Effects of Coumestrol on Estrogen Receptor Function and Uterine Growth in Ovariectomized Rats

Barry M. Markaverich, Brett Webb, Charles L. Densmore, and Rebecca R. Gregory

Department of Cell Biology and Center for Comparative Medicine, Baylor College of Medicine, Houston, TX 77030 USA

Isoflavonoids and related compounds such as coumestrol have classically been categorized as phytoestrogens because these environmentally derived substances bind to the estrogen receptor (ER) and increase uterine wet weight in immature rats and mice. Assessment of the binding affinities of isoflavonoids for ER and subsequent effects on uterine growth suggest these compounds are less active estrogens than estradiol and therefore may reduce the risk of developing breast or prostate cancer in humans by preventing estradiol binding to ER. With the renewed interest in the relationships between environmental estrogens and cancer cause and prevention, we assessed the effects of the phytoestrogen coumestrol on uterotropic response in the immature, ovariectomized rat. Our studies demonstrated that in this animal model, coumestrol is an atypical estrogen that does not stimulate uterine cellular hyperplasia. Although acute (subcutaneous injection) or chronic (multiple injection or orally via drinking water) administration of coumestrol significantly increased uterine wet and dry weights, the phytoestrogen failed to increase uterine DNA content. The lack of true estrogenic activity was characterized by the inability of this phytoestrogen to cause cytosolic ER depletion, nuclear ER accumulation, or the stimulation of nuclear type II sites which characteristically precede estrogenic stimulation of cellular DNA synthesis and proliferation. In fact, subcutaneous or oral coumestrol treatment caused an atypical threefold induction of cytosolic ER without corresponding cytosolic depletion and nuclear accumulation of this receptor, and this increased the sensitivity of the uterus to subsequent stimulation by estradiol. These results in the immature, ovariectomized rat contrast with studies of intact, immature animals and suggest that ovarian estrogens may be a component in the estrogenic response to phytoestrogens such as coumestrol in intact animals. Consequently, the potential estrogenicity of phytoestrogens requires careful reassessment in intact and ovariectomized animals before the impact of these environmentally derived substances on reproductive function and cancer can be realized. *Key words*: coumestrol, estrogen receptor, phytoestrogen, rat uterine growth, type II [^{3}H]estradiol binding sites. *Environ Health Perspect* 103:574–581 (1995)

Bioflavonoids represent a class of naturally occurring plant pigments that humans consume daily in gram quantities (*1,2*). A high correlation exists between the intake of bioflavonoid-rich diets and a lower incidence of stomach, colon, breast, and prostate cancer in man (*3–7*). For these reasons, some investigators have suggested that consumption of weakly estrogenic isoflavonoids such as equol, daidzein, and coumestrol may prevent estrogen-dependent breast and prostate cancers by competing with more active, endogenous estrogens such as estradiol for estrogen receptor (ER) in these target tissues (*5–7*). Conversely, studies by our laboratory and others have shown that flavonoids such as luteolin and quercetin bind with high affinity (K_d ~1–5 nM) to nuclear type II [^{3}H]estradiol binding sites, but not ER, and this is correlated with the antagonism of estrogenic response in the rat uterus, the inhibition of breast, ovarian, pancreatic, and colon cancer cell proliferation *in vitro* and estrogen-independent mammary tumor growth in mice (*8–13*). Therefore, it is not surprising that a naturally occurring flavonoid metabolite, methyl *p*-hydroxyphenyllactate (MeHPLA), has been identified as an endogenous cell-growth-regulating agent and the natural ligand for the type II site (*14*). On the basis of these studies demonstrating mixed agonist/antagonist (estrogenic/antiestrogenic) activities of flavonoids and isoflavonoids in estrogen-responsive tissues, it is likely that dietarily derived flavonoids and/or their metabolites which interact either with ER or type II sites may profoundly affect reproductive function and the incidence of estrogen-dependent breast and prostate cancer (*3–7*).

Although isoflavonoids such as coumestrol have demonstrated estrogenic activity in a variety of experimental systems, most of these studies involved feeding these compounds to intact animals for periods of time ranging from days to weeks and subsequently determining uterine wet and/or dry weights (*15–19*). Consequently, the uterotropic response profiles to phytoestrogens have not been extensively evaluated in ovariectomized animals, in which the contribution of ovarian steroids to the overall net uterine hypertrophy, hyperplasia, and DNA synthesis has been eliminated. Recent studies have shown that oral administration of coumestrol to intact, immature rats failed to antagonize estrogenic stimulation of uterine growth, and, in fact, coumestrol treatment increased uterine weight in these studies, suggesting that this isoflavonoid possessed estrogenic activity (*15–17*). However, because these studies involved the treatment of intact, immature female mice with coumestrol over a period of days, it is possible that coumestrol modulated gonadotropin secretion and/or ovarian steroidogenesis, which may have been partially responsible for the observed estrogenic response. A recent report by Yamazaki (*20*) demonstrating that the isoflavonoid ipriflavone is estrogenic in intact, but not ovariectomized, immature rats also supports this hypothesis (*20*).

Our studies described here were designed to assess the estrogenic activity of parenterally and orally administered coumestrol in the immature, ovariectomized rat to rule out the effects of ovarian estrogen on uterotropic response patterns. These data demonstrate that at the dose levels and treatment conditions used, coumestrol behaved as an atypical estrogen, failing to cause significant cytosolic-depletion and nuclear accumulation of ER or uterine hyperplasia and DNA synthesis in the ovariectomized rat, even though uterine wet and dry weights were elevated above control levels. Therefore, the true estrogenic activity of coumestrol, and perhaps other well-known phytoestrogens, requires careful evaluation in ovariectomized animals, particularly in view of the fact that emphasis is currently being directed toward defining the relationships between phytoestrogen exposure and neoplasia in estrogen-responsive tissues such as the mammary gland, uterine endometrium, and prostate.

Materials and Methods

Chemicals. Coumestrol was purchased from Eastman Kodak (Rochester, New York) and genestein and daidzein were purchased from Indofine (Somerville, New

Address correspondence to B.M. Markaverich, Department of Cell Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030 USA.

This research was supported by grants from the National Institute for Environmental Health Science (ES-05477) and the National Cancer Institute (CA-35490 and CA-55590).

Received 3 October 1994; accepted 27 February 1995.

Jersey). The purity of the flavonoids was determined to be greater than 99% by HPLC analysis using a μBondapak C_{18} column (Waters/Millipore, Milford, Massachusetts) eluted with water:methanol by standard procedures in our laboratory (*9*). Estradiol and diethylstilbestrol were obtained from Sigma (St. Louis, Missouri) and 2, 4, 6, 7-[^{3}H]estradiol (112 Ci/mmole) was purchased from Amersham Radiochemicals (Boston, Massachusetts).

