Influence of phytoestrogen diets on estradiol action in the rat uterus

Patricia L. Whitten,*† Elizabeth Russell,* and Frederick Naftolin‡

** Laboratory of Reproductive Ecology and Environmental Toxicology, Department of Anthropology, and † Department of Biology, Emory University, Atlanta, Georgia, USA; ‡ Department of Obstetrics and Gynecology, Yale University School of Medicine, New Haven, Connecticut, USA*

The influence of coumestrol on the action of estradiol was examined in oral and parenteral tests. Coumestrol did not antagonize the uterotrophic action of estradiol when administered either prior to, or jointly with, E_2 treatment, or when administered orally or parenterally. Additive effects on estradiol stimulation of uterine weight and reduction of cytosolic estrogen receptor binding were observed following oral, but not parenteral, administration of coumestrol. On the other hand, coumestrol pretreatment did appear to dampen estradiol's induction of progestin receptors, uterine protein, and nuclear estrogen receptor binding. However, even at those endpoints where coumestrol pretreatment did dampen estradiol action, coumestrol itself produced an estrogenic response. These findings contradict the assumption that all phytoestrogens are necessarily antiproliferative agents and argue for specific identification of the actions of each chemical. (*Steroids* **59**:443–449, 1994)

Keywords: steroids; estrogen antagonism; coumestrol; phytoestrogen, estrogen receptor; isoflavonoid

Introduction

Diet appears to play an important role in the etiology of Western, hormone-dependent diseases.[1] These effects may be mediated not only through the influence of nutrients on sex hormone metabolism but also through the antiestrogen action of isoflavonoids,[2,3] plant phenolics found in soybeans and other legumes[4] that also have been reported in human urine.[5] Asian women and vegetarians, both groups at low risk for breast cancer, have considerably higher urinary levels of isoflavones, presumably due to the high soy content of their diets, than have women consuming a typical Western diet, whereas breast cancer patients exhibit lower urinary levels, suggesting that isoflavones may exert a chemopreventative effect.[6,7] Experimental support is provided by the observation that soybean diets cause regression of mammary tumors in rats.[8,9] Although these effects have been attributed to trypsin or protease inhibitors, the persistence of antitumor activity in soy protein lacking trypsin inhibitor and the correlation of tumor regression with isoflavone content suggest that the biologically active agents in soy may be isoflavonoids.[9] However, direct evidence for isoflavonoid action is mixed. For example, the isoflavonoid coumestrol stimulates DMBA-induced mammary tumors when provided parenterally[10] and both coumestrol and the isoflavone genistein stimulate the growth of MCF-7 cells at nanomolar concentrations,[11] whereas genistein inhibits their growth at micromolar concentrations.[12]

Due to their affinity for estrogen receptors,[13] soy flavonoids have been assumed to act by inhibiting estrogen receptor action. However, suppression of cell growth has been observed in flavonoids that do not bind to the estrogen receptor, whereas some that do appear to lack suppressive action.[14,15] Moreover, a recent report indicates that the isoflavone genistein can inhibit cell growth in a human breast cancer line lacking estrogen receptors.[12] In vitro studies indicate that isoflavonoids interact not only with estrogen receptors but also with other steroid receptors,[16] Type II binding sites,[17] and protein kinases involved in the action of growth factors.[18,19] Therefore, it is important to understand whether any of their antiproliferative actions are related to their estrogen receptor affinity or to some other attribute. In fact, few studies have actually demonstrated that isoflavonoids are estrogen inhibitors. Early studies of in vivo action in mice found that some isoflavonoids inhibited the uterotrophic action of estrone or estradiol at some dose ratios.[20] These studies, however, used high

Address reprint requests to P.L. Whitten, Ph.D., Laboratory of Reproductive Ecology and Environmental Toxicology, Department of Anthropology, 1557 Pierce Drive, Emory University, Atlanta, GA 30322, USA.
Received July 16, 1992; accepted February 15, 1994.

parenteral dosages that cannot be readily translated into the chronic, low-dose regimens characteristic of dietary exposure. Moreover, because the majority of in vivo studies were conducted prior to the development of modern concepts of steroid action, little information is available on their effects on steroid receptors and the similarity of their action to that of classical estrogens and antiestrogens.

To address these issues, we examined the estrogenic effects of natural dietary levels of isoflavonoids. We previously showed that the isoflavonoid coumestrol induces uterine growth and progestin receptor biosynthesis at concentrations well within its natural concentrations in human foods.[21,22] In this paper, we address the potential antagonistic actions of isoflavonoids by examining the effects of dietary coumestrol on uterine responses to estradiol.

Experimental

Chemicals

Sephadex LH-20 was obtained from Pharmacia Fine Chemicals (Piscataway, NJ, USA). 17β[2,4,6,7-^{3}H]estradiol ([^{3}H]E_2, 112 Ci/mmol), [^{3}H]R5020 (86.9 Ci/mmol), and unlabeled R5020 were purchased from New England Nuclear (Boston, MA, USA). Unlabeled estradiol (E_2) and diethylstilbestrol (DES) were obtained from Sigma (St. Louis, MO, USA). Coumestrol was obtained from Eastman Kodak Co., Rochester, NY, USA. Purity was confirmed by melting point determinations.

