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Characterization of the Estrogenic Properties of a Nonsteroidal Estrogen, Equol, Extracted from Urine of Pregnant Macaques[1]

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ABSTRACT

Recent reports of substantial urinary levels of equol in pregnant macaques and humans pose a concern, because equol poisoning in the ovine is characterized by an often permanent failure of reproductive processes. Equol (Fig. 1), a metabolite of phytoestrogens, is thought to act through estrogen receptors. The present study made a direct comparison of the estrogenic activity of equol from macaque urine, (±) equol and 17β-estradiol (E_2) in vitro and in vivo. Relative binding affinity of equol for rat uterine receptor was 1% that of E_2, and the dissociation rate of equol from the receptor was very high. Consistent with equol's binding properties in vitro, it was ineffective in stimulating rat uterine weight gain and possessed limited ability to increase progesterone receptor. Uterine nuclear receptors after doses of equol sufficient to produce depletion and replenishment of cytosol estrogen receptor were not measurable by exchange assay. No antiestrogenic activity of equol could be demonstrated. Equol's weak potency and lack of antiestrogenic activity are difficult to reconcile with its ability to induce ovine infertility. We conclude species differences at some level other than classical estrogen receptor as defined in the rat model are responsible for variability in equol's impact.

INTRODUCTION

Recently, substantial quantities of equol (Fig. 1) were reported in macaque urine (μg/mg creatinine) at late pregnancy (Monfort et al., 1984). This poses a concern because equol, an isoflavin metabolite, is the molecule responsible for clover disease in sheep (female infertility, fetal death, uterine inertia, prolapse of the uterus, and cystic hyperplasia of the uterus and of the endometrium: Bennetts et al., 1946; Underwood, 1951; reviewed in Moule et al., 1963). Yet pregnancy in the macaque did not appear affected by presence of equol. There have been conflicting reports of equol's estrogenic activity in other species as well. Equol [7 hydroxy-3-(4′ hydroxyphenyl) chroman] was first isolated from the ketohydroxyoestrin fraction of pregnant mare's urine by Marrian and Haslewood (1932). Then it was considered an inactive compound, because it had no estrus-producing action in mice. In 1982, Axelson et al. reported substantial quantities of equol in human urine and suggested there may be some value in quantitatively screening for plant estrogens in women with unexplained infertility.

Systemic studies of the effects of exogenous compounds across species are complicated by many metabolic variables. To avoid these complications the present study was restricted to assessing the potency and intrinsic activity of equol at a local tissue level in vivo and in vitro. Two questions were asked: *1*) was the biological activity of equol extracted from macaque urine comparable to that of synthetically produced (±) equol? *2*) Could some aspect of (±) or macaque equol's receptor binding in the rat model provide information characterizing its variable estrogenic impact. Results indicate equol from macaque urine was identical to that produced in the ovine rumen, that it is a weak estrogen with no antiestrogenic activity, and

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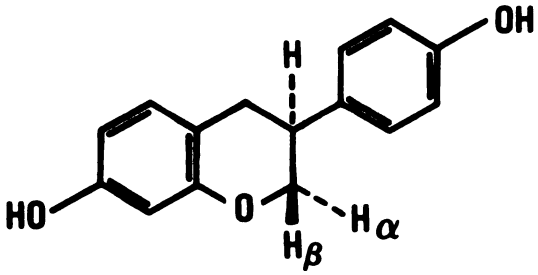
that species sensitivity to equol is regulated by mechanisms in addition to classical target tissue estrogen receptor. Because equol is found in a wide variety of food plants (Farnsworth et al., 1975) and can be introduced through normal diet, it may prove a useful probe with which to study species differences in control of reproductive processes.