Animals and treatment. Immature (21-day-old) Sprague-Dawley female rats (Holtzman Laboratories, Madison, Wisconsin) were ovariectomized under Metofane anesthesia by standard procedures and allowed to recover 7–10 days before treatment. Animals were housed in stainless-steel cages under controlled conditions consisting of 12 hr of light daily (lights on at 0700 hr), and food and water were provided *ad libitum*. We separated the rats into treatment groups consisting of five or six animals per group and injected them subcutaneously or treated them orally with the indicated dose levels of estradiol or coumestrol dissolved in saline-2.0% Tween 80 (injection) or tap water-2.0% Tween 80 vehicle (oral dosing studies) under the conditions described in the text and figure legends. This method of oral administration is remarkably consistent throughout the treatment period, and phytoestrogens were delivered for weeks without significant effects on body weights or other signs of generalized, nonspecific systemic cytotoxicity. In these experiments, the 30- to 40-day-old rats typically consumed 25.8 ± 4.4 mL of vehicle or coumestrol solution per day. At a concentration of 50 μg coumestrol/mL of tap water-Tween-80 vehicle, this represented a dose of 1.29 ± 0.22 mg of coumestrol per day per animal and doses in excess of ~13 mg/rat/day (500 μg/mL drinking water; ~286 mg/kg body weight) can be readily delivered by this procedure. Animals were sacrificed by cervical dislocation and the uteri were removed, stripped of extraneous tissue, weighed, and stored in saline at 4°C for biochemical analysis. To obtain dry weights, uteri from some of the animals (five to six per treatment group) were dried in an oven at 70°C for 16–24 hr until constant weights were obtained.

Tissue homogenization and fractionation. For biochemical analyses, we homogenized uteri from the control and treated animals in ice-cold TE buffer (10 mM Tris, 1.5 mM EDTA, pH 7.4 at 22°C) in Kontes ground-glass homogenizers in a volume equivalent to 100 mg fresh uterine wet weight equivalents per milliliter and centrifuged the homogenate at 800*g* for 20 min in a Beckman GH-3 rotor to obtain the low-speed cytosol (supernatant) and nuclear pellet fractions (*21,22*). The cytosol was centrifuged at 40,000*g* for 30 min in a Beckman JA 20 rotor to obtain the high-speed cytosol fraction, and equivalent results are routinely obtained with 200,000*g* cytosol preparations. We washed the nuclear pellet fraction three times by resuspension and centrifugation (800*g* for 7 min) in TE buffer before resuspension in the same buffer and analysis for ER or type II sites by [^{3}H]estradiol exchange as described below.

Measurement of cytosolic and nuclear ER by [^{3}H]estradiol exchange. We diluted cytosol and nuclear fractions from control and treated animals to 20 mg fresh uterine wet weight equivalents/mL in TE buffer and brought them to 10 mM with dithiothreitol (*21,22*). The preparations were incubated at 4°C for 140 min in the presence of the reducing agent to eliminate interference from type II sites (*21,22*). Aliquots (250 μL) of the cytosol or nuclear suspensions were incubated (cytosol, 30°C for 30 min; nuclei, 30°C for 30 min) in the presence of a wide range of [^{3}H]estradiol concentrations (0.4 to 10 nM, total binding) ± 300-fold excess diethylstilbestrol (0.12–3.0 μM, nonspecific binding). After this incubation, we incubated cytosol fractions (4°C for 15 min) with hydroxylapetite (HAP) and washed the HAP bound protein by resuspension and centrifugation (800*g* for 5 min) to separate bound and free [^{3}H]estradiol. Nuclear suspensions were also washed by resuspension and centrifugation to remove free [^{3}H]estradiol (*21,22*). Bound [^{3}H]estradiol was extracted from the final, washed HAP or nuclear pellets with ethanol and specific [^{3}H]estradiol binding evaluated by Scatchard analysis (*21,22*). Results were expressed as ER sites per cell assuming that mammalian tissues contain approximately 7 pg of DNA per cell nucleus (*23*). Uterine DNA content was estimated by the method of Burton (*24*).

In studies where the binding affinity of the various phytoestrogens for ER was assessed, uterine cytosol fractions from ovariectomized rats were prepared exactly as described above and diluted to 20 mg/mL in TE buffer containing 10 mM dithiothreitol. After preincubation (140 min at 4°C) in the presence of reducing agent to eliminate interference from cytosol type II sites (*21*), aliquots of the cytosol were incubated (37°C for 30 min) in triplicate in the presence of 10 nM [^{3}H]estradiol ± the indicated concentrations (0.1 nM–10.0 μM) of daidzein, genistein, coumestrol, or diethylstilbestrol, and bound and free steroid were separated by HAP adsorption as described above. We determined [^{3}H]estradiol binding to ER in these studies in the absence (100% bound) or presence of the competitor as previously described (*8*). In a typical experiment, 100% bound represented approximately 5000 cpm.

Assessment of isoflavonoid binding affinity for nuclear type II sites. Since nuclear type II site stimulation appears to be involved in target cell response to estrogenic hormones, and flavonoids such as luteolin, quercetin, and pelargonidin appear to inhibit cellular proliferation through type II site binding interactions (*8*), we determined the binding affinities of isoflavonoids such as coumestrol, daidzein, and genistein for type II sites in rat uterine nuclei. For these studies, uterine nuclear fractions from estradiol-implanted rats (*8*) were prepared in TE buffer, diluted to a final volume equivalent to 20 mg uterine wet weight equivalents/mL, and incubated (4°C for 60 minutes) in the presence of 20 nM [^{3}H]estradiol ± the indicated concentrations of competitor (1.0 nM–20.0 μM) as described in the figure legends. After incubation, the nuclear suspensions were washed by resuspension in TE buffer and centrifugation to remove free steroid, and bound [^{3}H]estradiol was determined by liquid scintillation counting exactly as previously described (*8*). A value of 100% bound in the absence of competitor represented approximately 25, 000 cpm.

Measurement of cytosolic and nuclear type II sites by [^{3}H]estradiol exchange. In experiments where it was necessary to quantitate treatment effects on the levels of cytosolic and nuclear type II binding sites, these subcellular fractions were prepared exactly as described above for the type II site competition assays (*21,22*). We quantified cytosolic type II sites by saturation analysis using the hydroxylapetite adsorption–[^{3}H]estradiol exchange assay (HAA–[^{3}H]estradiol exchange) previously developed by our laboratory for measurement of type II sites without interference from endogenous ligands such as MeHPLA (*22,25*). Briefly, cytosol preparations from controls and coumestrol-treated animals were incubated with hydroxylapetite (HAP) at 4°C for 15 min to allow the type II site to bind to the HAP. The pellet bound protein was washed three times by resuspension in TE buffer and centrifugation (800*g* for 7 min), and the final washed pellet was resuspended in TE buffer and aliquots incubated (22°C for 16 hr) in the presence of a wide range (0.4 nM–40 nM) of [^{3}H]estradiol concentrations in the absence (total binding) or presence (nonspecific binding) of 300-fold excess diethylstilbestrol (*22,25*). Similarly, the washed nuclear pellet fractions from these uteri were incubated (4°C for 60 min) with [^{3}H]estradiol ± diethylstilbestrol, and specific binding to type II sites in cytosol and nuclear fractions was deter-

mined on the basis of uterine DNA content (sites/cell) as previously described in detail (*23*). DNA was estimated by the method of Burton (*24*).

Statistical analyses. Where indicated, the experimental results are expressed as the mean ± SEM. The data presented in the various figures in this manuscript were analyzed statistically by the appropriate one-way or two-way analysis of variance (fixed treatment models) and Duncan's new multiple range test on the treatment means as described in detail (*26*).

Results

To correlate phytoestrogen binding interactions with biological response, we evaluated the binding affinities of these compounds with ER and type II sites in rat uterine nuclear fractions. The data in Figure 1 demonstrate that coumestrol (K_d ~180 nM), daidzein (K_d >1000 nM), and genistein (K_d ~180 nM) bind to the ER (Fig. 1A) with relatively low affinities as described by numerous laboratories for various tissues (*16,26*). This is consistent with the fact that these compounds are weak or short-acting estrogens with substantially less biological activity than long-acting estrogens such as estradiol (*28*). Coumestrol (K_d ~10 nM), daidzein (K_d ~5 nM), and genistein (K_d ~5 nM) displayed higher binding affinities for type II sites than for the ER (Fig. 1), and the apparent binding affinities of these three isoflavonoids for nuclear type II sites are similar to those determined for luteolin and quercetin, which bind to nuclear type II sites (but not ER) with very high affinity (K_d ~1–5 nM) and inhibit estrogen stimulation of uterine growth in the rat and mammary tumor growth in the mouse (*8,9*).