Animals

Subjects were immature female rats (20–21 days 30–40 g), Sprague-Dawley strain (Camm:Wayne, NJ, USA; Harlan: Indianapolis, IN, USA). Animals were housed in groups of seven in large plastic cages (8 × 50 × 20 cm) under a 12 h light, 12 h dark cycle. Food and water were freely available. Animal care and treatment was in accordance with animal care guidelines established by the Emory Institutional Animal Care and Use Committee.

Tests of estrogen antagonism

Estrogen antagonism was tested by administering coumestrol parenterally or orally and examining its effect on estrogen-stimulated growth in the 3-day uterine weight test. In all experiments, E_2 was administered each morning for 3 days at doses of 0.05–1 μg/day (s.c. in 0.1 mL saline). Uterine weights were determined on the fourth day, 24 h after the last E_2 injection. In parenteral tests, coumestrol was administered with E_2 in the same injection, using a dose that doubled uterine weight (100 μg/day). In oral tests, coumestrol was administered prior to (pretreatment) or during (conjoint treatment) E_2 administration. In pretreatment experiments, coumestrol or a control diet was administered for 90 h prior to the initiation of E_2 treatment; all animals were then placed on a control diet for the period of E_2 treatment. In conjoint treatment experiments, treatment with control or coumestrol diets began on the evening before the first E_2 injection and continued throughout the experiment. All treatment groups contained seven animals.

Dietary administration of coumestrol

Because commercial soy-based diets contain significant levels of phytoestrogens,[23] feeding experiments were conducted with a semipurified formulation (American Institute of Nutrition semipurified rat–mouse diet, US Biochemical, Cincinnati, OH, USA) containing the following ingredients: casein-high nitrogen 20%; DL-methionine 0.3%; cornstarch 15%; sucrose 50%; fiber-celufil 5%; corn oil 5%; mineral mixture 3.5%; vitamin mixture 1%; and choline bitartrate 0.2%. Prior experiments have shown that the phytoestrogen excretion of rats is rapidly and markedly reduced with this purified diet.[23] Coumestrol was incorporated into the semipurified diet at a range of natural dietary concentrations (0.001–0.1%). Control animals received the semipurified diet alone.

Cytosol progestin binding assay

Progestin binding was measured by saturation assays in uterine cytosols from coumestrol-treated rats. Aliquots (100 μL) were incubated with 50 μL TEGM[21] containing [^{3}H]R5020 concentrations ranging from 0.05 to 12 nM plus or minus a 100-fold excess of unlabeled R5020. Following an overnight incubation at 0–4 C, bound and free steroid were separated by gel filtration on Sephadex LH-20 mini columns as described above. The cytosol protein content was determined by the method of Bradford,[24] using bovine serum albumin for the standards. Specific binding was calculated by subtracting binding in the presence of an excess of unlabeled R5020 (nonspecific binding) from that measured in the absence of unlabeled R5020 (total binding). Binding maxima were calculated with the Packard Combicept program (Packard, Downers Grove, IL, USA). Results were expressed as femtomoles of [^{3}H]R5020 bound per mg protein.

Cytosolic estrogen binding assay

Initially, estrogen binding was measured by exchange in uterine cytosols. In single-point binding assays, aliquots (100 μL) of uterine cytosol were incubated with 50 μL TEGM containing 2 nM [^{3}H]E_2 plus or minus a 100-fold excess of DES. Following a 4.5 h incubation at 25 C, bound and free steroid were separated by gel filtration on Sephadex LH-20 mini columns.[25] The columns (7 × 32 mm) were equilibrated with TEGM buffer at 2–4 C. Aliquots (100 μL) of incubates were loaded onto the columns and washed into the column bed with TEGM buffer. Thirty minutes after sample application, the macromolecular bound fraction was eluted into scintillation vials with 0.4 mL TEGM. Radioactivity in the eluent was measured in Opti-fluor in a Beckman LS8000 spectrometer with 50% efficiency. The cytosol protein content was determined by the method of Bradford, using bovine serum albumin for the standards. Specific binding was calculated by subtracting binding in the presence of an excess of unlabeled DES (nonspecific binding) from that measured in the absence of unlabeled DES (total binding). Results were expressed as femtomoles of [^{3}H]E_2 bound per mg protein.

Saturation analyses of estrogen binding

In subsequent assays of cytosolic estrogen receptors, unoccupied estrogen receptors were measured using saturation assays. Uterine cytosols were obtained as described above, and aliquots (100 μL) were incubated with 50 μL TEGM containing [^{3}H]E_2 concentrations ranging from 0.01 to 10 nM plus or minus a 100-fold excess of unlabeled DES. Following an 18 h incubation at 2 C, bound [^{3}H]E_2 was separated from free as described above. Radioactivity in the eluent was measured in Opti-Gold in a Packard 1900CA liquid scintillation spectrometer with 50% efficiency. Binding maxima were calculated with the Packard Combicept program (Packard, Downers Grove, IL, USA).

