies of other estrogen-sensitive organ systems, such as cardiovascular, bone, and brain (30–34). This evidence suggests that OCs affect not only immediate ovarian hormone production but also long-term production or catabolism of steroid hormones within target tissues. Some studies indicate that steroid hormones, both from endogenous and exogenous sources, can affect the expression of genes through epigenetic modification within various target tissues (35–37). This process is thought to occur mainly through alterations in methylation and demethylation patterns of promoter elements of estrogen-producing enzymes. In subsequent studies, it would be highly informative to investigate differences in methylation patterns of OC-treated and nontreated subjects to determine if long-term usage of OCs may affect target systems through tissue-specific gene imprinting.

Results from this study related to dietary isoflavone effects on urinary estrogen metabolite excretion are generally consistent with previous results from our lab (16), which used commercial immunoassays and was limited to the non–OC-treated subset of animals. Dietary isoflavones decreased urinary excretion of E_1 and increased urinary $2OHE_1$ although the magnitudes of percent difference somewhat vary (−27% versus −54% for E_1 and +50% versus +72% for $2OHE_1$). However, there was variability in the direction and magnitude of change in urinary $16\alpha OHE_1$ levels between the previous publication and the current analysis. We report a 56% increase in $16\alpha OHE_1$ in isoflavone-fed monkeys whereas the previous findings indicate a 26% decrease. This led to a significant increase in the ratio of $2\alpha OHE_1/16\alpha OHE_1$ reported previously, where this study did not make a similar finding (data not shown). Whereas the prior study involving these monkeys did not include the OC+ treated animals, these differences are most likely attributable to the modification in methods used to quantitate the metabolites; we used high-performance liquid chromatography-tandem mass spectrometry whereas an ELISA, which exhibits a great deal of variability in sensitivity and precision, was used previously.

In contrast to premenopausal OC exposure, dietary soy isoflavones tended to have varied effects on different metabolite concentrations. A possible mechanism for this observation may relate to modulation of cytochrome P450 isoforms. There are few published studies, however, investigating isoflavone effects on cytochrome P450 activity. Khan and colleagues (38) have reported soy isoflavone inhibition of cytochrome P450 activation of benzo[a]pyrene into a genotoxic carcinogen in Swiss albino mice through decreasing benzo[a]pyrene–induced expression and activity of cytochrome P450 1A1 and 1A2. Other investigators have reported similar findings using *in vitro* models, but these studies found the significant effects to arise only at very high and supraphysiologic doses of isoflavones (39–41). The relevance of these effects to dietary isoflavone exposure is thus unclear.

The lack of OC effect on Soy+ treated animals' urinary $2MeOE_1$ excretion suggests that these two interventions may balance each other along this pathway of E_1 metabolism. $2MeOE_1$ is produced by COMT with $2OHE_1$ as its substrate. We have shown that prior OC use is associated with reduced $2OHE_1$ levels, thereby reducing the substrate for COMT. Others have published that soy isoflavones inhibit COMT expression and activity at physiologically relevant concentrations (42,43). This suggests that the effects of the Soy+ treatment not only counteract the OC effects on $2OHE_1$ but also hinders its phase II O-methylation, resulting in no net difference in $2MeOE_1$ excretion between OC−Soy+ and OC+Soy+ monkeys. The ability for soy isoflavones to partially reverse potentially negative outcomes of previous OC exposure warrants further investigation. Future studies will focus on cytochrome P450 1A1, 1B1, and

3A4 activities as these are three isoforms expressed in the breast that are involved in estrogen metabolism (44). The mechanism behind isoflavones increasing $2OHE_1$ while decreasing $4OHE_1$ or increasing hydroxylated estrogens while decreasing methoxylated estrogens is a matter for further investigation.

There was no statistical difference in the levels of $4OHE_1$ between Soy+ and Soy− animals because none was detected in the urine of the isoflavone-fed animals, yet there was no statistically significant difference in the probability of its detection between these groups either. The tendency for isoflavone-fed animals to excrete no $4OHE_1$ suggests that dietary soy isoflavones may suppress the activity and/or expression of cytochrome $P450$ 1B1, the primary enzyme responsible for hydroxylation of E_1 at the C4 position (45). The validity of this potential effect should be examined in the context of subjects with higher endogenous estrogen levels.