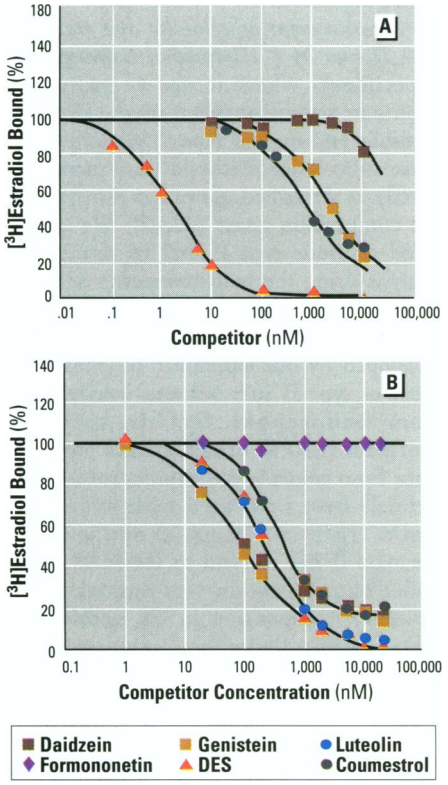

Figure 1. Bioflavonoid competition for (A) estrogen receptor (ER) and (B) type II binding sites in rat uterine cytosol and nuclei. Uterine cytosol and nuclear fractions were incubated in the presence of [^{3}H]estradiol (10 nM, ER; 20 nM, type II) plus or minus the indicated concentrations of competitor under conditions optimum for each of the respective binding sites as described in methods. DES, diethylstilbestrol.

Because coumestrol displayed higher affinity for ER than daidzein and has recently been described as an estrogen in the intact, immature female rat, we focused our efforts on the characterization of the estrogenic activity of coumestrol in the immature, ovariectomized rat uterine model system. Dose–response studies demonstrated that a single injection of coumestrol in doses ranging from 50 to 200 µg resulted in a significant increase in uterine wet weight relative to control (Fig. 2), and the response obtained with 100–200 µg of coumestrol was equivalent to that obtained after a single injection of 1 µg estradiol-17ß. Therefore, it appeared that in the immature, ovariectomized rat, coumestrol treatment increased uterine wet weight 24 hr after a single injection in a manner similar to that described for long-acting estrogens such as estradiol (*28,29*).

The data in Figure 3 represent the temporal effects of coumestrol on uterine wet weight and DNA content after the injection of 100 µg of this phytoestrogen. As expected, based on previous studies suggesting that coumestrol has estrogenic activity (*15–17*), uterine wet weight was increased within 4 hr after coumestrol treatment, and this response was sustained for 24 hr, declining to control levels by 62 hr after injection. This observation was consistent with that reported for active estrogens such as estradiol, where sustaining uterine wet weight beyond 24 hr after injection is typically associated with the stimulation of cellular DNA synthesis and true uterine growth (*29*). Much to our surprise, even though coumestrol treatment increased uterine wet and dry weights in a manner similar to that obtained with estradiol, it failed to increase uterine DNA content at 24 hr after injection. These findings suggest that in the immature, ovariectomized rat, coumestrol behaves as an atypical estrogen, which stimulates cellular hypertrophy and perhaps protein synthesis without stimulatins cellular hyperplasia, reflected by a doubling in DNA content (*29*). Therefore, coumestrol mimics estriol, estrone, and estradiol-17α in this model system by behaving as a short-acting estrogen, capable of stimulating cellular hypertrophy and not hyperplasia when administered as a single injection (*28–31*).

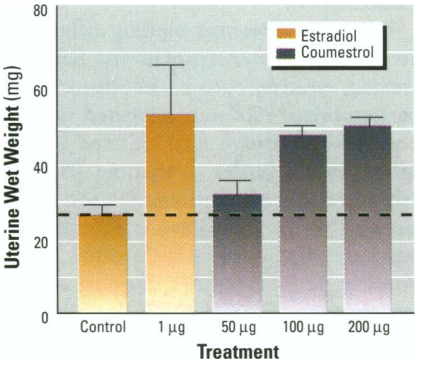

Figure 2. Effects of coumestrol and estradiol on uterine growth. Immature, ovariectomized rats received a single subcutaneous injection of vehicle (controls) or the indicated concentrations of estradiol or coumestrol. Uterine wet weights were determined 24 hr after injection (*40*). No greater response was subsequently obtained with a single subcutaneous injection of 500 µg coumestrol. Data are means ± SEM.

We also assessed the effects of multiple injections of this phytoestrogen on uterine wet weight and DNA content 24 hr after the last injection (Fig. 4) because estriol and estradiol-17α have been shown to stimulate uterine cellular hypertrophy and hyperplasia in the intact immature (*29*) or adult-ovariectomized (*28,30,31*) rats when administered by multiple injection or pellet implant. Therefore, we expected that similar results would be obtained after multiple injections of coumestrol. The data in Figure 4 clearly demonstrate that this was not the case. Although multiple injections of coumestrol increased uterine wet weight relative to control, this response was not equivalent to that after following a single injection of 1 µg estradiol (Fig. 4A). More importantly, estradiol treatment nearly doubled uterine DNA content (Fig. 4B), whereas neither single or multiple injections of this phytoestrogen increased uterine DNA content in this study. In fact, two injections of coumestrol may have slightly reduced uterine DNA content relative to control (Fig. 4B). These data further confirm that in the immature, ovariec-

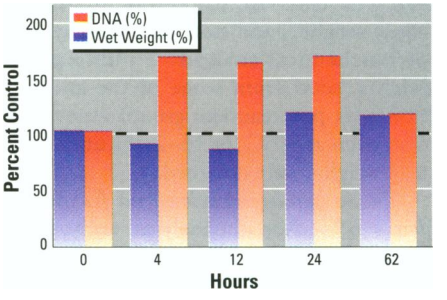

Figure 3. Temporal effects of coumestrol on uterine growth. Ovariectomized rats (four to six per group) were injected with vehicle (controls) or coumestrol (100 µg), and uterine wet weights and DNA content were determined at the indicated times following treatment. The data represent the means for two separate experiments.

tomized rat, coumestrol behaves as an atypical estrogen.

To explain the inability of coumestrol to stimulate uterine cellular hyperplasia (increased uterine DNA content) in the ovariectomized rat, we assessed the effects of this isoflavonoid on the intracellular compartmentalization of ER in the uterus at various times after treatment. Although it is likely that ER is localized in the nucleus and cytosolic ER is most likely an artifact generated during tissue homogenization (*32–34*), the assessment of ER dynamics (cytosolic ER depletion and nuclear ER accumulation) after estrogen administration can be used to assess the estrogenicity of a variety of compounds (*29,34*). In the present studies, we injected immature, ovariectomized rats with a dose (100 µg) of coumestrol, which substantially increased uterine wet weight without causing cellular hyperplasia (Fig. 4) and the levels of cytosolic and nuclear ER were measured as a function of time after treatment (Fig. 5). Again, coumestrol behaved as an atypical estrogen, failing to cause measurable cytosolic ER depletion or significant nuclear ER accumulation. In fact, cytosolic ER levels were elevated above the time zero control level within 4 hr after coumestrol treatment and reached a maximum two- to threefold induction by 24 hr.