Exchange assay for cell nuclear estrogen binding

Cell nuclear estrogen binding was measured by the one-tube exchange method of Kranzler et al.[26] and Brown et al.[27] Uteri were homogenized 1 mL BI buffer (1 mM KH_2PO_4, 3 mM $MgCl_2$, 0.32 M sucrose, pH 6.5), followed by 1 mL BII (1 mM KH_2PO_4, 1 mM $MgCl_2$, 2.4 M sucrose, 0.25% Triton X-100, pH 6.5). Buffer BIII (1.5 mL, containing 1 mM KH_2PO_4, 1 mM $MgCl_2$, 1.8 M sucrose, pH 7.0) was layered on top, and the mixture was centrifuged for 35 min at 20,000 × *g* in a Sorvall HB-4 swinging bucket rotor. The resultant supernatant was discarded and the sides of the tubes were dried with a lint-free tissue. Nuclear bound estrogen binding sites in the purified pellets were salt extracted using the method of Roy and McEwen.[28] Nuclear pellets were resuspended in 350 μL TEGMB (TEGM plus 0.5 mM bacitracin) and an equal amount of $TEGMBK_{0.8}$ (TEGMB plus 0.8 M KCl) was added to give a final salt concentration of 0.4 M. Following a 30 min extraction period, the extracted pellets were centrifuged at 20,000 × *g* for 10 min. Aliquots (200 μL) of the supernatant were incubated for 4.5 h at 25 C with 50 μL $TEGMBK_{0.4}$ (TEGMB with 0.4 M KCl) containing 10 nM [^{3}H]E_2 with and without a 500-fold excess of unlabeled DES. Uterine nuclear pellets were digested in 0.3 N KOH, and the DNA content was determined by the method of Burton.[29] Results are expressed as femtomoles of [^{3}H]E_2 bound per mg of DNA.

Saturation binding analyses of cell nuclear estrogen binding

Cell nuclear extracts from control and coumestrol-treated rats were obtained as described above and diluted with $TEGMBK_{0.4}$ to a volume of 2.5 mL. Aliquots (100 μL) were incubated with 50 μL $TEGMBK_{0.4}$ containing [^{3}H]E_2 concentrations ranging from 0.01 to 10 nM. Incubations were carried out for 4.5 h at 25 C. Incubates were cooled to 0 C, and bound [^{3}H]E_2 was separated from free as described above.

Data analyses

Analysis of variance was used to assess the relative contributions of coumestrol and estradiol to the observed effects of treatments. When significant treatment effects were found, differences between treatment groups were further tested by two-tailed *t*-tests using the multivariate *t*-distribution calculated by Dunnett for the simultaneous comparison of several treatments with a control or standard.[30] Saturation assays were analyzed with the nonlinear regression program LIGAND.[31] The significance of differences between binding constants estimated by these curve-fitting programs for the different treatments were assessed by constraining the curves to share a common value, as recommended by Munson and Rodbard.[31] The significance of the difference in goodness of fit between the separate and combined fits was tested by an *F* ratio test using the extra sum of squares principle.

Results

Effects of oral and parenteral coumestrol on E_2-induced uterine growth

Figure 1 shows the effect of conjoint parenteral (A) and oral (B) coumestrol on the uterotrophic response to E_2. No evidence of antagonism of uterine growth was observed by either route. In fact, oral coumestrol markedly augmented the response to E_2; analysis of variance indicated a significant effect of diet [$F_{3,49} = 148.879$, $P = 0.000$] and E_2 [$F_{1,49} = 67.908$, $P = 0.0000$] and an interactive effect [$F_{3,49} = 3.527$, $P = 0.0212$].

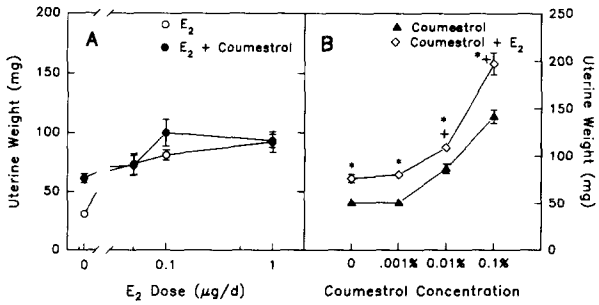

Figure 1 Effect of parenteral and oral coumestrol on E_2-stimulated uterine growth in immature Sprague-Dawley rats. (A) Dose-response curves for E_2 (0–1 μg/day), in the (●) presence and (○) absence of coumestrol (100 μg/day) showing no inhibition by coumestrol. (B) (◇) Effect of a range of coumestrol diets (0.001–0.1%) on the uterine response to a maximal physiologic dose of E_2 (0.01 μg/day), showing an additive effect of coumestrol on the response to E_2. (▲) These data are compared to the dose-response curve for coumestrol alone. Bars represent the mean ± SEM of seven observations per treatment group. (*) Indicates a significant difference between E_2 versus animals receiving the same diet; (+) indicates a significant difference between control diet versus coumestrol diet groups, Student's *t*-test

Effect of pretreatment with a coumestrol diet on uterine response to estradiol

Figure 2A shows the effect of pretreatment with a 0.05% coumestrol diet on the subsequent response to 3 days of treatment with 0.1 μg estradiol. Two-way analysis of variance indicated a significant effect of diet [$F_{1,24} = 14.956$, $P = 0.001$] and E_2[$F_{1,24} = 33.946$, $P = 0.0000$] but no interactive effect [$F_{1,24} = 0.257$, $P = 0.6224$]. Coumestrol-treated animals exhibited an increase in uterine weight similar to that produced by E_2, even though this determination was made 4 days after the end of dietary treatment. The coumestrol pretreatment did not significantly inhibit the uterotrophic response to E_2; the increase in uterine weight after E_2 (+45 mg) in coumestrol-treated animals was not significantly different from the response of animals receiving the control diet (+54 mg, $t = 0.864$, $P > 0.20$).