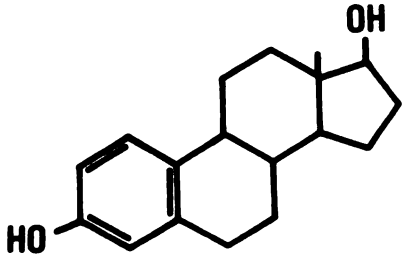
MATERIALS AND METHODS

Chemicals

(-) Equol (macaque equol) was isolated from urine of pregnant *Macaca silenus*. Urine samples were obtained as previously described by Monfort et al. (1984) at late gestation. Macaque equol was extracted and measured by methods described in Erb et al. (1982). Briefly, 0.5-ml aliquots of urine were adjusted to pH 5 and enzymatically hydrolyzed with excess 0.02 ml β-glucuronidase-arysulfatase (Boehringer-Mannheim, West Germany) for 24 h at 37°C. Samples were extracted twice with a ratio of 1:8 (v/v), urine to anhydrous diethyl ether (Mallinckrodt Inc., St. Louis, MO). The ether extract was dried under a stream of nitrogen and was reconstituted in 55 μl chromatographic grade methanol (Fisher Scientific, Wilton, NH) in preparation for high-performance liquid chromatography (HPLC). Equol was detected in HPLC elution fractions by radioimmunoassay. Nonlabeled steroids, with the exception of 17α-methyl-promegestrone (R5020), were purchased from Sigma (St. Louis, MO). Radioactive [2,4,6,7-^{3}H(N)] 17β-estradiol ([^{3}H] E_2, 104–115 Ci/mmol) and [17α-methyl-^{3}H] promegestrone (87 Ci/mmol), [^{3}H] R5020, were purchased from New England Nuclear Corp. (Boston, MA), as was nonlabeled R5020. Synthetic (±) equol was kindly supplied by Dr. J. A. Lamberton, CSIRO, Australia. It was produced by catalytic hydrogenation of daidzein (Lamberton et al., 1978), the immediate precursor of (-) equol in the ovine rumen (Batterham et al., 1965; Shutt and Braden, 1968).



EQUOL



ESTRADIOL - 17β

FIG. 1. Comparison of the structures of equol and 17β-estradiol.

MCF-7 Cell Culture and Harvest

MCF-7 cells were supplied by the Breast Cancer Animal and Human Tumor and Human Cell Culture Bank, National Cancer Institute, NIH, Bethesda, MD. Cell culture and experimental conditions were as described in Horwitz and McGuire (1978) with minor modification. Briefly, culture medium consisted of Eagle's medium essential medium prepared in Earle's salts supplemented with nonessential amino acids and 2 mM L-glutamine (MEM, Gibco, Grand Island, NY) plus 100 U Regular Iletin I (insulin, Eli Lilly and Co., Indianapolis, IN), 10% fetal bovine serum (Sterile Systems Inc., Logan, UT) and 25 μg/ml gentamicin (Schering, Kenilworth, NJ). Experimental medium was the same as culture medium with the exceptions that it contained 10 nM hydrocortisone in addition to other supplements, and 5% fetal bovine serum stripped of endogenous hormones with dextran-coated charcoal (DCC) was substituted for regular fetal bovine serum. Cells were grown in Corning T150 plastic flasks in 5% CO_2 in air at 37°C. Estrogens prepared in ethanol were added to experimental media (0.5 ml ethanol per 500 ml of medium) to obtain final concentrations described in text. Flasks were treated 4 days with experimental media and fresh media was provided every 48 h. Confluent or near confluent cells were removed from the flasks by 30 min incubation at 37°C with 1 mM EDTA in Ca^{++}/Mg^{++} free Hank's balanced salt solutions buffered with bicarbonate.

Progesterone Receptor Assay MCF-7 Cells

MCF-7 cells were washed once with 10 ml phosphate buffer (5 mM sodium phosphate, pH 7.4, 10 mM thioglycerol and 10% glycerol) after removal from culture flasks. All procedures were carried out at 0–4°C. Equivalent of one confluent 150 cm^2 flask was homogenized in 1.0 ml buffer in a Kontes glass homogenization tube with a Teflon pestle. Homogenates were centrifuged at 20,000 × *g* for 60 min. Two hundred μl of cytosol was incubated 2 h at 4°C in duplicate with a single saturating dose, 5 nM [^{3}H] R5020 (25 μl), in the absence and presence of 100-fold molar excess R5020 (25 μl). Incubation was terminated with 0.5 ml DCC, 15 min (Korenman, 1969) to remove free steroid. The charcoal was sedimented by centrifugation, the supernatant decanted directly into scintillation vials and counted. Protein in samples was measured by the Coomassie binding technique (Bradford, 1976).

Animals and Surgical Preparation

Adult female Sprague-Dawley rats (Simonsen Labs., Gilroy, CA) were housed under ambient light conditions at 22°C. Rat chow and water were available ad libitum. Female rats weighed 220–250 g, estrous cycles were monitored and ovariectomy performed at diestrus under ether anesthesia. All in vivo estrogen and vehicle treatments were locally applied by injection into the lumen of the uterine horn while the rat was under ether anesthesia. Experimentation was performed 5–7 days after ovariectomy. A total volume of 50 μl was applied per uterine horn. All rats were sacrificed by decapitation. Uteri were removed rapidly, stripped of adhering fat and lumenal fluid, if any, was expressed. Uteri were then weighed or homogenized as described below.