To ensure that the atypical effects of coumestrol on ER dynamics were not a characteristic of the model system, we compared the temporal effects of estradiol and coumestrol on ER compartmentalization in the rat uterus in immature, ovariectomized rats. The data in Figure 6 clearly demonstrate that the cytosolic ER depletion and nuclear ER retention patterns after estradiol injection were exactly as described by our laboratory and others for the rat uterine model system under a variety of experimental conditions (*29,35–41*) and the level of cytosolic ER did not return to control levels (0 hours) until 16–24 hr after treatment. It is this sustained cytosolic ER depletion and nuclear ER occupancy that correlates with estrogenic stimulation of cellular hyperplasia and DNA synthesis (*28,29*). The failure of coumestrol to mimic these ER dynamics is likely responsible for the inability of this phytoestrogen to stimulate DNA synthesis in these studies. However, again, in this second experiment, coumestrol treatment did not cause cytosolic depletion or nuclear ER accumulation/retention even though cytosolic ER levels were elevated two- to threefold above time zero controls by 24 hr.

The aforementioned experiments suggested that coumestrol was an atypical estrogen when administered subcutaneously. Therefore, we evaluated the sustained effects of this compound on uterine growth in the rat after oral administration in the drinking water. These studies demonstrated that 96 hr after treatment, orally administered coumestrol resulted in a dose-dependent increase in uterine wet and dry weight relative to controls at dose levels ranging from 5 to 100 µg/mL. Uterine wet and dry weights were essentially doubled by treatment with 100 µg of coumestrol/mL drinking water (~60 mg/kg body weight/day; Fig. 7). Time studies with the higher dose level of coumestrol (100 µg/mL drinking water) demonstrated that the uterotropic response peaked between 72 and 96 hr after treatment, and uterine wet and dry weights were increased five- to sixfold relative to the time-zero vehicle controls (Fig. 8). More important, however, was the observation that uterine DNA content 72 hr after coumestrol treatment (242 µg/uterus) was nearly equivalent to the control value (217 µg/uterus), suggesting that coumestrol failed to stimulate significant cellular hyperplasia and DNA synthesis even when administered in a sustained fashion under these experimental conditions.

To further characterize the uterotropic response patterns to orally administered coumestrol, we also assessed the effects of this phytoestrogen on cytosolic and nuclear ER and nuclear type II binding site levels 72 hr after oral administration (Fig. 9). These data essentially confirmed the injection studies (Figs. 5 and 6) in that oral administration of coumestrol also failed to cause accumulation of nuclear ER or deplete cytosolic ER. In fact, coumestrol treatment resulted in a three- to fourfold induction in the level of cytosolic ER, as was the case for the coumestrol injection studies. That nuclear type II sites were not stimulated by coumestrol treatment (Fig. 9) is consistent with the observation that uterine hyperplasia and DNA synthesis was not stimulated by coumestrol under these conditions (see legend to Figure 8). Estrogen stimulation of nuclear type II sites in the rat uterus is directly correlated with the induction of uterine cellular DNA synthesis and true uterine growth under a wide variety of experimental conditions (*28–30,35*). That cytosolic type II sites appeared to be slightly increased following coumestrol treatment (Fig. 8) is interesting; however, the relationship between this soluble [^{3}H]estradiol binding site and uterotropic

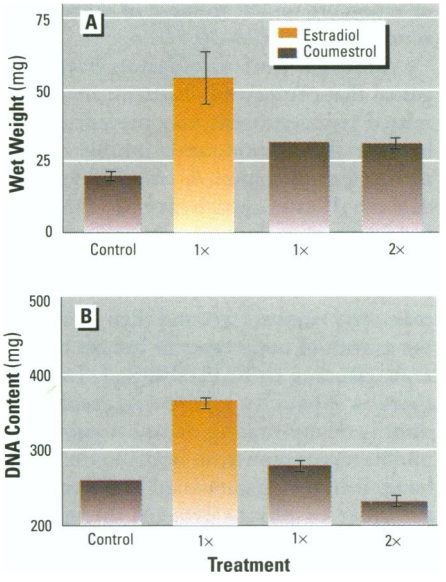

Figure 4. Effects of multiple coumestrol injections on uterine growth in the rat. Ovariectomized rats received one daily injection of 10 µg estradiol or one or two daily injections of 100 µg coumestrol as indicated. Controls were injected with 2% Tween 80 in 0.9% saline vehicle. (A) Uterine wet weights and (B) DNA content were determined 24 hr after the last injection. Data are means ± SEM.

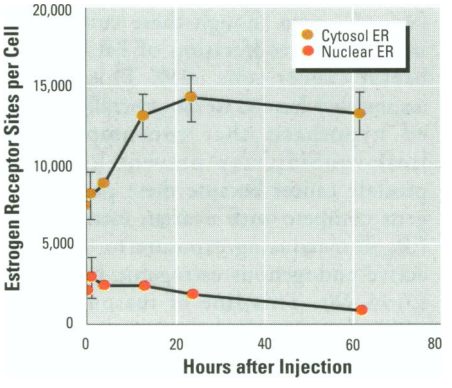

Figure 5. Effects of coumestrol on rat uterine estrogen receptor (ER) dynamics. Ovariectomized rats were injected subcutaneously with 100 µg coumestrol or vehicle (time 0 controls) and uterine cytosol and nuclear estrogen receptors were assayed by [^{3}H]estradiol exchange. Results are expressed as sites per cell (*23*). Data are means ± SEM.

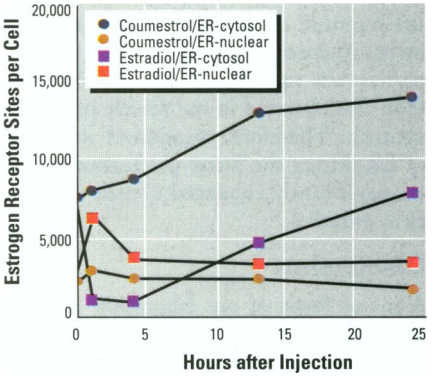

Figure 6. Effects of estradiol and coumestrol on estrogen receptor (ER) dynamics in the rat uterus. Ovariectomized rats received a single injection of estradiol (5 µg) or coumestrol (100 µg), and estrogen receptors (ER) were measured in cytosol and nuclear fractions by [^{3}H]estradiol exchange at the indicated times after injection. Results were based on DNA content and were expressed as sites/cell as described. In separate experiments essentially identical ER responses were observed following a 500 µg injection of coumestrol.