Figure 2B shows that both diet [$F_{1,52} = 6.658$, $P = 0.0122$] and E_2[$F_{1,52} = 33.492$, $P = 0.0000$] also increased uterine protein. Unlike the uterine weight response, however, the uterine protein response to E_2 was not augmented by coumestrol pretreatment ($t = 1.109$, $P = 0.272$). Although the protein level was higher than that produced by the coumestrol diet alone, the proportionate increase was lower ($t = 2.372$, $P = 0.024$) following coumestrol pretreatment (149.4% ±0.1) than following treatment with the control diet (190.6% ± 0.1).

Effect of coumestrol pretreatment on progestin receptor induction

Figure 2C shows the effect of diet and E_2 treatments on cytosolic progestin receptor concentrations. In contrast to the effects observed with uterine weight, where the effects of coumestrol diet and E_2 were additive, here the estrogens modulated each other's effects. Analysis of variance indicated a significant interactive effect of diet

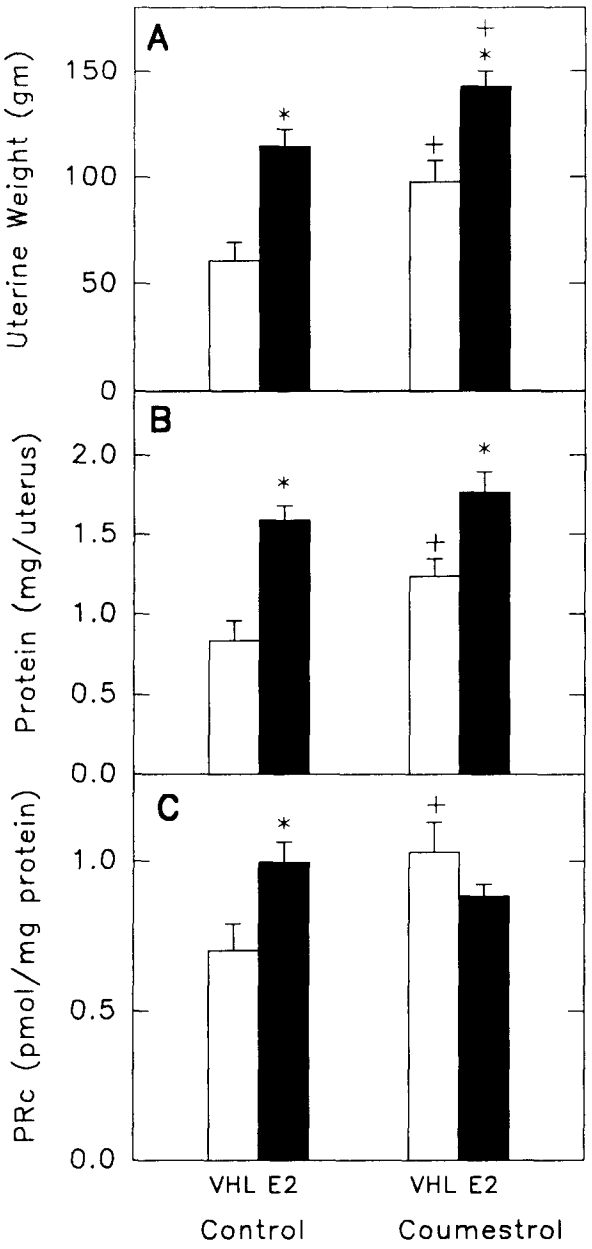

Figure 2 Effect of pretreatment with a coumestrol diet on uterine response to E_2. Immature female rats were given a control or a coumestrol (0.05%) diet for 90 h; subsequently, animals received a control diet while receiving daily injections of saline or E_2 (0.1 μg s.c.). Uteri were obtained 24 h after the last E_2 injection for determination of (A) uterine weight, (B) uterine protein content, and (C) cytosolic progestin receptors levels (PRc). Bars represent the mean $\pm$ SEM of seven observations per treatment group. (*) Indicates a significant difference between E_2 versus saline-injected animals receiving the same diet, Student's t-test; (+) indicates a significant difference between control versus coumestrol-treated animals receiving the same s.c. injections

and E_2 treatment [$F_{1,24} = 8.322$, $P = 0.008$] but no significant effects of either diet [$F_{1,24} = 1.942$, $P = 0.1732$] or E_2[$F_{1,24} = 0.902$, $P = 0.3541$] alone. Although both coumestrol and E_2 treatment elevated progestin receptor levels, the combined treatment reduced receptor concentrations to control levels ($t = 1.054$, $P = 0.181$).

