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Estrogenicity of the Isoflavone Metabolite Equol on Reproductive and Non-Reproductive Organs in Mice[1]

Vimal Selvaraj,[3] Melissa A. Zakroczymski,[3] Afia Naaz,[3] Motoko Mukai,[3] Young H. Ju,[4] Daniel R. Doerge,[7] John A. Katzenellenbogen,[5] William G. Helferich,[2,4,6] and Paul S. Cooke[2,3,6]

Departments of Veterinary Biosciences,[3] Food Science and Human Nutrition,[4] and Chemistry[5] and Division of Nutritional Sciences,[6] University of Illinois at Urbana-Champaign, Urbana, Illinois 61802
National Center for Toxicological Research,[7] Food and Drug Administration, Jefferson, Arkansas 72079

ABSTRACT

Equol, a metabolite of the phytoestrogen daidzein, is present at significant levels in some humans who consume soy and in rodents fed soy-based diets. Equol is estrogenic in vitro, but there have been limited studies of its activity in vivo. We evaluated equol effects on reproductive and non-reproductive endpoints in mice. Ovariectomized age-matched (30-day-old) female C57BL/6 mice were fed phytoestrogen-free diets and given a racemic mixture of equol by daily injections (0, 4, 8, 12, or 20 mg [kg body weight]$^{-1}$ day^{-1}) or in the diet (0, 500, or 1000 ppm) for 12 days. Mice were killed, and serum concentrations of total and aglycone equol were measured. Total serum equol concentrations ranged from 1.4 to 7.5 μM with increasing doses of injected equol, but uterine weight increased significantly only at 12 and 20 mg (kg body weight)$^{-1}$ day^{-1}. Dietary equol at 500 or 1000 ppm produced total serum equol concentrations of 5.9 and 8.1 μM, respectively, comparable with those in rodents consuming certain high-soy chows; the proportion of equol present as the free aglycone was much lower with dietary administration than injections, which may be a factor in the greater biological effects induced by injections. Dietary equol did not significantly increase uterine weight. Increasing dietary and injected equol doses caused a dose-dependent increase in vaginal epithelial thickness. Uterine epithelial proliferation was increased by equol injections at 8–20 mg (kg body weight)$^{-1}$ day^{-1} and 1000 ppm dietary equol. Neither dietary nor injected equol decreased thymic or adipose weights. In conclusion, equol is a weak estrogen with modest effects on endpoints regulated by estrogen receptor α when present at serum levels seen in rodents fed soy-based diets, but quantities present in humans may not be sufficient to induce estrogenic effects, although additive effects of equol with other phytoestrogens may occur.

female reproductive tract, steroid hormone receptors, uterus, vagina

INTRODUCTION

There is a large body of literature describing potential health benefits of soy and soy products in humans and animal models. Soy phytoestrogens, which may play a major role in these effects, have been extensively investigated and reviewed [1–5]. The two major soy phytoestrogens, genistein and daidzein, are isoflavones that have a structural similarity to 17β-estradiol and elicit or selectively modulate estrogenic responses by binding to both estrogen receptor (ER) α and ERβ [6, 7], with higher affinity for ERβ [8]. Studies on soy isoflavones have focused primarily on genistein because it is a more potent estrogen than daidzein [9, 10], is typically present in higher quantities than daidzein, and has other properties such as inhibition of protein tyrosine kinases [11] and topoisomerase II [12, 13], as well as induction of TGFβ [9].

Despite the limited estrogenicity of daidzein, some recent evidence suggests that its metabolite equol may play a critical role in phytoestrogen effects [2]. Daidzein is converted by microbial biotransformation in the intestine to the isoflavan equol, a biologically active metabolite [14]. Equol has a significantly longer half life in the circulation than genistein or daidzein [15] and binds to both ERα and ERβ, although similar to genistein and daidzein its binding affinity for ERβ is greater than that for ERα [16]. Approximately one third of humans produce equol; this may reflect differences in the intestinal microbial flora of this group compared with humans who do not produce equol [17, 18]. Setchell et al. [2] have postulated that the beneficial responses from soy consumption may correlate with the equol-producing status of individuals, suggesting a major role for equol as a biomarker for the effectiveness of soy [2]. Further, rodents on commercial diets formulated with soy meal are continuously exposed to isoflavones [19] and have sustained high serum equol concentrations [20]. Such high equol levels may potentially have important implications in experiments measuring endpoints influenced by isoflavones [21].