Progesterone Receptor Assay Rat Uterus

Each rat uterus was homogenized in 1 ml cold phosphate buffer (5 mM sodium phosphate, 10 mM thioglycerol, 10% glycerol, pH 7.4) in a Duall ground-glass homogenizer with motor-driven pestle. The homogenization tube and pestle were rinsed three times with 1 ml buffer and rinses were added to the homogenate. Homogenates were centrifuged at 20,000 × *g* for 1 h. Aliquots of cytosol (400 μl) were incubated 2 h at 4°C in triplicate with a saturating dose, 20 nM, [^{3}H] R5020 (50 μl) plus 100-fold molar excess corticosterone in the presence and absence of 100-fold molar excess R5020 (50 μl). Incubations were terminated with 1.0 ml DCC to remove free steroid. Charcoal was sedimented by centrifugation and the supernatant counted. All procedures were performed at 0–4°C.

Cytosol Estrogen Receptor Assay

Cytosol for in vitro measurement of estrogen receptor after treatment in vivo was prepared by homogenization of rat uteri (1 uterus/ml) in ice-cold TED buffer (10 mM Tris-HCl, 1.5 mM EDTA, 1 mM dithiothreitol, pH 7.4) in a Duall ground-glass homogenizer with a motor-driven pestle. The homogenization tube and pestle were rinsed twice with 1 ml buffer and rinses were added to the homogenate. For saturation analysis experiments, homogenate was centrifuged at 20,000 × *g* for 1 h. Cytosol was diluted to a total volume of 15 ml and 400-μl aliquots were incubated with progressively increasing amounts of [^{3}H] E_2 (50 μl) in the presence or absence of 100-fold molar excess diethylstilbesterol (DES, 50 μl) for 18 h at 4°C. For single saturating dose assays cytosol was not further diluted and 200-μl aliquots were incubated with 10 nM [^{3}H] E_2 in the presence and absence of 100-fold molar excess DES for 2 h at 0–4°C. Cytosol for exchange assay was prepared and the assay performed as described by Katzenellenbogen (1973). Cytosol for saturation analyses in the presence and absence of equol was prepared in the same manner as for the cytosol exchange assay.

[^{3}H] E_2 Nuclear Exchange Assay

Saturation analyses of nuclear estrogen receptors were performed on crude chromatin by the method of Kelner et al. (1980, 1981). Uteri were homogenized in ice-cold Tris-EDTA buffer (10 mM Tris, 1.5 mM EDTA, pH 7.4) with a Duall ground-glass homogenizer with motor-driven pestle. Crude chromatin was obtained by layering 1 ml dilute homogenate (0.05 adult uterus/ml) onto 1.0-ml pads of 1.2 M sucrose in 12 × 75-mm plastic culture tubes and centrifuging at 1500 × *g* for 3 h. The supernatant and sucrose pad were then aspirated, and the pellet was incubated with various concentrations of [^{3}H] E_2 in the absence and presence of 100-fold molar excess DES in a total volume of 250 μl. Incubation of the reaction mixtures was at 37°C for 1 h, and was terminated by chilling on ice for 5 min. Free steroid was separated from receptor-steroid complexes by precipitation of the complexes with an equal volume of protamine sulfate solution (1 mg/ml) for 10 min. Two ml cold wash buffer (Tris-EDTA buffer + 1% Tween-80; pH 7.4) was added to each tube, and samples were centrifuged at 1500 × *g* for 15 min. The pellet was washed a second time with 2 ml wash buffer, and the second pellet was extracted with 100% ethanol. Ethanol extracts were counted in Ready-Solv (Beckman Instr., Fullerton, CA).

Data Analysis

In experiments testing the effect of estrogen treatment on levels of progesterone receptor and rat uterine weight the significance of differences among means was determined by analysis of variance, with post hoc comparisons by the least significance of differences method. Scatchard regression lines were tested for similarity of intercept and/or of slope according to the method of Kleinbaum and Kupper (1978). Differences in maximal number of nuclear binding sites (B_{max}) by Scatchard analysis and dissociation constants (K_d) after vehicle or estrogen treatment in vivo were also determined by *t* test. All *t* tests were two-tailed.