Long-term CEE treatment of these macaques resulted in significantly higher ratios of both $2\alpha OHE_1/16\alpha OHE_1$ and $2OHE_1/4OHE_1$ (data not shown), as well as drastically increased levels of all estrogen metabolites compared with animals fed the control diet. Most likely, this is a reflection of the clearance of exogenous estrogens. However, the uniquely heterogeneous mix of highly estrogenic compounds present within CEE may be stimulating 2-hydroxylase enzyme expression or activity, creating potential for altered ratios of E_1 metabolites. This phenomenon would require additional investigation comparing treatment with CEE versus 17β-estradiol. CEE alone was used in this study, however, because it is the most commonly prescribed estrogen replacement therapy in the United States, and one major aim of this investigation was to model the female experience with exogenous hormone exposure in non-human primates.

Apart from OCs, several other lifestyle factors have been associated with alterations in circulating estrogen levels. Increased body mass index, fat intake, and alcohol consumption have been strongly correlated with increased estrogen levels, whereas smoking, increased parity, soy food intake, and exercise have been associated with modest decreases in estrogen concentrations in postmenopausal women (46). Various mechanisms have been proposed for these effects, including increased local aromatization of androgens with increased body mass index (47), isoflavone-induced changes in hepatic conjugation of estrogens (48), and alterations in hepatic production of SHBG and/or enzymes responsible for catabolism of circulating estrogens with smoking (49).

Prior OC treatment may affect subsequent target tissue response to estrogens. Although prior OC use produced many significant changes in estrogen metabolite concentrations, these changes did not translate to widespread robust effects on markers of estrogen exposure and cell proliferation in macaque mammary gland and endometrium. Prior OC administration decreased markers of proliferation in the uterus while increasing proliferation markers in the mammary gland, which corroborates the findings of early epidemiologic studies reporting marked long-lasting reductions in endometrial cancer risk but a slight increase in breast cancer risk associated with recent use of OC (50,51). Mechanisms for long-term regulation of mRNA expression effects by prior OC use are unknown but may include induction of terminal differentiation of epithelial progenitor cells and possibly modulation of DNA methylation patterns.

In conclusion, these data provide evidence that prior OC therapy and dietary soy isoflavones may exert effects on estrogen exposure. Moreover, our current findings support previous epidemiologic studies about OC effects on uterine and breast proliferation. Analyses included in this study support the hypothesis that hormonal and dietary effects on estrogen-responsive tissues occur, in part, by alterations in estrogen metabolism. Direct effects of these treatments on mammary gland and endometrium cannot and should not be ruled out based on these statistical descriptions of mediation. However, these results suggest that OC and isoflavone

influences on estrogen metabolite production are significant components of their physiologic actions.

Because this was not a longitudinal prospective investigation of hormonal and dietary treatment effects on endogenous estrogen metabolism, we cannot account for any baseline differences in urinary estrogen excretion that may have existed between treatment groups. However, these animals were randomized on characteristics that are highly correlated with endogenous estrogen concentrations (body weight and total plasma cholesterol/high-density lipoprotein cholesterol ratio). The groups of animals were all of the same mean approximate age and multiparous, so it is reasonable to assume that interindividual variations in baseline estrogen excretion were not major factors in these results. More work is necessary to elucidate mechanisms behind these effects and to determine if these effects are specific to certain contraceptive formulations or attributable to specific isoflavones. These findings indicate that there may be additional and novel effects of prolonged exposures to exogenous hormones and phytochemicals.