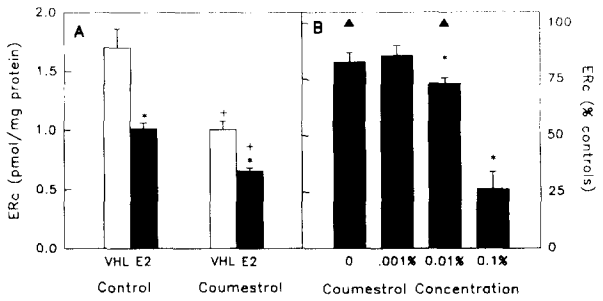

Figure 3 Effects of coumestrol pretreatment or conjoint treatment on estrogen regulation of cytosolic estrogen receptors (ERc). (A) Immature rats were given a control diet (left bars) or a 0.05% coumestrol diet (right bars) for 90 h followed by 3 days of treatment with saline (open bars) or 0.1 μg E_2 (solid bars) for 3 days. Unoccupied ERc were determined by saturation assays. (B) Daily s.c. injections of saline or 0.1 μg E_2 were administered for 3 days while females received control or a range of coumestrol diets. ERc were determined by exchange in single-point binding assays. Data are expressed as a proportion of the mean value for animals receiving the control diet and saline. The bars represent the responses (mean $\pm$ SEM, N = 7) of animals receiving control or the coumestrol diets plus E_2; (▲) represents the responses of animals receiving the control or coumestrol diet alone. (*) Indicates a significant difference from diet controls, Student's t-test

Effect of coumestrol on estrogen regulation of estrogen receptors

Figure 3 illustrates the additive effects of coumestrol on cytosolic estrogen receptor (ERc) concentrations observed in the pretreatment (A) and conjoint treatment (B) experiments. Analysis of variance (A) indicated that ERc were significantly influenced by both diet [$F_{1,24} = 21.801$, $P = 0.0002$] and E_2 treatment [$F_{1,24} = 20.950$, $P = 0.0003$]. There was no interactive effect [$F_{1,24} = 0.896$, $P = 0.3558$]. E_2 produced the same decline in ERc in the presence of coumestrol (35%) as in its absence (34%); the greater decline ($t = 2.632$, $P = 0.014$) observed in the presence of coumestrol (57%) was due to the additive effects of the coumestrol diet. The fact that uterine protein in E_2-treated animals was the same in the presence and absence of coumestrol (see above) indicates that this additive effect was not simply a side effect of growth and receptor dilution. Figure 3B shows that conjoint E_2 and coumestrol treatment also reduced ERc concentrations. Increasing dietary concentrations of coumestrol augmented the ERc reduction induced by E_2 alone (0 dose). Marked reductions in ERc content were observed with the 0.1% coumestrol diet. In contrast to the results obtained with the 0.05% coumestrol diet in Figure 3A, the 0.01% coumestrol diet did not significantly reduce ERc in the absence of estradiol treatment (▲).

Saturation analysis of estrogen receptor binding

An alternative explanation for the apparent reductions in estrogen receptors (ER) might be that high levels of coumestrol and/or E_2 present in cytosolic preparations interfered with the binding assay. This issue was addressed by examining binding affinity and binding capacity in saturation assays. Our previous studies

showed that coumestrol reduces binding affinity when added to ER preparations.[22] Therefore, reductions in affinity should accompany reductions in ER binding if they were an artifact of interference by endogenous ligand. To test this possibility, a 0.05% coumestrol diet was administered along with 3 days of E_2 treatments (0.1 μg/d). This treatment increased uterine weight threefold and reduced cytosolic estrogen receptors by approximately 50% (see Figure 4A). Saturation analyses of uterine cytosols were carried out to determine whether the observed loss of uterine ER was associated with any changes in binding affinity. The mean binding dissociation constants (K_d) were 0.12 nM ±0.02 and 0.18 nM ±0.03 for the control and coumestrol-plus-E_2-treated uteri, respectively. The parallelism and widely separated x-intercepts in Scatchard plots (Figure 2B) of representative analyses from the two treatment groups are indicative of changes in maximum binding (B_{max}) but not in dissocation constant (K_d). Attempts to fit a two-site model to represenative control and coumestrol-treated uteri did not significantly improve the fit over a one-site model [$F = 0$, $P = 1$] and estimates of K_d(0.2 nM), in fact, converged to a single binding site, indicating that the observed reductions in ER binding were a consequence of receptor depletion rather than changes in binding affinity.