RESULTS

Figure 2 presents a double-reciprocal plot of

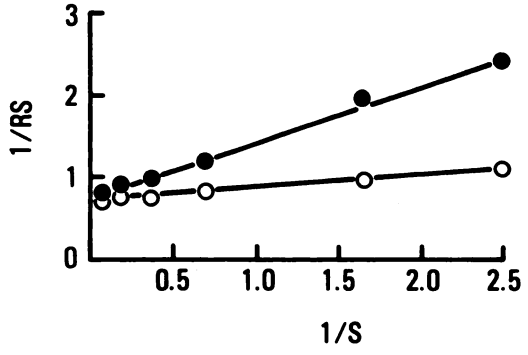


FIG. 2. Double reciprocal plot of saturation analysis of [^{3}H] E_2 binding to rat uterine cytosol receptor in the presence (●) and absence (○) of macaque equol. Concentration of [^{3}H] E_2 (*S*) ranged from 0.4 to 12 nM. Receptor-mediated binding of [^{3}H] E_2 (*RS*), i.e., DES blockable binding, was calculated in pmol/uterus.

saturation analysis of [^{3}H] E_2 binding to rat uterine cytosol in the presence and absence of macaque equol (1 μM). Data confirm that the interaction between macaque equol and [^{3}H] E_2 for the rat uterine estrogen receptor binding site was competitive. Similar analyses of [^{3}H] E_2 binding in the presence of (±) equol showed 100 μM (±) equol displaced greater than 99% of the specific [^{3}H] E_2 binding whereas 10 nM (±) equol had no effect (data not shown). An estimate of the relative molar binding affinity of (±) equol for the rat receptor from these data would be 1% that of E_2 which is consistent with the 0.2–1% reported by Shutt and Cox (1972) for ovine uterine receptor.

The effect of macaque equol on long-term (48 h) rat uterine weight gain was compared to the effect of E_2 and several other steroidal estrogens (Fig. 3). All estrogens chosen for this study were also observed at late pregnancy in macaque urine (Monfort et al., 1984). Treatments were applied locally within the lumen of the uterine horn of ovariectomized mature rats. Vaginal lavage indicated estrogen did not leave the uterus in significant quantities as the vaginal epithelium, a tissue highly sensitive to estrogen (Martin and Claringbold, 1960) was not stimulated. E_2 was the most effective treatment with a maximal weight elevation, 219.4 ± 8.2 mg (mean ± SEM: n=4), elicited by the lowest (5 ng) dose. Higher doses of estrone (E_1, 24 ng) and estriol (E_3, 300 ng) were required to elevate uterine weight above that of vehicle injected (144.9 ± 4.9 mg, n=5). All three doses (0.5, 1.5, 5.0 μg) of macaque equol failed to elevate uterine weight. Additionally, the highest dose of macaque equol, administered simultaneously with a similar dose of E_2, failed to modify the effect of E_2 on uterine weight (214.0 ± 14.9 mg, n=4). Histological examination of uteri under the light microscope after fixation and staining revealed tissue morphology similar to that observed during proestrus after E_2 administration and during diestrus after equol administration. Simultaneous treatment with E_2 and equol produced histology similar to that observed with E_2 alone.

Figure 4A demonstrates uterine progesterone receptor levels in ovariectomized mature rats 24 h after a single intralumenal injection of vehicle, macaque equol or E_2. Macaque equol stimulated an increase in progesterone receptor, an early response to estrogen (Feil et al., 1972), compared to vehicle, but the response to macaque equol (1 μg) was only 42% that to E_2 (5 ng). Similarly, Fig. 4B demonstrates progesterone receptor levels increased in a dose-dependent manner in MCF-7 cells treated 4 days with increasing concentration of macaque equol (0.1 to 100 nM) and E_2 (0.001 to 10 nM). Progesterone receptor levels were maximal in those cultures with 1.0 nM E_2. In cultures with