Acknowledgments

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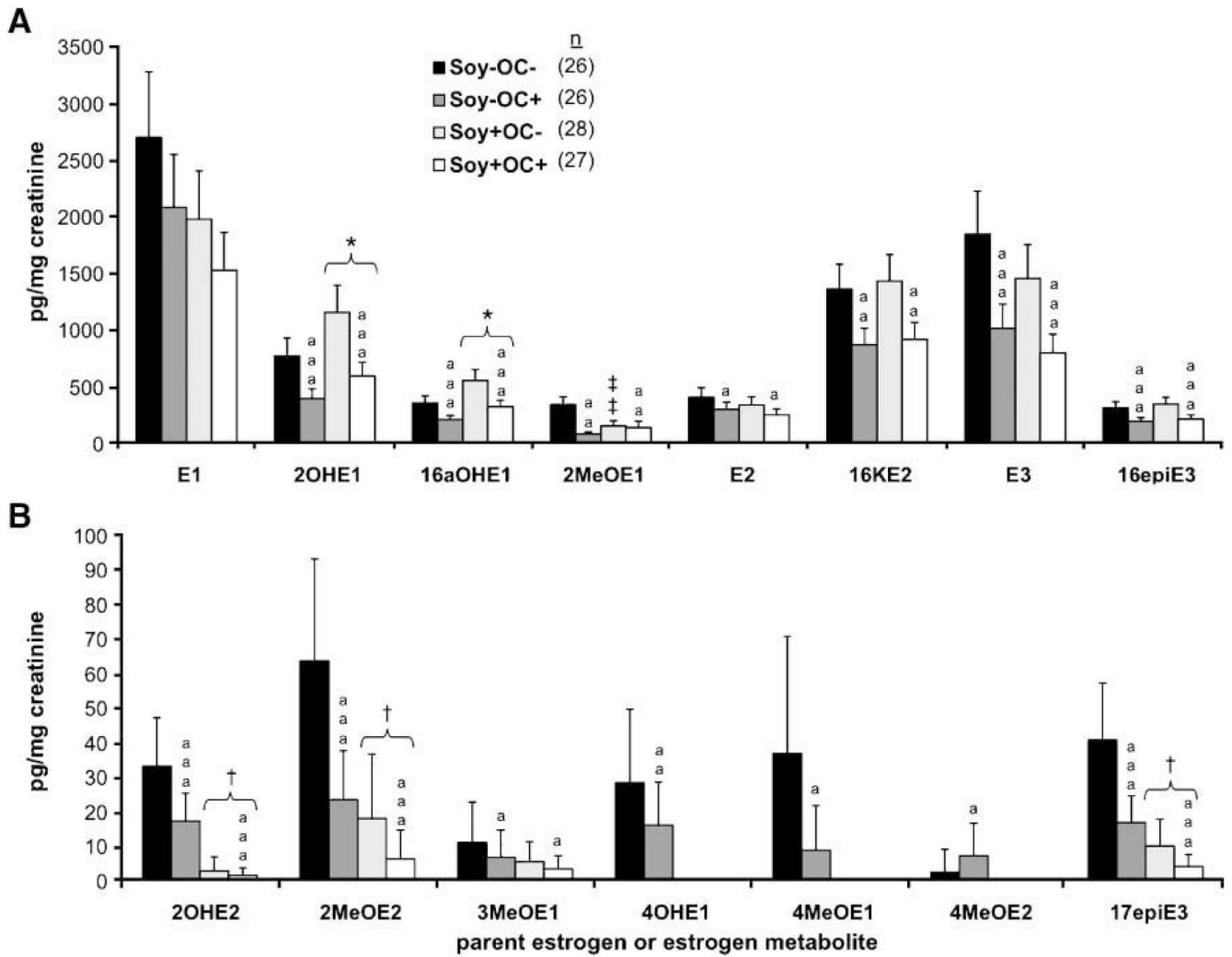
Figure 1.
Postmenopausal urinary estrogen metabolites from control, OC, and soy isoflavone–treated monkeys. Columns, geometric mean urinary content (corrected to creatinine) of the most abundant (**A**) and least abundant (**B**) estrogen metabolites; bars, SE. *, $P < 0.05$, versus Soy− group (two-way ANOVA); ***, $P < 0.0001$, versus Soy− group (two-way ANOVA); †, $P < 0.05$, versus Soy− group (two-part regression); † † † , $P < 0.0001$, versus Soy− group (two-part regression); ‡ ‡ ‡, $P < 0.0001$, interactive effect of OC+ and Soy+ (versus OC−Soy−); a, $P < 0.05$, versus OC−; aa, $P < 0.01$, versus OC−; aaa, $P < 0.0001$, versus OC−.