Despite the potential importance of equol, there have been limited studies of equol effects in vivo because of the high cost of this compound and its limited availability. In addition, studies of equol effects in vivo [22, 23] have utilized equol injection, rather than the more physiological administration of equol in the diet, again because of the high cost of equol precluding dietary studies. Furthermore, due to difficulties in quantitating equol in biological fluids [2], there have been no studies correlating various serum equol concentrations with estrogenic or other biological effects.

Development of a technique for producing high quantities of a racemic mixture of equol [16] has provided a unique opportunity to more fully characterize the effects of equol in vivo on a variety of estrogen-responsive endpoints.

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[2]Correspondence: William Helferich, Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, Urbana, IL 61802. FAX: 217 244 2455; e-mail: helferic@uiuc.edu. Paul S. Cooke, Department of Veterinary Biosciences, 2001 South Lincoln Avenue, University of Illinois, Urbana, Illinois 61802. FAX: 217 244 1652; e-mail: p-cooke@uiuc.edu

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In this study, we examined effects of injected equol and dietary equol exposure that produce levels of equol exposure similar to those reported in animals consuming standard soy-based diets. Our results show that this racemic equol can induce estrogenic effects on various parameters by acting through ERα when present at concentrations routinely obtained in rodents fed soy-based diets, although its potency is substantially less than genistein.

MATERIALS AND METHODS

Animals

Juvenile age-matched female C57BL/6 mice (21-day-old weanlings; 12–14 g) were purchased from Harlan (Indianapolis, IN). Individually caged mice were ovariectomized on Day 26 to minimize endogenous estrogen production and placed on a phytoestrogen-free diet (AIN-93G purified rodent diet; Dyets Inc., Bethlehem, PA); treatment was started on Day 30. Mice were housed under controlled lighting (12L:12D) and temperature (21°–22°C) and maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Illinois.

Treatments

The effects of a racemic mixture of equol [16] on a number of estrogen-sensitive endpoints were evaluated in this study. Mice were given daily subcutaneous injections of vehicle (control) or equol at 4, 8, 12, and 20 mg (kg body weight [BW])$^{-1}$ day^{-1} in a DMSO–corn oil suspension (50 μl DMSO in 1 ml corn oil). Equol dissolved in DMSO was used as a suspension in oil for the injections to create a more sustained release and to avoid rapid increases in serum followed by a log decline as observed in previous experiments with injected phytoestrogens [24]. As a positive control for the effects of estrogen on uterine weight, one group of animals was given daily injections of 1.5 μg E_2/kg BW. To determine the effects of dietary equol, mice were fed ad libitum with AIN-93G purified rodent diet (control) or this diet supplemented with equol at 500 or 1000 ppm for 12 days (n = 5 per group). Individual feed intake was measured during the treatment period to control for possible changes in consumption levels of equol-supplemented feed compared with control.

Tissue Collection

At the end of treatment, mice were weighed and killed by CO_2 overdose. Blood was collected by cardiac puncture, and the serum was separated following coagulation at 4°C and frozen at −20°C for subsequent equol assay. Mice were killed 2 h after the last injection [25]. Mice given dietary equol were killed at lights on (0800 h) on Day 12 to get a representative serum measurement of equol [26]. The right parametrial and inguinal fat pads were removed and weighed, and uterus, vagina, and thymus were removed, weighed, and fixed in neutral buffered formalin (NBF) for histological processing.

Histology and Immunohistochemistry

Tissues were embedded in paraffin and sectioned at 4 μm. For routine histological examination, sections were stained with hematoxylin and eosin. To quantify the effect of equol on uterine epithelial proliferation, uterine cross-sections were immunostained with anti-mouse Ki-67 monoclonal antibody (BD Pharmingen, San Diego, CA). Paraffin sections were hydrated and subjected to antigen retrieval by immersing in 0.01 M citrate buffer and microwaving for 20 min. Endogenous peroxidase was inactivated using 0.3% H_2O_2 in methanol. Nonspecific binding of antibodies was blocked using 5% normal goat serum, and then samples were incubated with anti-mouse Ki-67 antibody (1:1200) in a moist chamber overnight. After incubation with a biotinylated secondary antibody, the signal was amplified by using the streptavidin-biotin system with the Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA). The samples were then incubated with substrate Tris-HCl/H_2O_2/DAB for 8 min. The sections were mounted after slight counterstaining with hematoxylin. Vaginal epithelial thickness was measured after digital imaging using the software package Image J, 1.30v (National Institutes of Health, Bethesda, MD).