Consequences of cytosolic receptor content for receptor activation

The biological significance of the apparent ERc reductions was investigated by examining the consequences for ER activation. A 0.05% coumestrol diet was administered along with 3 days of E_2 treatments (0.1 μg/d). Basal ERn were determined 24 h after the last E_2 injection in seven animals from each group. Subsequently, a physiologic (0.1 μg) or a supraphysiologic (5 μg) E_2 dose was administered to the remaining animals, and ERn were determined 3 h later. Parallelling the ERc reductions seen in Figure 4, Figure 5 shows that both basal ERn and estrogen-stimulated ERn levels were

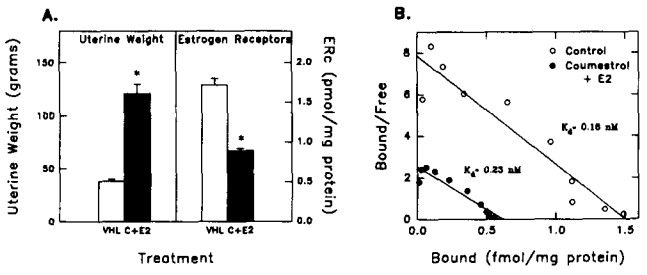

Figure 4 Effects of coumestrol-plus-E_2 treatment on estrogen receptor binding constants. (A) Uterine weight and cytosolic estrogen receptor response in the presence and absence of the 0.05% coumestrol diet. (B) Scatchard plots of representative saturation analyses of control and coumestrol-treated uteri. Immature females were fed the respective diets for 90 h along with s.c. E_2 (0.01 μg/day in 0.1 mL saline). Bars represent mean ± SEM of seven observations per treatment group. Asterisks indicate significant differences ($P < 0.05$, Student's t-test) between treatment groups. Estimates of K_d are the best-fit values obtained when the two curves were fit separately by LIGAND

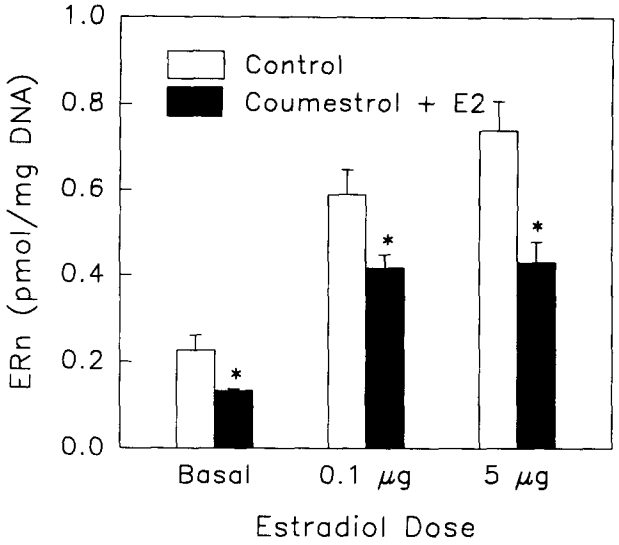

Figure 5 Effects of coumestrol-induced estrogen receptor depletion on the response to E_2 in immature female rats. Animals received control or coumestrol-plus-E_2 treatments described in Figure 2 for 90 h before injection of a physiologic (0.1 μg) and supraphysiologic (5 μg) dose of E_2, and animals were sacrificed 0 and 3 h later. Estrogen binding in salt-extracted nuclear preparations was determined in single-point binding assays. Bars represent the mean ± SEM of seven observations per treatment group. (*) Indicates $P < 0.05$ versus controls, Student's t-test

reduced by the combined treatment. Moreover, although control animals exhibited an increased response to the higher E_2 dose, animals receiving the combined treatment responded similarly to both E_2 doses.

Saturation analysis of nuclear receptor binding

The possibility of interference of coumestrol or endogenous ligand with ERn determinations was addressed by saturation analysis of nuclear binding in uteri that received the 5 μg E_2 stimulus. Once again, these analyses provided no evidence of changes in binding affinity: The mean dissociation constants (K_d) for control and coumestrol plus E_2-treated uteri were 0.38 ± 0.04 nM and 0.40 ± 0.04 nM, respectively.

Discussion

These findings contradict the assumption that all phytoestrogens are necessarily antiproliferative agents. Coumestrol did not antagonize the uterotrophic action of estradiol when administered either prior to, or jointly with, E_2 treatment, or when administered orally or parenterally. The fact that coumestrol produces significant uterine growth and increases ERn binding over the same period of time[22] indicates that this time frame was ample for observation of inhibitory effects mediated by blockage of steroid binding sites.

Additive effects were observed following oral, but not parenteral, administration of coumestrol, possibly a consequence of the more prolonged half-lives of flavonoids following oral administration.[32] Coumestrol diets enhanced estradiol's effect on uterine weight and on estrogen receptor binding. In the latter case, estradiol

reduced ERc binding over a 3-day period, and coumestrol treatment produced further reductions when provided prior to, or along with, estradiol treatments. Scatchard assays suggested that the observed decline did not reflect changes in binding affinity or interference of endogenous ligand with binding assays.

On the other hand, coumestrol pretreatment did appear to dampen estradiol's induction of PRc, uterine protein, and ERn binding. We previously showed that 0.01% and 0.005% coumestrol diets induce progestin receptors;[22] similar results were apparent here following treatment with the 0.05% coumestrol diet. However, when coumestrol treatment was followed by an additional 3 days of E_2 treatment, not only was there no further response to E_2 but PR levels also were reduced to control levels. In addition, both basal levels and estradiol-induced ERn levels were reduced when estradiol treatment was accompanied by the coumestrol diet. We previously showed that a 0.01% coumestrol diet alone increased ERn after 90 h of treatment.[22] The difference between those findings and the present findings appear to be a consequence of the combined estradiol and coumestrol treatments. For example, ERc binding was not altered by the 0.01% coumestrol diet, whereas it was altered by the combined treatments.