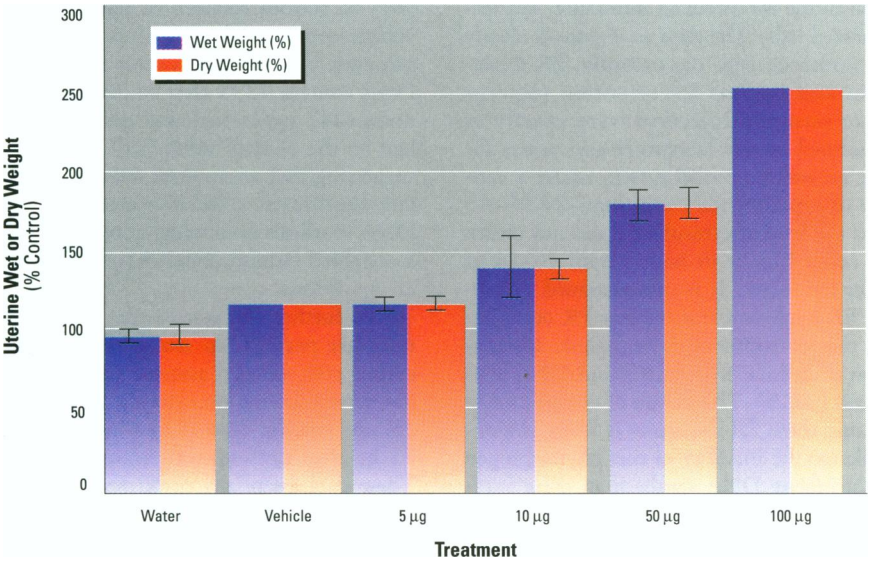

Figure 7. Dose response of rat uterus to coumestrol. Ovariectomized rats were given the indicated concentrations of coumestrol dissolved in drinking water containing 2% Tween 80. Controls received water or the 2% Tween-80 vehicle and uterine wet and dry weights were determined 96 hr after treatment. Data are means ± SEM.

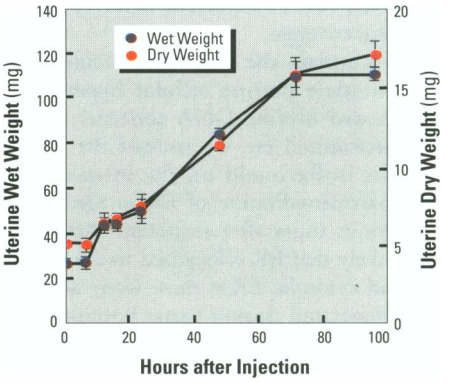

Figure 8. Effects of orally administered coumestrol on uterine growth in the rat. Ovariectomized rats were treated with 100 µg/mL coumestrol dissolved in drinking water containing 2% Tween 80 (vehicle). Controls received water-Tween 80 vehicle and uterine wet and dry weights were determined at the indicated times (0–96 hr) after treatment. The uterine DNA content (not shown in graph) after 72 hr of coumestrol treatment (242 µg/uterus) was not different from that measured in uteri from controls (217 µg/uterus). Data are means ± SEM.

response has not been evaluated.

On the basis of the aforementioned studies demonstrating that coumestrol treatment will stimulate cytosolic ER levels in the rat uterus (Figs. 5, 6, and 9), we suspected that subcutaneous or oral exposure to this phytoestrogen may alter the uterotropic response to estrogenic steroids. To evaluate this possibility, immature, ovariectomized rats were treated orally with vehicle (controls) or coumestrol (250 µg/mL) for 5 days before receiving three daily subcutaneous injections of various doses of estradiol (0.01-10 µg) and uterine weight was determined 24 hr after the last injection. Coumestrol pretreatment increased uterine sensitivity to estradiol as the response of the coumestrol pretreated uterus to doses of estradiol greater than 0.1 µg/day was significantly (p <0.05) greater than that observed in the vehicle pretreated controls. Therefore, coumestrol induction of ER during the 5-day pretreatment period significantly enhanced uterine sensitivity to estradiol.

Discussion

A major focus of our laboratory over the past decade has been estrogen regulation of normal and abnormal cell growth and proliferation. Our efforts have led to the identification of a bioflavonoid metabolite (MeHPLA) as an important cell growth regulating agent (*9,14*). MeHPLA is an endogenous ligand for nuclear type II sites (*14*), and occupancy of this site by MeHPLA and bioflavonoids such as luteolin and quercetin appears to inhibit estrogen stimulation of uterine growth in the rat and mammary tumor growth in mice (*8,9,14*). Subsequent studies demonstrate a direct correlation exists between the occupancy of type II sites by flavonoids such as luteolin, quercetin, and dihydroxybenzylidine acetophenone and the inhibition of breast (*8–10*), colorectal (*11*), pancreatic (*12*), and ovarian cancer (*13*) as well as the inhibition of leukemia (*42*) and lymphoblastoid cell proliferation (*43*). These bioflavonoids do not bind to the ER (*8*), suggesting that the antiestrogenic and/or inhibitory effects of these compounds on cellular proliferation are mediated through type II sites. Therefore, it is likely that dietarily derived bioflavonoids inhibit normal and abnormal cell growth through this mechanism as well.

Conversely, isoflavonoids such as coumestrol, genistein, and daidzein have been described as estrogens, antiestrogens, and anticarcinogens because of their abilities to bind to ER in estrogen target cells (*44–48*), even though these compounds inhibit the proliferation of ER negative breast cancer cells (*49*). This former assumption has led to the generally accepted hypothesis that consumption of isoflavonoids may prevent breast or prostate cancer because these phytoestrogens compete with ovarian estradiol for ER, thus reducing exposure to the more active endogenous estrogenic hormones (*7,46,48*). This line of reasoning was offered as an explanation as to why Japanese women, who have higher circulating levels of less potent, short-acting estrogens such as estriol and estrone, have a lower incidence of breast cancer than their American counterparts (*50*). Although these hypotheses regarding the protective effects of the so-called weak or impeded estrogens are logical, they are not necessarily supported by animal studies. It is well documented that estriol and estradiol-17α are very weak estrogens incapable of stimulating cellular hyperplasia, when exposure is acute (single injection). However, chronic exposure to estriol or estradiol-17α via multiple injection or subcutaneous implant causes uterine cellular hypertrophy, hyperplasia, and mammary cancer in rodents in a manner analogous to that achieved with estradiol (*28–31,51*). This is a likely paradigm for coumestrol, daidzein, and genistein if their pharmacology and mechanism of action are similar to those of other short acting estrogens (*28–30,51*).

Although some investigators have suggested that coumestrol, daidzein, and other related phytoestrogens may prevent cancer because these compounds inhibit malignant cell proliferation *in vitro* (*52,53*) and dimethylbenz[*a*]anthracene (DMBA) induction of mammary tumors in the rat (*54*), these phytoestrogens do not inhibit the growth of established DMBA-induced mammary tumors (*55*), and their effects on the growth of other types of tumors in animals remains to be established. In fact, there is a paucity of experimental data directly demonstrating that phytoestrogens inhibit tumor growth *in vivo*. On the other hand, it is well documented that sustained exposure to phytoestrogens during the neonatal period is associated with persistent vaginal cornification, cervico-vaginal pegs and downgrowths, and uterine squamous metaplasia, which mimics that observed after exposure to diethylstibestrol (*56,57*). These results demonstrate that phytoestrogens may hyperestrogenize target tissues under certain experimental conditions *in vivo*. Therefore, although it is

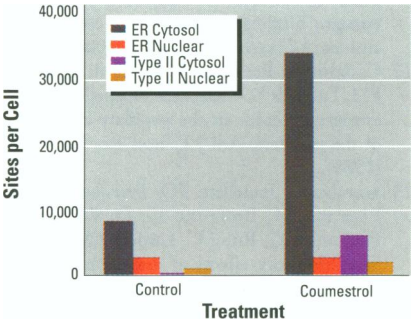

Figure 9. Effects of oral coumestrol administration on cytosolic and nuclear estrogen receptor (ER) and type II sites in the rat uterus. Uteri from ovariectomized rats treated with vehicle (controls) or coumestrol for 72 hr as described in Figure 8 were assayed for cytosolic and nuclear ER and type II sites by [^{3}H]estradiol exchange. Results are expressed as binding sites/cell.

certainly possible that acute exposure to phytoestrogens may block carcinogenic insult to prevent cancer (*46–48,54*), these compounds may also hyperestrogenize ER-containing target tissues after chronic exposure, and none of the parameters (dose and duration of exposure) involved in these response profiles have been adequately defined.