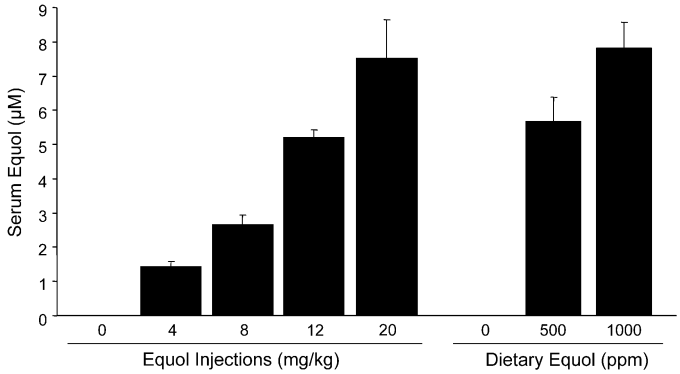

FIG. 1. Serum equol concentrations in mice following exposure to injected or dietary equol. Ovariectomized 30-day-old mice were injected daily with equol at 0 (control), 4, 8, 12, or 20 mg (kg BW)$^{-1}$ day^{-1} or fed equol at 0 (control), 500, or 1000 ppm for 12 days. Serum was collected 2 h after the last equol injection or at lights on in groups given dietary equol. Data are shown as mean ± SEM, and n = 5 for all groups. All values within the injected group are significantly different from the control and each other, and all values in the dietary equol group are significantly different.

Serum Equol Measurements

Total equol and the concentration of the aglycone form of equol were measured in the serum using a validated isotope dilution liquid chromatography electrospray mass spectrometry (LC-ES/MS) method [27].

Statistical Analyses

Data were analyzed by ANOVA and Dunnet post hoc two-sided comparisons using Systat version 10 (SPSS Inc., Chicago, IL; 2000). Data are represented as mean ± SEM. Differences were considered significant at $P < 0.05$.

RESULTS

Serum Concentrations of Equol

There was a dose-dependent increase in total serum equol levels following equol injection or dietary equol exposure (Fig. 1). Serum equol was measured 2 h after the last injection, and the concentrations of equol were 1.4 ± 0.1, 2.6 ± 0.3, 5.1 ± 0.2, and 7.5 ± 1.1 μM for the 4, 8, 12, and 20 mg (kg BW)$^{-1}$ day^{-1} groups, respectively (n = 5/group; Fig. 1). In the feeding study with dietary equol exposure, serum concentrations of equol measured at lights on were 5.9 ± 0.7 and 8.1 ± 0.8 μM for the 500 and 1000 ppm groups, respectively (n = 5/group; Fig. 1). Neither injection nor feeding controls had measurable serum equol (n = 5/group).

Equol aglycone concentrations showed dose-dependent increases in the injected mice (Fig. 2). The concentration of the aglycone form of equol in the serum was approximately 20% that of total equol in the injected mice. This was 10-fold higher than the relative percentage of equol present as the aglycone following dietary equol exposure at 1000 ppm, which was only 2% of the total serum equol (Fig. 2).

Equol added in the diet did not affect feed consumption in the mice over the study period. Total average food consumption was 33.8 ± 3.5 g (control), 33.3 ± 2.4 g (500 ppm equol), and 32.4 ± 1.7 g (1000 ppm equol) over the 12-day experimental period. Similarly, body weights were not significantly different in any of the injected or dietary equol groups compared with controls (data not shown).