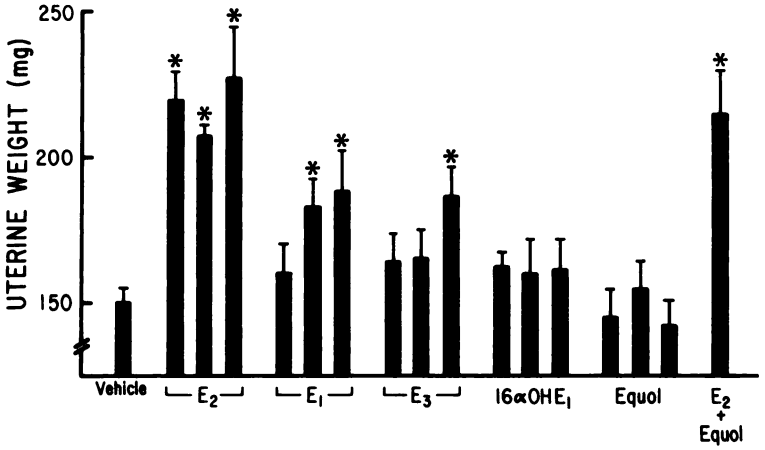


FIG. 3. Uterine weight of mature ovariectomized rats measured after estrogen treatment. Vehicle (0.1% BSA-physiological saline) or estrogen in vehicle was applied locally to uteri of ether-anesthetized rats. Three doses of estrogen were administered as described in *Results* to each uterus. Treatment doses were: E_2, 5, 15, 50 ng; E_1, 8, 24, 80 ng; E_3, 30, 120, 300 ng; 16α-OHE_1, 0.2, 0.6, 2.0 μg; and macaque equol, 0.5, 1.5, 5.0 μg. The initial treatment was followed at 24 h with a second treatment at the same dose. Forty-eight h after the initial treatment animals were sacrificed as described in *Materials and Methods*. *Vertical lines* above bars indicate standard error of the mean. *Asterisks* indicate means significantly different from those of vehicle injected ($P<0.05$, n=3–6).

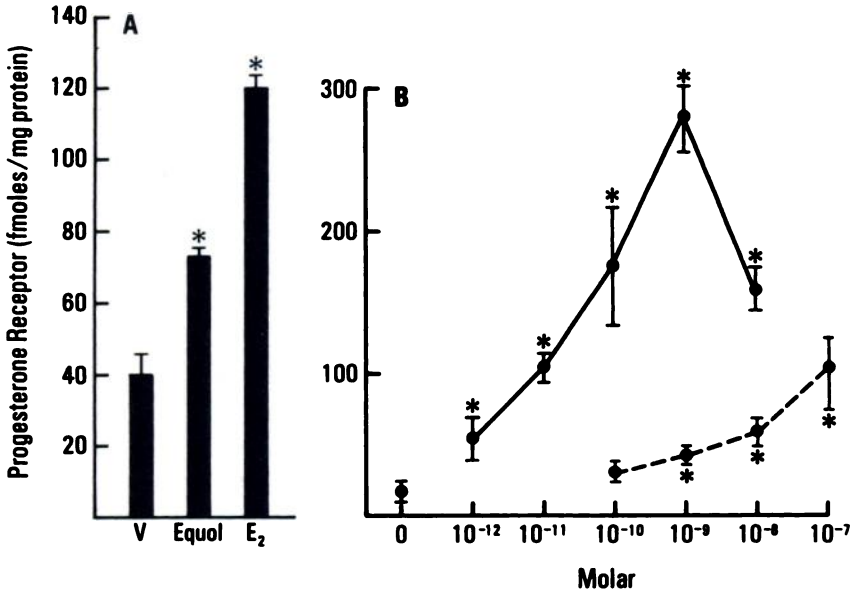


FIG. 4. Progesterone receptor levels after treatment with macaque equol or E_2. *A*) Ovariectomized rats were locally treated (intralumen) with vehicle (10% ethanol-saline) or estrogen (E_2, 5 ng; macaque equol, 1 μg) 24 h prior to sacrifice, (n=3) for all treatments. *Asterisks* indicate values significantly above vehicle injected, $P<0.001$. *B*) Effect of E_2 (●—●) or macaque equol (●– – –●) on progesterone receptor levels in MCF-7 cells. T-150 culture flasks (n=5–8 flasks per treatment group) were treated 4 days with increasing concentrations of E_2 or macaque equol. Control flasks received experimental medium without estrogen (0 M). *Asterisks* indicate progesterone receptor levels significantly above control (0 M) levels, $P<0.02$. Progesterone receptor was measured by a single saturating dose assay in triplicate as described in *Materials and Methods*. Data shown are corrected for nonspecific binding. *Vertical lines* represent the standard error of the mean.

macaque equol, progesterone receptor was elevated above control values at 1 nM. However, even at 100 nM, macaque equol was only 37% effective as 1.0 nM E_2, 0.103 ± 0.029 fmol/mg protein (n=8) compared to 0.279 ± 0.024 fmol/mg protein (n=10).