The present studies were performed to more completely define the interactions of isoflavonoids with ER and type II sites in the ovariectomized rat uterus and evaluate subsequent effects on uterine growth and DNA content after acute or sustained exposure to these phytoestrogens. The findings confirmed our hypothesis that isoflavonoids bind to the ER with low affinity and therefore should demonstrate very little estrogenic activity when administered acutely. In fact, the apparent dissociation constants of coumestrol and daidzein for rat uterine nuclear ER were approximately 180 nM and 1000 nM, respectively, whereas little competition was obtained with genistein (Fig. 1). Based on these binding affinities for ER, one has to wonder whether endogenous levels of either coumestrol or genistein will reach concentrations capable of occupying ER and eliciting estrogenic response under normal physiological conditions. Although we previously noted that some isoflavonoids and lignans failed to compete significantly for nuclear type II sites before becoming insoluble in the binding assays (*8*), we used dimethylsulfoxide to solubilize these compounds in the present studies to enhance their solubility in the aqueous binding assay buffer. Under these conditions coumestrol and daidzein competed for [^{3}H]estradiol binding to nuclear type II sites and displayed much higher affinities for this protein (Fig. 1). That coumestrol and daidzein displayed much higher affinities for nuclear type II sites (K_d 5–10 nM) than for the ER suggests that type II sites might be occupied *in vivo* at concentrations where these compounds will not bind to ER. This is currently being evaluated.

The ability of isoflavonoids to interact with both the ER and nuclear type II sites suggest that these compounds may display mixed agonist/antagonist activities. At lower concentrations (<100 nM) coumestrol may occupy nuclear type II sites (K_d 10 nM) and inhibit cell proliferation, as we have shown for luteolin and quercetin (*8*), whereas at higher concentrations (>100 nM) coumestrol may occupy ER (K_d ~180 nM), resulting in the stimulation of cellular proliferation. This concept is consistent with our observations that the binding of the ER complex in the nucleus results in the estrogen-induced dissociation of MeHPLA from nuclear type II sites, and similar events may occur after the binding of isoflavonoid-ER complexes in the nucleus as well (*14,58*). However, it is important to consider that although supraphysiological concentrations (μM) of coumestrol stimulate ER-dependent reporter gene transcription in MCF7 breast cancer cells or HeLa cervical cancer cells *in vitro* (*59,60*), whether endogenous levels of coumestrol reach concentrations required for ER (100–1000 nM) binding *in vivo* under physiological conditions remains to be resolved.

Although recent studies demonstrate that oral administration of coumestrol increased uterine wet and dry weight, nuclear ER levels, and uterine progesterone receptor content in intact, immature rats, uterine DNA content was not determined in these studies (*15–17*), and whether coumestrol stimulated cellular hyperplasia remains to be resolved. Therefore, even though coumestrol appeared to behave as a complete estrogen in these experiments, the animals were dosed with the phytoestrogen over a number of days, and it is possible that ovarian-derived estrogen contributed to the observed stimulation of uterine growth and progesterone receptor content as these animals reached puberty. The present studies using the immature, ovariectomized rat support this contention. Although coumestrol administration by single or multiple injection (Figs. 2–4) or orally in the drinking water (Figs. 7 and 8) increased uterine wet and dry weights relative to control, even sustained exposure to high doses of this phytoestrogen failed to increase uterine DNA content, suggesting that uterine hyperplasia was not observed. This is a significant finding demonstrating that increases in uterine wet and dry weight are not always indicative of uterine hyperplasia as reflected by a doubling in DNA content (*28,29*). It is more likely that the coumestrol-induced increase in uterine wet and dry weight in our studies in ovariectomized animals reflected increases in water and protein content. Studies by Yamazaki demonstrating that the isoflavonoid ipriflavone is estrogenic in intact, but not ovariectomized animals (*20*) support our findings with coumestrol and confirm the hypothesis that ovarian estrogens contribute to the net estrogenic response of the uterus to isoflavonoids.

Further evidence that coumestrol may be an atypical estrogen is provided by studies where the effects of this phytoestrogen on ER function were assessed. In two separate experiments designed to evaluate the temporal effects of coumestrol on ER dynamics and compartmentalization in the ovariectomized rat uterus, injection of 100 μg (or 500 μg; not shown) of coumestrol failed to deplete cytosolic ER or cause nuclear ER accumulation (Figs. 5 and 6) even though increases in uterine wet and dry weight were observed. These results are in sharp contrast to the data in Figure 6 where injection of immature, ovariectomized rats with estradiol resulted in the classical cytosolic ER depletion and nuclear ER accumulation and retention patterns which precede estradiol stimulation of uterine growth in immature (*28,29*) or adult, ovariectomized rats (*29–31,35*). Since sustained nuclear occupancy by the ER-estrogen complex is generally thought to be required for cellular hyperplasia and DNA synthesis (*28–31*), it is not surprising that coumestrol failed to stimulate uterine cellular hyperplasia (DNA content) under these experimental conditions in ovariectomized rats.

Even more surprising was the observation that subcutaneous injection (Figs. 5 and 6) or oral administration of coumestrol (Fig. 9) increased cytosolic ER levels two- to threefold without causing significant cytosolic depletion and nuclear accumulation of ER or induction of nuclear type II sites, which are characteristic responses to estrogenic hormone administration (*22,23,28*). These latter two nuclear events are typically correlated with estrogenic stimulation of cellular DNA synthesis and proliferation (*28–30,35*). These findings also imply that coumestrol may be an atypical estrogen which does not modulate ER or type II site function in a manner analogous to that of estradiol (Figs. 6 and 9), estriol, or estradiol-17α (*28–31*) in the ovariectomized rat uterus. Consequently, although recent studies suggest nuclear ER are elevated in coumestrol-treated, intact, immature rats (*15–17*), this ER could have been occupied by ovarian estrogen and not necessarily coumestrol.

Although it is certainly possible that much higher doses of coumestrol would

have significantly altered ER dynamics to stimulate true uterine growth in the present studies, injection of 500 µg of coumestrol under the conditions described in Figures 5 and 6 also failed to cause cytosolic ER depletion and/or nuclear ER accumulation and DNA synthesis (not shown). Nevertheless, 500 µg of coumestrol stimulated cytosolic ER in a manner similar to that shown obtained with 100 µg of this phytoestrogen (Fig 5). Therefore, increasing the dose level of coumestrol fivefold (~10 mg/kg body weight) failed to alter the response profiles at these short times (1–3 days) after injection. Whether this increase in cytosolic ER concentration after coumestrol treatment reflects phytoestrogen-induced ER activation, ER phosphorylation, and/or the stimulation of ER gene transcription remains to be resolved (*61,62*).