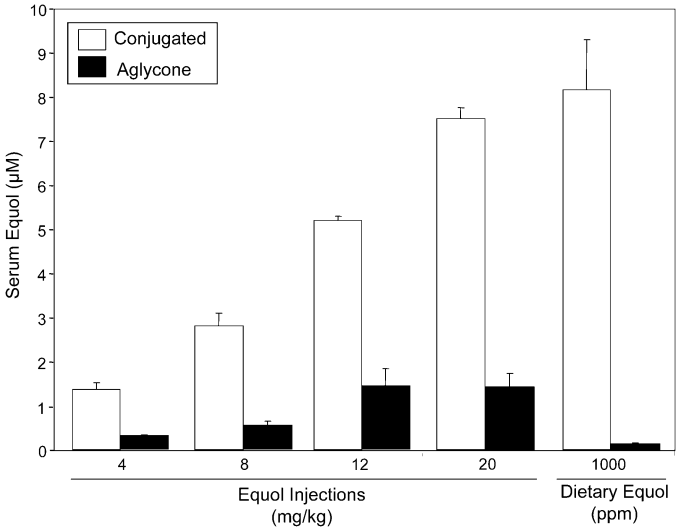

FIG. 2. Concentrations of conjugated and aglycone equol in ovariectomized mice. Data were obtained from the same mice shown in Figure 1 and are presented as mean ± SEM; n = 5 for all groups. Relative percent of equol present as the free aglycone was approximately 10-fold greater in the injected animals compared with those given dietary equol (free aglycone as a percentage of total equol was 20%, 17%, 25%, and 17% in animals given the 4, 8, 12, or 20 mg/kg equol injections, respectively, compared with 2% in the dietary equol group).

FIG. 3. Effect of equol on uterine wet weight. Uterine weights were determined in ovariectomized 30-day-old mice treated with equol injections at 0–20 mg (kg BW)$^{-1}$ day^{-1} or fed equol at 0–1000 ppm for 12 days. Estradiol treatment (1.5 μg E2/kg BW) was used as a positive control. Data are shown as mean ± SEM, and n = 5 for all groups. *$P <$ 0.01 vs. controls.

Uterotropic and Vaginal Response

Equol was uterotropic at high doses. Uterine weights at 12 and 20 mg (kg BW)$^{-1}$ day^{-1} were 2- and 3.5-fold that of the controls, respectively (Fig. 3). The uterine weights even with the highest dose of injected equol were less than that seen in the E_2-treated group, which functioned as a positive control. Although equol injected at 8 mg (kg BW)$^{-1}$ day^{-1} produced a trend toward increased uterine weights, this did not reach statistical significance. Uterine weight was not significantly increased at either the 500 or 1000 ppm equol feeding doses, although again there was a trend toward an increase (P = 0.132 and 0.063 compared with control, respectively).

There was a dose-dependent increase in uterine epithelial proliferation with equol injections (Fig. 4), which was significantly different from control at 8 mg (kg BW)$^{-1}$ day^{-1} and higher. Proliferation was lower following dietary equol treatments compared with the groups given the higher injected doses of equol (Fig. 4A), although dietary equol produced a significant increase in labeling index compared with the control at 1000 ppm (Fig. 4B). This observation was consistent with the increases in uterine weights seen following equol treatment, where even the highest dietary equol concentration (1000 ppm) produced less pronounced responses than the higher dose injections.

Equol also produced dose-dependent increases in vaginal epithelial height consistent with an estrogenic response (Fig. 5A). Vaginal epithelial height increased from 7.3 ± 0.5 μm in the control to 8.5 ± 0.6, 20.3 ± 1.1, 27.7 ± 2.4, and 39.3 ± 4.1 μm with equol injections of 4, 8, 12, and 20 mg (kg BW)$^{-1}$ day^{-1}, respectively, and to 21.5 ± 2.8 and 30.1 ± 2.4 μm with dietary equol at 500 and 1000 ppm, respectively (Fig. 5B). This increase was significant for the 8, 12, and 20 mg (kg BW)$^{-1}$ day^{-1} equol injections and at both dietary equol concentrations.

Adipose Tissue and Thymus

Both adipose tissue and thymus express ERα and ERβ, and administration of phytoestrogens such as genistein produces thymic atrophy, immune suppression, and a decrease in adipose tissue [24, 28, 29]. However, neither the parametrial nor inguinal fat pads were significantly decreased in weight at any of the injected or dietary doses of equol (data not shown). Similarly, a decrease in thymic weight was not observed with any equol treatment (data not shown).