Free cytosol estrogen binding sites were measured 1 h after treatment with vehicle, E_2, macaque equol or E_2 plus macaque equol (Fig. 5A) by saturation analysis. Macaque equol (1 μg) depleted cytosol receptor 76% (by Scatchard analysis) from the 1.71 pmol/uterus for vehicle treated to 0.41 pmol/uterus, which was similar to 0.60 pmol/uterus measured after E_2 (5 ng). Simultaneous treatment with the combined doses of equol and E_2 did not result in any further depletion of the cytosol receptor. Figure 5B shows levels of free rat uterine cytosol estrogen binding sites at several times after macaque equol or E_2 measured by a single saturating dose assay. Neither estrogen completely depleted cytosol receptor at the doses employed at any time. Maximal depletion was observed at 2 h and recovery to near control level occurred by 8 h with both treatments. In similar experiments (±) equol produced data (not shown) which were not different than those obtained with macaque equol.

Figure 6 shows data obtained with nuclear exchange assay 1 h after treatment of rat uteri with macaque equol, E_2 or vehicle. Estrogen binding sites depleted from the cytosol fraction after macaque equol (1.5 μg), unlike after E_2 (10 ng), were not measurable in the nuclear fraction at 1 h (Fig. 6), 4 h or 8 h (data not shown) with exchange at 37°C or at 0°C in control experiments. Vehicle- and macaque equol-treated uteri demonstrated similar low levels of nuclear binding sites. Scatchard analyses of these data (Fig. 6B) estimated the maximal number of binding sites (B_{max}) at 1 h to have been 0.35 ± 0.08 pmol/uterus for vehicle-treated (n=3), 0.38 ± 0.09 pmol/uterus for macaque equol-treated (n=4) and 2.20 ± 0.21 pmol/uterus for E_2-treated (n=2).

Explanations for absence of the expected number of nuclear receptors in equol-treated uteri by [^{3}H]E_2 exchange might include: *1*)

nuclear receptor-macaque equol complex had a retention time shorter than 1 h, or *2*) macaque equol bound irreversibly to the receptor making exchange impossible. Qualitative estimates of the relative rate of dissociation of unlabeled ligands can be accomplished with the cytosol exchange assay described by Katzenellenbogen et al. (1973). Figure 7A presents results of an experiment which measured receptor binding by [^{3}H] E_2 exchange assay of samples of cytosol saturated prior to assay with 30 nM E_2, 3 μM macaque equol, or 3 μM (±) equol. Apparent exchange of [^{3}H] E_2 for unlabeled macaque equol and (±) equol was complete by 15 min compared to 2–4 h for unlabeled E_2. Aliquots of these same estrogen-saturated

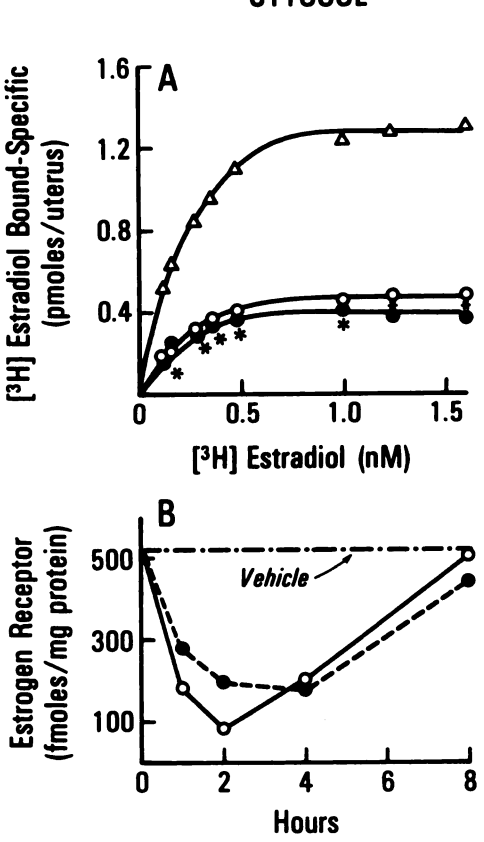


FIG. 5. Uterine cytosol receptor levels after treatment with macaque equol or E_2. *A*) Saturation analysis of [^{3}H] E_2 binding to uterine cytosol prepared 1 h after local administration of vehicle [10% ethanol-saline (Δ)] or estrogen [E_2, 5 ng (○); macaque equol, 1 μg (●); E_2, 5 ng + macaque equol, 1 μg (*)] to mature ovariectomized rats. Cytosol was incubated at 4°C for 18 h with [^{3}H] E_2 (0.1 to 1.8 nM) in the absence or presence of 100-fold molar excess DES to evaluate nonspecific binding. *B*) Cytoplasmic levels of free uterine estrogen receptor at various times following E_2 [5 ng (○)] or macaque equol [1 μg (●)] treatment of mature ovariectomized rats. Animals were sacrificed at the times indicated. Estrogen binding sites were measured with a single saturating dose assay in the absence and presence of 100-fold molar excess DES as described in *Materials and Methods*. Values are the average of two separate evaluations, each performed in triplicate. Data shown (*A* and *B*) are corrected for nonspecific binding.