These responses resemble the refractory condition produced by repeated[33] or prolonged[34,35] high-dose estrogen exposure, a condition that has been attributed to ER down-regulation,[34] as distinguished from the more transitory loss of uterine ER following a single E_2 injection.[36–39] In these experiments, coumestrol diets elevated and extended estrogen exposure. Because the E_2 dose used here is a physiologic dose known to produce a near maximal uterotrophic response,[40] these treatments are likely to have produced a supraphysiologic stimulus capable of inducing estrogen refractoriness. The accompanying reductions in ERc are unlikely to reflect the transient changes characteristic of ER processing, previously reported for coumestrol in cultured MCF-7 cells,[11] given the paradigm of repeated treatments, coumestrol pretreatment, and the assessment of ER 24 h following the last E_2 treatment. Nor are the reductions simply a side effect of increased protein content and receptor dilution since coumestrol actually reduced the uterine protein response to E_2. Since only receptor binding was measured here, the observed reductions cannot be categorically attributed to reductions in receptor protein, but they do suggest that it may be productive to pursue further investigation of the influence of phytoestrogens on estrogen receptor regulation.

On the whole, however, these investigations provide no support for an antiproliferative or antiestrogen role for coumestrol. Even at those endpoints where coumestrol pretreatment did dampen estradiol action, coumestrol itself produced an estrogenic response. Thus, it is difficult to see how this isoflavonoid could act as a protective agent in the etiology of estrogen-dependent carcinoma. Certainly, it does not conform to the classical picture of an antiestrogen. These findings do not preclude more antagonistic actions by other phytoestrogens nor do they rule out the possibility of antiproliferative actions of phytoestrogens through other mechanisms such as growth-factor mediated action or through down-regulatory effects on steroid receptors and other estrogen-responsive proteins. However, they do argue for caution in the global attribution of antiproliferative action to this diverse group of plant chemicals.

Acknowledgments

This research was supported by the National Institute of Environmental Health Sciences, NIH (ES04297:PLW), and Emory University.

References

1. Trowell HC, Burkitt DP (1983). *Western Diseases: Their Emergence and Prevention.* Edward Arnold, London.
2. Adlercreutz H (1990). Western diet and western diseases: some hormonal and biochemical mechanisms and associations. *Scand J Clin Lab Invest* **50**(Suppl 201):3–23.
3. Whitten PL, Naftolin F (1991). Dietary estrogens: a biologically active background for estrogen action. In: Hochberg RB, Naftolin F (eds), *The New Biology of Steroid Hormones.* Raven Press, New York, pp. 155–167.
4. Harborne J (1979). Flavonoid pigments. In: Rosenthal GA, Janzen DH (eds), *Herbivores: Their Interaction with Secondary Plant Metabolites.* Academic Press, New York, pp. 619–655.
5. Axelson M, Kirk DN, Farrant RD, Cooley G, Lawson AM, Setchell KDR (1982). The identification of the weak oestrogen equol [7-hydroxy-4-(4′-hydroxyphenyl)-chroman] in human urine. *Biochem J* **201**:353–357.
6. Adlercreutz H, Fotsis T, Heikkinen R, Dwyer JT, Woods M, Goldin BR, Gorbach SL (1982). Excretion of the lignans enterolactone and enterodiol and of equol in omnivorous and vegetarian women and in women with breast cancer. *Lancet* **ii**:1295–1299.
7. Adlercreutz H, Fotsis T, Bannwart C, Wahala K, Makela T, Brunow G, Hase T (1986). Determination of urinary lignans and phytoestrogen metabolites, potential antiestrogens and anti-carcinogens, in urine of women on habitual diets. *J Steroid Biochem* **25**:791–797.
8. Troll W, Wiesner R, Shellabarger CJ, Holtzmann S, Stone JP (1980). Soybean diet lowers breast cancer incidence in irradiated rats. *Carcinogenesis* **1**:469–472.
9. Barnes S, Grubbs C, Setchell KDR, Carlson J (1990). Soybeans inhibit mammary tumors in models of breast cancer. In: Pariza M (ed), *Proceedings Mutagens and Carcinogens in the Diet.* Wiley-Liss, New York, pp. 239–253.
10. Verdeal K. Brown RR, Richardson J, Ryan DS (1980). Affinity of phytoestrogens for estradiol-binding proteins and effect of coumestrol on growth of 7,12-dimethylbenz(a)anthracene induced rat mammary tumours. *J Natl Cancer Inst* **64**:285–290.
11. Martin PM, Horwitz KB, Ryan DS, McGuire WL (1978). Phytoestrogen interaction with estrogen receptors in human breast cancer cells. *Endocrinology* **103**:1860–1867.
12. Peterson G, Barnes S (1991). Genistein inhibition of the growth of human breast cancer cells: independence from estrogen receptors and the multi-drug resistance gene. *Biochem Biophys Res Commun* **179**:661–667.
13. Shutt SA, Cox RI (1972). Steroid and phyto-oestrogen binding to sheep uterine receptors in vitro. *J Endocrinol* **52**:299–310.
14. Hirano T, Oka K, Kawashima E, Akiba M (1989). Effects of synthetic and naturally occurring flavonoids on mitogen-induced proliferation of human peripheral-blood lymphocytes. *Life Sci* **45**:1407–1441.
15. Hirano T, Oka K, Akiba M (1989). Anitproliferative effects of synthetic and naturally occurring flavonoids on tumor cells of the human breast carcinoma cell line, ZR-75-1. *Res Commun Chem Pathol Pharmacol* **64**:69–78.
16. Whitten PL, Naftolin F. Steroid receptor affinities of plant flavonoids (under revision).