DISCUSSION

Despite its potential biological importance for both humans and rodents, limited studies have been performed with equol because of the high cost of commercial equol and difficulties in establishing accurate equol assays. Our recently developed method for producing equol [16] allows cost-effective preparation of large amounts of a racemic mixture of equol. This makes it feasible to more completely determine the effects of equol in vivo, and also makes it possible to examine the effects of dietary administration of equol, an approach that is the most physiological way to study this compound but has previously been cost-prohibitive. In the present study, we have examined the effects of injected and dietary equol on various reproductive and nonreproductive parameters. By concomitantly measuring serum equol in all groups, we have also been able to determine the relationship between the equol dose and route of administration and the resultant serum equol concentrations. This allows us to not only correlate the serum equol concentrations in our experimental animals with serum equol concentrations in rodents and humans exposed to phytoestrogens, but also makes it possible to directly determine effects of various serum concentrations on the reproductive, adipose, and immune endpoints used in this study.

Equol is optically active and exists as R and S enantiomers. Equol produced during intestinal digestion in humans and other animals is the S-form, which has greater ER binding affinity and increased ERβ binding compared with the R-form [16]. However, overall ERα and ERβ binding of a racemic mixture produced by the new synthesis procedure is comparable with that of the naturally occurring S enantiomer [16], and this racemic mixture was used for all of our studies.

Increases in uterine weight and epithelial proliferation are classical estrogen effects mediated by ERα, and this effect is used to measure estrogenicity of environmental estrogens [30–32]. Equol was initially reported to be bio-