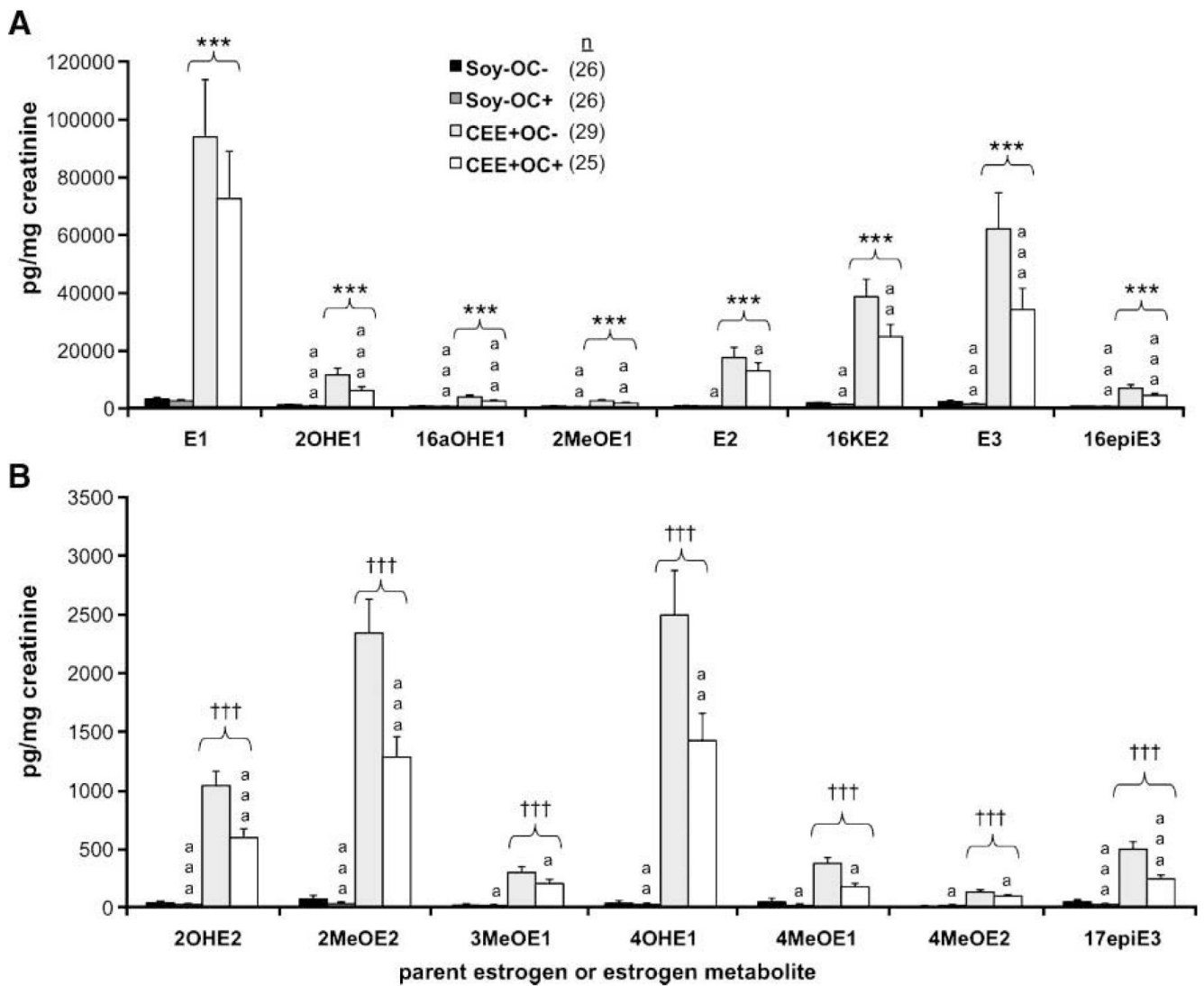
Figure 2.
Postmenopausal urinary estrogen metabolites from control, OC-treated, and CEE-treated monkeys. Columns, geometric mean urinary content (corrected to creatinine) of the most abundant (**A**) and least abundant (**B**) estrogen metabolites; bars, SE. *, $P < 0.05$, versus Soy− group (two-way ANOVA); ***, $P < 0.0001$, versus Soy− group (two-way ANOVA); †, $P < 0.05$, versus Soy− group (two-part logistic regression); † † †, $P < 0.0001$, versus Soy− group (two-part regression); [a], $P < 0.05$, versus OC−; [aa], $P < 0.01$, versus OC−; [aaa], $P < 0.0001$, versus OC−.