Regardless of the mechanism by which coumestrol increases ER concentration in the uterus, it appears that this isoflavonoid may enhance the sensitivity of this target tissue to estradiol. The data in Figure 10 demonstrate that coumestrol pretreatment significantly shifted the dose–response curve for estradiol, and it is likely that coumestrol induction of cytosolic ER as shown in Figures 5 and 6 was responsible for this increased sensitivity of the uterus to estradiol. Therefore, the mechanisms by which phytoestrogens such as coumestrol, daidzein, and genistein modulate estrogenic response and uterine growth may be much more complex than generally thought and may involve binding to ER, increasing the ER binding capacity of estrogen target tissues such as the uterus

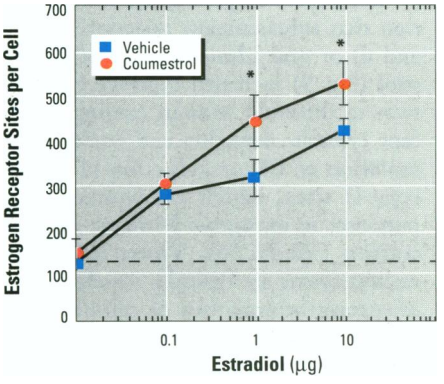

Figure 10. Coumestrol effects on estradiol stimulation of uterine growth in the rat. Adult, ovariectomized rats were treated orally with vehicle (2% Tween 80) or coumestrol (250 µg/mL) in the drinking water for 5 days before receiving three daily subcutaneous injections of the indicated doses (0.1–10 µg) of estradiol. Animals were sacrificed 24 hr after the last injection and uterine wet weights were determined. Results are expressed as the means ± SEM and the response to 1, 5, and 10 µg of estradiol in the coumestrol-treated animals was significantly different (*p <0.05) from the vehicle controls.

by causing ER activation or phosphorylation (*60,61*) or by enhancing ovarian release of estrogen. If this is the case, one might anticipate that the observed estrogenicity or antiestrogenicity of dietarily derived phytoestrogens such as coumestrol may be different in premenopausal and postmenopausal women. This being the case, it is difficult to speculate as to whether phytoestrogens such as coumestrol will prevent and/or protect against cancer by competing with ovarian estrogens for ER as suggested (*5,6*) or whether continuous consumption of antiestrogenic flavonoids such as luteolin and quercetin which inhibit the growth of a broad spectrum of rodent (*8,9*) and human cancers *in vitro* and *in vivo* (*10–13*) will reduce cancer incidence in humans by antagonism at the level of the type II site. Studies designed to accurately define the estrogenicity and antiestrogenicity of dietarily derived isoflavonoids and flavonoids and potential interactions with one another and endogenous estrogens and androgens in these experimental systems will be required to adequately address these issues.

References

1. Kuhnau J. The flavonoids. A class of semi-essential food components: their role in human nutrition. Wld Rev Nutr Diet 24:117–119 (1976).
2. Harborne JB, Mabry TJ, Mabry H. The flavonoids. New York:Academic Press, 1975.
3. Haenszel W, Locke FB, Segi MA. Case-control study of large bowel cancer in Japan. J Natl Cancer Inst 64:17–22 (1980).
4. Graham W, Dayal H, Swanson, M. Diet in the epidemiology of cancer of the colon and rectum. J Natl Cancer Inst 51:709–714 (1978).
5. Adlercreutz H, Mousavi Y, Hockerstedt K. Diet and breast cancer. Acta Oncol 31:175–181 (1992).
6. Sharma OP, Adlercreutz H, Strandberg JD, Zirkin BR, Coffey DS, Ewing LL. Soy of dietary source plays a preventive role against the pathogenesis of prostatitis in rats. J Steroid Biochem Molec Biol 43:557–564 (1992).
7. Adlercreutz H, Markkanen H, Watanabe S. Plasma concentrations of phyto-oestrogens in Japanese men. Lancet 342:1209–1210 (1993).
8. Markaverich BM, Roberts RR, Alejandro MA, Johnson GA, Middleditch BS, Clark JH. Bioflavonoid interactions with rat uterine type II binding sites and cell growth inhibition. J Steroid Biochem 30:71–78 (1988).
9. Markaverich BM, Gregory RR, Alejandro MA, Kittrell FS, Medina D, Clark JH, Varma M, Varma RS. Methyl p-hydroxyphenyllactate and nuclear type II binding sites in malignant cells: metabolic fate and mammary tumor growth. Cancer Res 50:1470–1478 (1990).
10. Scambia G, Ranelletti FO, Benedetti Panici P, Piantelli M, Bonanno G, DeVincenzo R, Ferrandina G, Pierelli L, Capelli A, Mancuso S. Quercetin inhibits the growth of multidrug-resistant estrogen-receptor negative MCF-7 human breast cancer cell line expressing type II estrogen-binding sites. Cancer Chemother Pharmacol 28:255–258 (1991).
11. Piantelli M, Ricci R, Larocca LM, Capelli A, Rizzo S, Scambia G, Ranelletti FO. Type II estrogen binding sites in human colorectal carcinoma. J Clin Pathol 43:1004–1006 (1990).
12. Carbone A, Ranelletti FO, Rinelli A, Vecchio FM, Lauriola L, Piantelli M, Capelli A. Type II estrogen receptor in the papillary cystic tumor of the pancreas. Am J Cancer Res 92:572–576 (1989).
13. Scambia G, Ranelletti FO, Benedetti Panici P, Piantelli M, Bonanno G, DeVincenzo R, Ferrandina G, Rumi C, Larocca LM, Manusco SX. Inhibitory effects of quercetin on OVCA 433 cells and the presence of type II oestrogen binding sites in primary ovarian tumors and cultured cells. Br J Cancer 62:942–946 (1989).
14. Markaverich BM, Gregory RR, Alejandro MA, Clark JH, Johnson GA, Middleditch BS. Methyl p-hydroxyphenyllactate: an inhibitor of cell proliferation and an endogenous ligand for nuclear type II binding sites. J Biol Chem 263:7203–7210 (1988).
15. Whitten PL, Naftolin F. Effects of phytoestrogen diet on estrogen-dependent reproductive processes in immature female rats. Steroids 57:56–61 (1992).
16. Whitten PL, Russel E, Naftolin F. Effects of a normal, human-concentration, phytoestrogen diet on rat uterine growth. Steroids 57:98–106 (1992).
17. Whitten PL, Russell E, Naftolin F. Influence of phytoestrogen diets on estradiol action in the uterus. Steroids 59:443–449 (1994).
18. Kaziaro R, Dennedy JP, Cole ER, Sothwel-Keely PT. The oestrogenicity of equol in sheep. J Endocrinol 103:395–399 (1984).
19. Shutt DA. The effects of plant oestrogens on animal reproduction. Endeavor 35:110–113 (1976).
20. Yamazaki I. Effect of Ipriflavone on the response of the uterus and thyroid to estrogen. Lancet 342:1209–1210 (1993).
21. Markaverich BM, Upchurch S, Williams M, Clark JH. Heterogeneity of nuclear estrogen-binding sites in the rat uterus: a simple method for the quantitation of type I and type II sites by [^{3}H]estradiol exchange. Endocrinology 109:62–69 (1981).
22. Markaverich BM, Adams NR, Roberts RR, Alejandro MA, Clark JH. Cytosol type II sites in the rat uterus: interaction with an endogenous ligand. J Steroid Biochem 28:599–608 (1987).
23. Markaverich BM, Roberts RR, Alejandro MA, Clark JH. The effect of low dose continuous exposure to estradiol on the estrogen receptor (type I) and nuclear type II sites. Endocrinology 114:814–820 (1984).
24. Burton K. A study on the conditions and mechanism of the diphenylamine reaction for colorimetric estimation of deoxyribonucleic acid. Biochem J 62:315–323 (1956).
25. Markaverich BM, Roberts RR, Alejandro MA, Clark JH. An endogenous inhibitor of [^{3}H]estradiol binding in normal and malignant tissues. Cancer Res 44:1515–1519 (1984).
26. Steele RGD, Torrie JH. Principles and procedures of statistics. New York:McGraw Hill, 1960.
27. Tang BY, Adams NR. Effect of equol on oestrogen receptors and on synthesis of DNA and protein in the immature rat uterus. J Endocrinol 85:291–297 (1980).
28. Clark JH, Paszko Z, Peck EJ Jr. Nuclear binding and retention of the receptor estrogen complex: relation to the agonistic and antagonistic properties of estriol. Endocrinology 100:91–96 (1977).