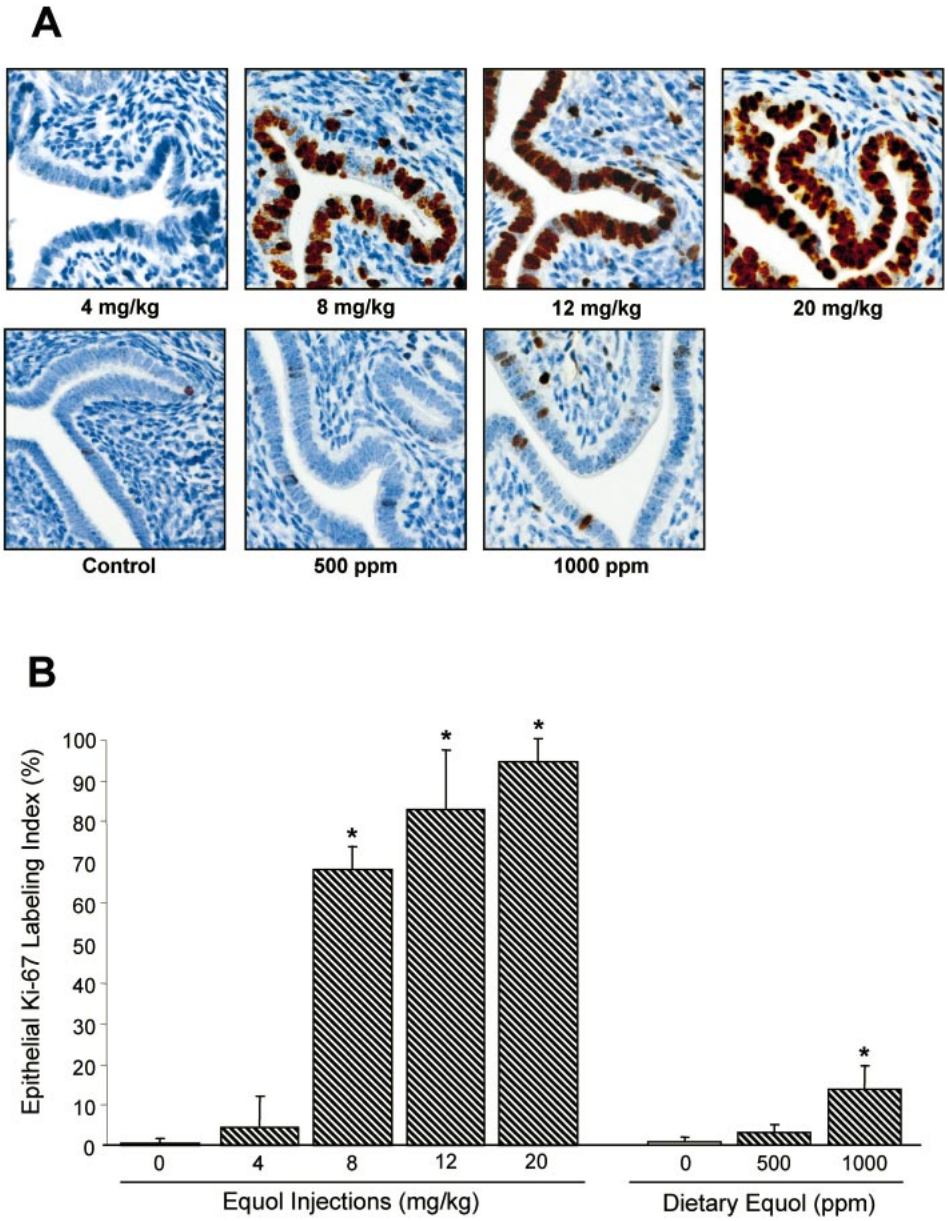

FIG. 4. Effect of equol on uterine epithelial proliferation. **A**) Ki-67 immunostaining in uteri of mice given dietary or injected equol; a dose-responsive increase in epithelial proliferation is seen with either dietary or injected equol, with the injected equol producing higher levels of epithelial proliferation. Injection control was similar to the dietary control and is not shown. Original magnification ×400. **B**) Ki-67 labeling index in the uterine epithelium (Ki-67 positive uterine epithelial cells/total number of uterine epithelial cells) with various doses of equol treatment. Data are shown as mean ± SEM, and n = 5 for all groups; $*P < 0.01$ vs. controls.

logically inactive [33], but in vitro experiments have shown that equol binds both ERα and ERβ [6, 16]. In addition, previous work has indicated that injected equol is uterotropic in mice and sheep [22, 34], a finding confirmed by our present results.

Equol injections produced greater increases in uterine weight and epithelial proliferation than dietary equol, although even injections with the highest equol dose did not increase uterine weight to the degree seen in the positive controls, which received E_2. Dietary equol at 500–1000 ppm produced a weak estrogenic response with moderate increases in uterine weight that did not reach significance and a low Ki-67 epithelial labeling index that was significantly greater than control only at 1000 ppm. Mice fed commercial soy-based diets have high isoflavone intake and total plasma isoflavone levels ranging from 4.4 to 8.5 μM have been reported [20]. Rodents efficiently convert daidzein to equol, and up to 90% of circulating isoflavone in rodents fed commercial chow is equol, emphasizing the importance of equol in isoflavone action and its potential ability to alter processes regulated by estrogens [19–21]. Our study demonstrates the first estrogenic effects of dietary equol, and our results show that equol is weakly uterotropic at serum concentrations comparable with those in rodents fed soy-based diets.

Induction of vaginal epithelial proliferation and cornification is a classical estrogenic effect involving both stromal and epithelial ERα [35]. Without estrogen exposure, vaginal epithelium is atrophic, consisting of two to three layers of flattened squamous epithelial cells. Estrogenic stimulation causes epithelial proliferation, stratification, and apical cornification. We observed dose-dependent increases in vaginal epithelial thickness with both dietary and injected equol. Vaginal epithelial thickness was the most sensitive estrogenic endpoint examined. Dietary doses of equol that produced slight uterotropic effects caused clear increases in vaginal epithelial height and stratification, although even at the highest equol doses vaginal stratification was less than seen with E_2 treatment.

Adipose tissue expresses ER and is highly estrogen-responsive. Increases in adipose tissue occur in ovariectomized animals and postmenopausal women, and these in-