Table 1

Precision (percent coefficient of variation) of quality control sample estrogen measurement

Estrogen metabolite	0.12 ng EM/mL	0.96 ng EM/mL	6.4 ng EM/mL
E_3	3.16	0.09	0.47
16-ketoE_2	2.18	0.32	0.54
$16a\text{-OHE}_1$	2.08	0.44	2.61
16-epiE_3	1	0.71	1.02
17-epiE_3	1.12	0.68	1.23
3-MeOE_1	1.4	0.53	1.82
2-MeOE_1	0.46	1.36	0.2
4-MeOE_1	0.86	0.13	0.51
2-MeOE_2	2.46	1.64	1.35
E_1	2.4	0.44	0.55
4-MeOE_2	0.67	0.06	0.45
E_2	0.87	0.2	0.17
2-OHE_1	0.4	0.66	1.72
2-OHE_2	1.38	1.4	0.66
4-OHE_1	0.88	0.98	0.7

Abbreviation: EM, estrogen metabolite.

Table 2

Estrogen metabolite detection proportions by treatment group combination

	n	$17epiE_3$	$3MeOE_1$	$2MeOE_1$	$4MeOE_1$	$2MeOE_2$	$4MeOE_2$	$2OHE_2$	$4OHE_1$
OC−Soy−	26	6	0	19	0	4	0	5	1
OC−Soy+	28	3	2	15	0	2	0	1	0
OC−CEE+	29	28	20	28	24	27	24	27	28
OC+Soy−	26	6	1	19	0	3	0	5	3
OC+Soy+	27	1	0	6	0	0	0	0	0
OC+CEE+	25	25	18	25	18	25	25	24	23
totals	161	69	41	112	42	61	49	62	55
% detected		43	25	70	26	38	30	39	34

Table 3

Premenopausal and postmenopausal treatment effects on proliferation and estrogen exposure biomarkers

Treatment group	Endometrial			Lobular area (%)	PGR mRNA	Mammary	
	Avg thickness (mm)	Epithelial area (%)	Glandular area (%)			Ki67 mRNA	pS2 mRNA
OC−	1.4 (1.2–1.5)	10.1 (9.2–11.1)	14.0 (12.6–15.5)	0.78 (0.63–0.97)	16.8 (14.0–20.0)	0.014 (0.011–0.018)	0.50 (0.38–0.67)
OC+	1.1 (1.0–1.3)[*]	8.6 (7.8–9.5)[*]	12.4 (11.2–13.8)	0.90 (0.72–1.13)	17.1 (14.2–20.8)	0.029 (0.023–0.038)[*]	0.58 (0.43–0.79)
Soy−	0.89 (0.78–1.0)	7.3 (6.5–8.2)	9.3 (8.1–10.5)	0.64 (0.49–0.84)	9.7 (7.7–12.1)	0.019 (0.014–0.026)	0.25 (0.17–0.35)
Soy+	0.83 (0.72–0.94)	6.9 (6.1–7.7)	8.7 (7.7–9.9)	0.56 (0.43–0.73)	8.1 (6.4–10.2)	0.011 (0.008–0.015)[†]	0.20 (0.14–0.29)
CEE+	2.7 (2.4–3.1)[†‡]	16.1 (14.3–18.0)[†‡]	28.4 (15.0–32.2)[†‡]	1.64 (1.26–2.14)[†‡]	62.2 (49.6–78.0)[†‡]	0.041 (0.030–0.055)[†‡]	3.16 (2.21–4.52)[†‡]

NOTE: Values represent geometric means with 95% confidence intervals. Treatment group n values are the same as stated in Materials and Methods.

Abbreviation: PGR, progesterone receptor.

[*] $P < 0.01$, versus OC− group.

[†] $P < 0.01$, versus Soy− group.

[‡] $P < 0.0001$, versus Soy− group.

Table 4

Mediation effects of selected estrogens and their metabolites on treatment-induced changes in mammary gland and endometrial proliferation biomarkers

	Change in means (%)[*]	$P^{\dagger}$	Significant covariate[‡] estrogens/EM
Soy+ vs Soy−[§]			
Mammary Ki67 mRNA expression	−44.7	0.006	$16\alpha OHE_1$[‖]
OC+ vs OC−			
Mammary Ki67 mRNA expression	101.7	0.0004	None
Endometrial thickness	−23.1	0.027	E_1,[‖] E_2,[¶] $16\alpha OHE_1$,[**] $2OHE_1$,[**] $2MeOE_1$[¶]
Endometrial epithelial area (%)	−17.1	0.042	E_1,[‖] E_2,[¶] $16\alpha OHE_1$,[**] $2OHE_1$,[**] $2MeOE_1$[¶]
CEE+ vs Soy−			
Mammary lobular area (%)	153.9	1.26e−05	E_2[¶]
Endometrial thickness	211.2	4.70e−19	None
Endometrial epithelial area (%)	120.3	6.08e−15	E_1[¶], E_2[¶]
Endometrial glandular area (%)	206.7	2.36e−20	None

[*] Percentage change in unadjusted least square means from untreated to treated for designated biomarker.

[†] P value associated with change in unadjusted least square means.

[‡] Estrogen or estrogen metabolite which, on inclusion in regression model, abolished significant treatment effect.

[§] Treatment comparisons for the indicated proliferation biomarker(s).

[‖] Inclusion increases P value to >0.05.

[¶] Inclusion increases P value to >0.1.

[**] Inclusion increases P value to >0.5.