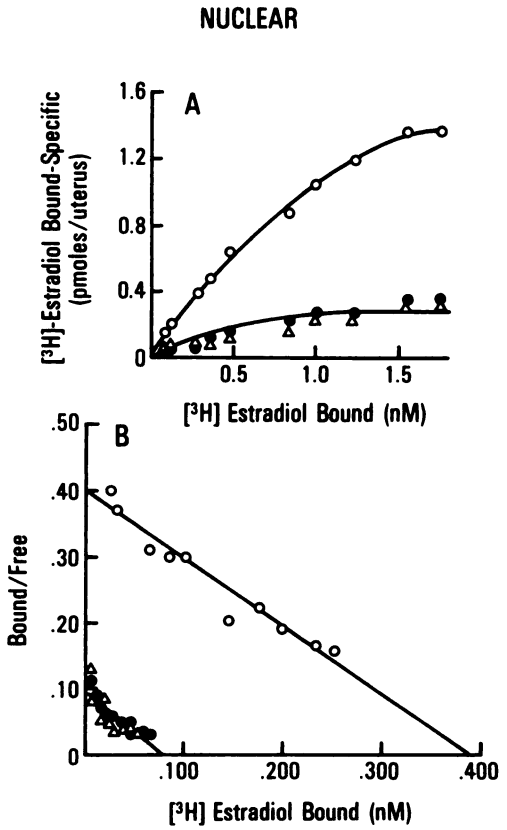


FIG. 6. Uterine nuclear receptor levels after treatment of ovariectomized rats with macaque equol or E_2. *A*) Saturation analysis of [^{3}H] E_2 binding (0.08 to 1.8 nM) was performed on crude uterine chromatin prepared as described in *Materials and Methods*. Mature ovariectomized rats were sacrificed 1 h after local administration of vehicle [10% ethanol-saline (Δ)] or estrogen [E_2, 10 ng (○); macaque equol, 1.5 μg (●)]. Total [^{3}H] E_2 binding was determined by protamine precipitation after exchange at 37°C for 90 min. A parallel set of tubes contained 100-fold molar excess of DES. Data shown are corrected for nonspecific binding. *B*) Scatchard analysis of data in *A*. For vehicle (Δ) and macaque equol- (●) treated B_{max}=0.50 pmol/uterus, K_d=0.83 nM. For E_2- (○) treated B_{max}=2.3 pmol/uterus, K_d=1.04 nM.

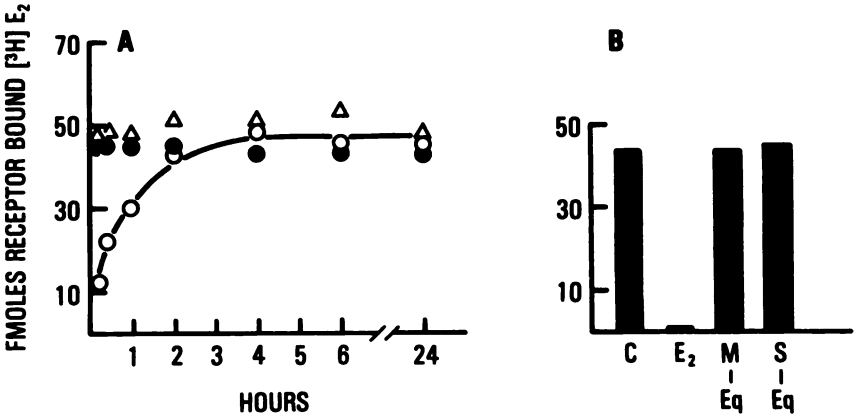


FIG. 7. [^{3}H] E_2 binding after cytosol exchange assay of receptor previously saturated with E_2, (±) equol or macaque equol. *A*) Evaluation of [^{3}H] E_2 binding with increasing time at 25°C of rat uterine cytosol previously saturated with either 30 nM E_2 (○), 3.0 M macaque equol (●), or 3.0 μM (±) equol (Δ). *B*) Measurement of unfilled estrogen binding sites at 0°C after removal of free estrogen with DCC in cytosol previously saturated with E_2, macaque equol (*M-Eq*) or (±) equol (*S-Eq*). Control samples (*C*) were not exposed to unlabeled estrogen. Measurement was made in triplicate with a single saturating dose (30 nM) [^{3}H] E_2. Data shown are corrected for nonspecific binding by subtraction of parallel values measured in the presence of 100-fold molar excess DES.