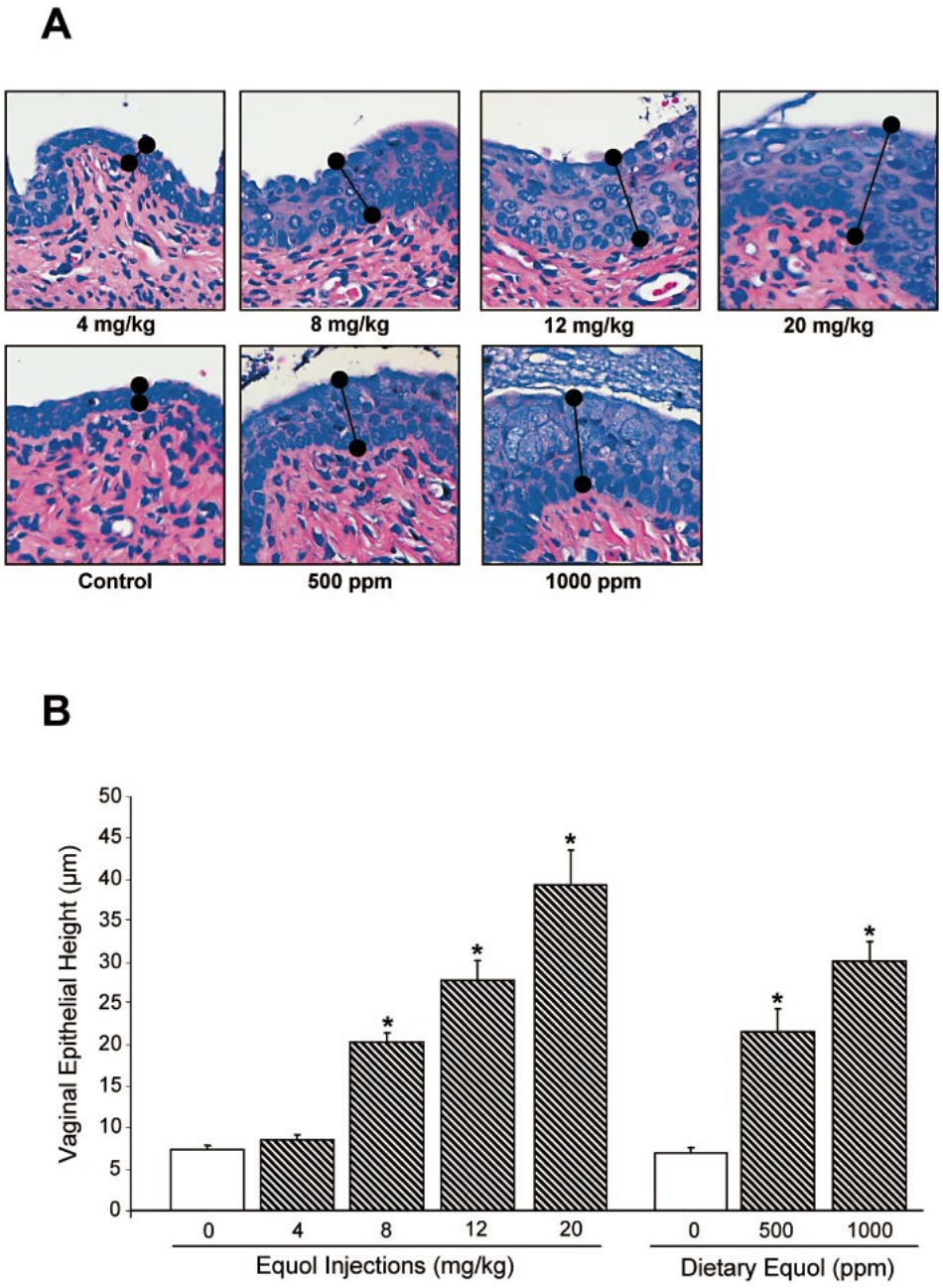

FIG. 5. Effect of equol on vaginal epithelium. **A**) Vaginal histology showing epithelial stratification and cornification with increasing doses of dietary or injected equol. Injection control was similar to the dietary control and is not shown. Magnification of all panels was ×400. **B**) Quantitation of vaginal epithelial height in mice given increasing doses of equol. Data are shown as mean ± SEM, and n = 5 for all groups; $*P < 0.01$ vs. controls.

creases are reversed with estrogen [36, 37]. Inhibitory effects of estrogens on adipose tissue are mediated by ERα, as shown by comparable increases in adiposity in ERα knockout mice [35] and aromatase-deficient mice [38]. Similarly, the thymus expresses ER, and estrogen treatment of young animals induces thymic atrophy. Genistein administered by injection or in the diet produces inhibitory thymic and adipose effects [24, 29]. The lack of effects on thymic or adipose weights following equol administration by injection or in the diet reflects the lower estrogenic potency of equol relative to genistein. Despite clear vaginal and uterine responses seen even with dietary equol, neither the injected nor dietary equol doses appear to reach a sufficient threshold for an estrogenic response in adipose tissue or thymus.