17. Adlercreutz H, Mousavi Y, Loukovaara M, Hamalainen E (1991). Lignans, isoflavones, sex hormone metabolism and breast cancer. In: Hochberg RB, Naftolin F (eds), *The New Biology of Steroid Hormones*. Raven Press, New York, pp. 145–154.
18. Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M, Fukami Y (1987). Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J Biol Chem* **262:**5592–5595.
19. Ogawara H, Akiyama T, Watanabe S, Ito N, Kobori M, Sedoa Y (1989). Inhibition of tyrosine protein kinase activity by synthetic isoflavones and flavones. *J Antibiot (Tokyo)* **41:**340–343.
20. Folman Y, Pope GS (1966). The interaction in the immature mouse of potent oestrogens with coumestrol, genistein and other utero-vaginotrophic compounds of low potency. *J Endocrinol* **34:**215–225.
21. Whitten PL, Naftolin F (1992). Effects of a phytoestrogen diet on estrogen-dependent reproductive processes in immature female rats. *Steroids* **57:**56–61.
22. Whitten PL, Naftolin F (1992). Effects of a normal, human-concentration, phytoestrogen diet on rat uterine growth. *Steroids* **57:**98–106.
23. Axelson M, Sjovall J, Gustafsson BE, Setchell KDR (1984). Soya—a dietary source of the non-steroidal oestrogen equol in man and animals. *J Endocrinol* **102:**49–56.
24. Bradford MM (1976). A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:**248–254.
25. Ginsburg M, Greenstein BD, MacLusky NJ, Morris ID, Thomas PJ (1974). An improved method for the study of high-affinity steroid binding: oestradiol binding in brain and pituitary. *Steroids* **23:**773–792.
26. Kranzler JD, Jones EE, MacLusky NJ, Sakamoto H, Naftolin F (1984). An exchange assay for the measurement of cell nuclear estrogen receptors in microdissected brain regions. *J Neurochem* **43:**895–898.
27. Brown TJ, MacLusky NJ, Shanabrough M, Naftolin F (1990). Comparison of age- and sex-related changes in cell nuclear estrogen-binding capacity and progestin receptor induction in the rat brain. *Endocrinology* **126:**2965–2972.
28. Roy EJ, McEwen BS (1977). An exchange assay for estrogen receptors in cell nuclei of the adult rat brain. *Steroids* **30:**757–769.
29. Burton K (1956). A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric determination of deoxyribonucleic acid. *Biochem J* **62:**315–323.
30. Dunnett CW (1964). New tables for multiple comparisons with a control. *Biometrics* **20:**482–491.
31. Munson PJ, Rodbard D (1989). LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* **107:**220–239.
32. Griffiths LA (1982). Mammalian metabolism of flavonoids. In: Harborne JB, Mabry TJ (eds), *The Flavonoids: Advances in Research*. Chapman and Hall, New York, pp. 681–718.
33. Stormshak F, Leake R, Wertz N, Gorski J (1976). Stimulatory and inhibitory effects of estrogen on uterine DNA synthesis. *Endocrinology* **99:**1501–1511.
34. Medlock KL, Lyttle CR, Kelepouris N, Newman ED, Sheehan DM (1991). Estradiol down-regulation of the rat uterine estrogen receptor. *Proc Soc Exp Biol Med* **196:**293–300.
35. Markaverich BM, Roberts RR, Alejandro M, Clark JH (1984). The effect of low dose continuous exposure to estradiol on the estrogen receptor (Type I) and nuclear Type II sites. *Endocrinology* **114:**814–820.
36. Jensen EV, Suzuki T, Numata M, Smith S, DeSombre ER (1969). Estrogen-binding substances of target tissues. *Steroids* **13:** 417–427.
37. Sarff M, Gorski J (1971). Control of estrogen binding protein concentration under basal conditions and after estrogen administration. *Biochemistry* **10:**2557–2563.
38. Mester J, Baulieu EE (1975). Dynamics of oestrogen-receptor distribution between the cytosol and nuclear fractions of immature rat uterus after oestradiol administration. *Biochem J* **146:**617–623.
39. Katzenellenbogen BS, Ferguson ER (1975). Antiestrogen action in the uterus: biological effectiveness of nuclear bound estradiol after antiestrogen. *Endocrinology* **97:**1–12.
40. Anderson JM, Peck JR JE, Clark JH (1973). Nuclear receptor-estrogen complex: relationship between concentration and early uterotrophic responses. *Endocrinology* **92:**1488–1495.