cytosol samples were incubated at 0°C after DCC treatment (Time 0 of the exchange assay) to remove free steroid (Fig. 7B). Control cytosol samples were treated the same as experimental samples except they were not exposed to unlabeled estrogen prior to incubation with [^{3}H] E_2. These data indicate dissociation of macaque equol and (±) equol from the estrogen receptor was very rapid after removal of free estrogen by DCC. At Time 0 of the exchange assay the cytosol binding sites previously saturated with macaque equol and (±) equol were unfilled.

DISCUSSION

Urinary levels of equol, similar to those which are severely detrimental to ovine pregnancy, were observed in a previous study of pregnant macaques by Monfort et al. (1984), yet all eight females included in the macaque study delivered healthy offspring without complication. In order to investigate this apparent resistance of macaques to equol poisoning, the present study compared in detail the potency and intrinsic activity of equol and E_2 in the ovariectomized adult rat uterus and MCF-7 human breast tumor cell cultures. To do this, milligram amounts of pure equol were obtained from macaque urine with HPLC separation, and standard in vivo protocols were adapted to include local application of estrogen to the uterine lumen. This approach was taken to minimize interspecies differences in systemic metabolism of equol, as such differences could be significant to interpretation of data.

Results of this study indicate that equol found in macaque urine is biochemically the same as synthetic equol. It is a weak estrogen with an apparently high dissociation rate from the estrogen receptor in vitro. In vivo, at sufficiently high dose, equol induced a pattern of cytosol receptor depletion and replenishment similar to E_2, yet nuclear retention of the receptor-equol complex was of short duration. The attenuated increase in progesterone receptor after equol treatment was consistent with its receptor binding properties both in the rat uterus and MCF-7 cultures. Any advantage the phenolic A ring may impart for tight binding of this molecule (Jordan et al., 1977) is apparently overridden by the lack of alkyl chain substitution in the chroman ring. Michell et al. (1962) have shown with such substitution the estrogenic potency of isoflavins approaches that of DES.

To reconcile equol's low potency and weak estrogenic activity with its documented intrinsic activity to induce infertility and cystic hyperplasia in the ewe, further studies should be carried out in that species. Some investigators have proposed that excessive steady-state levels of any estrogen will provide an environment in

which a predisposition to hyperplasia can manifest itself. However, it is possible the high urinary levels of equol observed in pregnant macaques by Monfort et al. (1984) reflected a very rapid clearance of phytoestrogen. Very efficient clearance may have lowered plasma equol to ineffectual levels in the monkey. This would make the monkey unlike the ewe on estrogenic pasture where plasma-free equol was measured to be about 30 ng/ml (Shutt and Braden, 1968). With a potency of 1% that of E_2, 30 ng/ml equol would present a greater than normal estrogen environment for the pregnant ewe (Rawlings and Ward, 1978), possibly equivalent to the potency of 1 nM E_2. In contrast, a similar or lower plasma concentration in the monkey would probably not be an excessive estrogen environment where 2–3 nM E_2 is commonly observed during pregnancy (Hodgen et al., 1972).

Interspecies differences beyond systemic metabolism may influence the estrogenic impact of equol. Local tissue metabolism may vary, and/or the ovine uterine receptor in vivo may bind equol differently than the rat. Another class of nonsteroidal compounds, the triphenylethylene derivatives, have mixed estrogenic-antiestrogenic effects on fertility and tissue responses which are species specific (reviewed in Clark and Peck, 1979). Binding studies of that group of compounds have demonstrated the importance of a second receptor binding site which has been designated the antiestrogen binding site (Sutherland et al., 1980). While it is unlikely equol's structure would permit it to compete at the antiestrogen binding site or to the estrogen receptor in the manner described for triphenylethylenes by Lieberman et al. (1983), it is too soon to conclude all possible binding sites important to estrogen action in various species are identical. Results of the present study indicate that equol from macaque urine is chemically identical to equol produced in the ovine rumen and that it is a low potency estrogen. These data suggest species differences at some level beyond classical estrogen receptor as defined in the rat model are responsible for the variability in equol's impact.

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