These findings with equol parallel our previous results with genistein. Although genistein had greater effects on all reproductive and non-reproductive endpoints than equol at similar doses [24, 29], higher genistein exposures were necessary to produce effects on adipose and thymus compared with uterus. For example, genistein produces uterotropic, adipose, and thymic effects at dietary concentrations of 300, 500, and 1000 ppm, respectively [24, 29]. These dietary genistein levels result in serum genistein concentrations of 1.0–2.5 μM. However, serum equol concentrations in this study of up to 8.1 μM in mice fed 1000 ppm equol had no effect on adipose tissue or thymus, had minimal effects on uterine epithelial proliferation, and did not produce the uterine weight increases seen with dietary genistein at 300 ppm or above and serum concentrations of 1 μM or greater.

Human epidemiological studies have correlated increased soy and phytoestrogen intake with decreased risk of certain diseases [39–41]. However, clinical effects of dietary phytoestrogens on parameters such as bone loss or hot flashes have been variable [42]. Equol producers, the

subset of humans who convert daidzein to equol when exposed to dietary soy, have highly elevated serum and urinary levels of equol [43]. Steady-state total serum equol concentrations in these individuals are as high as 1 μM in Japanese men [44], and urinary equol levels are 1000-fold higher than endogenous estrogens [41, 43]. Setchell et al. [2] recently suggested that health benefits of isoflavone supplements in postmenopausal women were correlated with equol production, with equol producers having the highest response to isoflavones. In contrast, rodents fed 500–1000 ppm of equol had limited estrogenic responses despite total circulating equol concentrations (up to 8.1 μM) that were several-fold greater than the approximately 1 μM concentrations in humans who are equol producers and consume high-soy diets [2, 17, 18]. Based on this, equol concentrations normally seen in humans would not be expected to have significant estrogenicity on the ERα-mediated parameters examined here, which is not consistent with a primary role for equol in the effectiveness of soy [2].

Based on feed intake, mice in the 500 and 1000 ppm dietary equol groups were exposed to approximately 60 or 120 (mg equol) (kg BW)$^{-1}$ day^{-1}, respectively, which was more on a weight basis than any of the injected equol doses. The comparable serum levels in the dietary vs. injected groups despite the higher overall exposure in the former likely reflects the relatively small portion of equol that is absorbed in the gut, as reported for genistein [41]. Although serum equol concentrations 2 h after injection provide some information about the magnitude of serum equol concentrations resulting from injection, these values also may not represent peak concentrations [25]. The relatively constant exposure to dietary equol throughout the day compared with one bolus injection would also contribute to the comparable serum equol levels obtained with each mode of administration, despite greater total exposure with dietary equol.

The relative proportion of free aglycone was much less with dietary equol compared with injection. Higher aglycone proportions following genistein injection compared with animals given dietary genistein has been reported [25] and may be the result of a significant portion of the aglycone being conjugated during absorption through the enteric epithelium. Because the free aglycone is considered the biologically active moiety, the increased proportion of circulating aglycone following equol injection could contribute to increased activity of injected vs. dietary equol on parameters such as uterine weight and Ki-67 labeling, and indeed our data indicate that the aglycone concentrations present with a given dietary or injected equol dose correlate well with the biological response. This may be critical in explaining the greater estrogenic effects of the injections despite our data indicating that the serum equol levels produced by the injections are comparable with those obtained with dietary administration. These results emphasize the important differences between injecting equol or other phytoestrogens compared with delivering them dietarily and indicate that results obtained from phytoestrogen injection studies must be extrapolated with extreme caution to understand effects resulting from normal dietary exposure.

Equol acts as a weak ERα agonist in vivo in the present study, but a complete understanding of its biological effects in humans or animals must take into account the gamut of its actions and the complexity of the in vivo environment where other phytoestrogens, as well as various other environmental estrogens, may be present. For example, equol could have additive effects in vivo with genistein and other phytoestrogens, and therefore would contribute to the overall estrogenicity of circulating phytoestrogens. Likewise, there could also be equol effects through its non–ERα-mediated antioxidant [45, 46] and antiproliferative properties [47, 48]; these types of effects might be more apparent with long-term exposure, rather than the relatively short-term exposure utilized in this study. Many phytoestrogens, including equol, bind with a higher affinity to ERβ than to ERα. ERβ-mediated responses were not examined in this study, and the possibility that equol could have important biological effects through these pathways remains untested. Further, the potential role of equol in nongenotropic signaling mediated by a putative membrane ER, which may be responsible for reversal of bone loss [49], remains to be investigated.

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