29. Clark JH, Peck EJ Jr. Female sex steroids, receptors and function. New York:Springer-Verlag, 1979.
30. Markaverich BM, Clark, JH. Two binding sites for estradiol in rat uterine nuclei: relationship to uterotropic response. Endocrinology 105:1458–1462 (1979).
31. Clark JH, Williams M, Upchurch S, Eriksson H, Helton E, Markaverich BM. Effects of estradiol-17α on nuclear occupancy of the estrogen receptor, stimulation of nuclear type II sites and uterine growth. J Steroid Biochem 16:323–328 (1982).
32. King WJ, Greene GL. Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. Nature 307:745–746 (1984).
33. Jordan VC, Tat AC, Lyman SD, Gosded B, Wolf MF, Bain RR, Welshons WB. Rat uterine growth and induction of progesterone receptor without estrogen receptor translocation. Endocrinology 116:1845–1857 (1985).
34. Welshons WV, Lieberman ME, Gorski J. Nuclear localization of unoccupied oestrogen receptors. Nature 307:747–749 (1984).
35. Markaverich BM, Upchurch S, Clark JH. Progesterone and dexamethasone antagonism of uterine growth: role for a second nuclear estrogen binding site for estradiol in estrogen action. J Steroid Biochem 14:125–132 (1981).
36. Ruh TS, Baudendistal LJ. Different nuclear binding sites for antiestrogen and estrogen receptor complexes. Endocrinology 100:420–426 (1977).
37. Ruh TS, Baudendistal LJ. Antiestrogen modulation of the salt-resistant nuclear estrogen receptor. Endocrinology 102:1838–1846 (1978).
38. Katzenellenbogen BS, Ferguson ER. Antiestrogen action in the uterus: biological ineffectiveness of nuclear bound estradiol after antiestrogen. Endocrinology 97:1–12 (1975).
39. Koseki Y, Zava D, Chamness GC, McGuire WL. Estrogen receptor translocation and replenishment by the antiestrogen tamoxifen. Endocrinology 101:1104–1110 (1977).
40. Ferguson ER, Katzenellenbogen BS. A comparative study of antiestrogen action: temporal patterns of antagonism of estrogen stimulated uterine growth and effects on estrogen receptor levels. Endocrinology 100:1242–1251 (1977).
41. Lan NC, Katzenellenbogen BS. Temporal relationships between hormone receptor binding and biological responses in the uterus: studies with short- and long-acting derivatives of estriol. Endocrinology 98:220–227 (1976).
42. Teofili L, Pierelli L, Iovino MS, Leone G, Scambia G, DeVincenzo R, Beneditti-Pancini P, Menichella G, Macri E, Piantelli FO, Larocca LM. The combination of quercetin and cytosin arabinoside synergistically inhibits leukemic cell growth. Leukemia Res 16:497–503 (1992).
43. Scambia G, Ranelletti FO, Beneditti-Pancini P, Piantelli M, Rumi C, Battaglia F, Larocca LM, Capelli A, Manusco S. Type II estrogen binding sites in a lymphoblastoid cell line and growth inhibitory effect of estrogen, antiestrogen and bioflavonoids. Int J Cancer 46:1112–1116 (1990).
44. Murphy PA. Phytoestrogen content of processed soybean products. Food Technol 36:62–64 (1982).
45. Xu X, Wang H-J, Murphy PA, Cook L, Hendrich S. Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. J Nutr 124:825–832 (1994).
46. Setchell KDR, Adlercreutz H. Mammalian lignans and phytoestrogens. Recent studies on their formation, metabolism, and biological role in health and disease. In: Role of the gut flora in toxicity and cancer (Rowland IR, ed). London:Academic Press, 1988;315–345.
47. Fisher S, Gameron GS, Baldwin JK, Jasheway DW, Patrick KE. Reactive oxygen in the tumor promotion stage of skin carcinogenesis. Lipids 23:592–597 (1988).
48. Adlercreutz H, Mousavi Y, Hockerstedt K. Diet and breast cancer. Acta Oncol 31:175–181 (1992).
49. Peterson G, Barnes SX. Genistein inhibition of the growth of human breast cancer cells: independence from estrogen receptors and the multi-drug resistence gene. Biochem Biophys Res Commun 179:661–667 (1991).
50. Siiteri PK, Nisker JA, Hammond GL. Hormonal basis of risk factors for breast and endometrial cancer. In: Hormones and cancer (Iacobeli S, ed). New York:Raven Press, 1980;499–505.
51. Noble RL, Hochachka BC, King D. Spontaneous and estrogen-produced tumors in Nb rats and their behavior after transplantation. Cancer Res 35:766–780 (1975).
52. Monti E, Sinha BK. Antiproliferative effect of genistein and adriamycin against estrogen-dependent and -independent human breast carcinoma cell lines. Anticancer Res 14: 1221–1226 (1994).
53. Pagliacci MC, Spinozzi F, Migliorati G, Fumi G, Smacchia M, Grignani F, Riccardi C, Nicoletti I. Genistein inhibits tumour cell growth *in vitro* but enhances mitochondrial reduction of tetrazolium salts: a further pitfall in the use of the MTT assay for evaluating cell growth and survival. Eur J Cancer 29A:1573–1577 (1993).
54. Lamartininiere CA, Moore J, Holland M, Barnes S. Neonatal genistein chemoprevents mammary cancer. Proc Soc Exp Biol Med 208:120–123 (1993).
55. Verdeal K, Brown RR, Richardson T, Ryan DS. Affinity phytoestrogens for estradiol binding proteins and effect of coumestrol on growth of 7,12-dimethylbenz[a]anthracene-induced rat mammary tumors. J Natl Cancer Inst 64:285–290 (1980).
56. Burroughs CD, Mills KT, Bern HA. Reproductive tract abnormalities in female mice exposed neonatally to various doses of coumestrol. J Toxicol Environ Health 30:105-122 (1990).
57. Burroughs CD, Bern HA, Stokstad EL. Prolonged vaginal cornification and other changes in mice treated neonatally with coumestrol, a plant estrogen. J Toxicol Environ Health 15:51–61 (1985).
58. Markaverich BM, Roberts RR, Finney RW, Clark JH. Preliminary characterization of an endogenous inhibitor of [^{3}H]estradiol binding in rat uterine nuclei. J Biol Chem 258:11663–11671 (1983).
59. Miksicek RJ. Commonly occuring plant flavonoids have estrogenic activity. Mol Pharmacol 44:37–43 (1993).
60. Makela S, Davis VL, Tally WC, Korkman J, Salo L, Vihko R, Santti R, Korach KS. Dietary estrogens act through estrogen receptor-mediated processes and show no antiestrogenicity in cultured breast cancer cells. Environ Health Perspect 102:572–578 (1994).
61. Orti E, Bodwell J, Monk A. Phosphorylation of steroid hormone receptors. Endocr Rev 13:105–128 (1993).
62. Migliaccio A, Pagano M, De Goed CJC, Di Domenico M, Castoria G. Phosphorylation and estradiol binding of estrogen receptor in hormone-dependent and hormone-independent GR mouse mammary tumors. Int J Cancer 51:733–739 (1992).