Relative Potency of Xenobiotic Estrogens in an Acute *in Vivo* Mammalian Assay

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The *in vivo* effects of xenoestrogens are of interest in relation to their potential health risks and/or beneficial effects on humans and animals. However, the apparent *in vivo* potency of the examined response can be confounded by a short half-life, and the metabolism of estrogens is very dependent on the nature of conversion and/or inactivation. To minimize such variables, we examined the estrogenic potency of a range of xenoestrogens in an acute *in vivo* assay—the stimulation of increased uterine vascular permeability in ovariectomized mice 4 hr after subcutaneous administration. While estradiol (E_2) and estriol (E_3; a relatively weak natural estrogen) readily induced vascular responses [median effective dose (ED_{50}) $<10^{-9}$ mol], much higher amounts of xenoestrogens were required. Bisphenol A was about 10,000-fold less potent than E_2 and E_3, and octylphenol and nonylphenol were about 100,000-fold less potent; dioctyl phthalate, benzyl butyl phthalate, dibutyl phthalate, and trichlorinated biphenol produced no effect. Coumestrol was the most active phytoestrogen, with an ED_{50} between 10^{-6} and 10^{-7} mol; genistein was about 10-fold less potent than coumestrol, and neither daidzein nor formononetin produced any marked effect, even at doses up to 10^{-5}mol. All increases in vascular permeability could be blocked by the pure antiestrogen ICI 182,780. There was no evidence that any of the compounds could act as an antiestrogen in this assay or that they could exert synergistic effects in combination. These results indicate that even short-term exposure to most of the xenobiotic estrogens can induce typical estrogenic effects *in vivo*, but their estrogenic potency is very weak even when assessed in an acute response.

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There is considerable public and scientific interest in the potential effects of the exposure of humans and other animals to environmental estrogens. These nonsteroidal estrogenic compounds essentially fall into two categories: they are derived from either dietary sources or industrial sources. The recent surge of interest in this area has largely come from the use of very sensitive *in vitro* assays, which have allowed the identification of weak estrogenic activity in many industrially derived chemicals (*1*). This has been coupled with natural and experimental observations of estrogenic effects in aquatic animals (e.g., fish) living under conditions of continuous exposure to the compounds (*2*). The possible health benefits of diets rich in phytoestrogens has provided an additional and alternative focus of attention (*3*).

While the existence of dietary and environmental compounds with estrogenic activity is clear, speculation linking them to epidemiological data on fertility, reproductive abnormalities, and disease is severely hampered by our very poor understanding of the *in vivo* biological activity of these compounds. *In vitro* assays have established that all the compounds are relatively weak estrogens, with both binding affinity to the estrogen receptor and *in vitro* biological activity usually <1/1,000 of estradiol (*4*). However, it is difficult to extrapolate from such *in vitro* data to the *in vivo* situation in which the biological activity is dependent on the complex *in vivo* pharmacokinetics associated with uptake, distribution, metabolism, and excretion.

There have been virtually no systematic, detailed studies of the nature and time course of the estrogenic effects of these chemicals *in vivo* in mammals. As a first stage to assessing the *in vivo* estrogenic activity, this study was undertaken to establish the relative potency of the compounds in an acute *in vivo* mammalian assay. *In vivo*, the complex pattern of estrogenic action on the uterus can be dissociated into a group of early responses (e.g., increased vascular permeability, uterine edema, uterine eosinophilia) and a group of later responses (a host of biochemical changes leading to cell division, differentiation, and uterine growth) (*5*).

Estimates of estrogenic potency are markedly affected depending on which of these two groups of responses are used as the baseline. Thus while estriol and estradiol are relatively similar when assessed in terms of the ability of single subcutaneous (sc) injections to induce early uterine responses, such single exposures to estriol are considerably less effective than estradiol in producing sustained effects due to the rapid dissociation of estriol from its receptor (*5*).

The rationale behind this study was to use one of the early specific estrogen-stimulated responses as the basis for comparing relative estrogenic potencies. This was based on the assumption that estimates of potency from early responses would minimize complications arising from metabolism, clearance, rapid dissociation from receptors, etc. The early response chosen was the rapid increase in uterine vascular permeability [apparent within 15–30 min, with very large responses (three- to fourfold increase) within 3–4 hr] of estrogen exposure (*5,6*). This response has the advantage that it can be readily quantified by monitoring the extravasation of intravenously administered [^{125}I]-labeled human serum albumin (*7*).

Methods

Animals. Female Swiss albino mice (A. Tuck & Son Ltd., Battlesbridge, Essex, U.K.), approximately 3 months of age and weighing 25–35 g, were maintained under constant conditions of lighting (lights on from 0600 hr to 1800 hr) and temperature (21 ± 1°C) and fed on a pelleted diet (Economy Rodent Maintenance, Essex, UK) *ad libitum*. All experiments were performed on ovariectomized animals. Ovariectomies were performed under tribromoethanol anesthesia at least 2 weeks before the start of each experiment. All test compounds were administered by sc injection in a volume of 0.1 ml.

Chemicals. 17β-Estradiol (E_2), genistein (4′,5,7-trihydroxyisoflavone), and daidzein (4′,7-dihydroxyisoflavone) were obtained from Sigma Chemical Co. Ltd. (Dorset, U.K.). Coumestrol (3,9-dihydroxy-6*H*-benzofuro-[3,2-*c*]-[1]benzopyran-6-one) and formononetin (7-hydroxy-4′-methoxyisoflavone) were obtained from Acros Organics (Springfield, NJ) and Extrasynthese (Genay, France), respectively. 4-Nonylphenol, 4-*tert*-octylphenol, bisphenol A [2,2-bis(4 hydroxyphenyl)propane, dioctyl phthalate, benzyl butyl phthalate, and dibutyl phthalate were obtained from Aldrich Chemical Co. Ltd. (Gillingham, U.K.). Trichlorinated biphenol (TCB; 3,4,3′,4′-tetra-chlorobiphenyl) was a gift from P. Darbre (University of Reading, Reading, U.K.). Stock solutions of the chemicals were normally dissolved in ethyl alcohol or DMSO. The injection vehicle was either arachis oil (phytoestrogens) or saline (others)

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containing 25% DMSO or alcohol. The pure antiestrogen ICI 182,780 (a gift from A. Wakeling, Zeneca Pharmaceuticals, Macclesfield, U.K.) was dissolved in alcohol to give a stock solution of 10 mg/ml ethanol and administered in an arachis oil vehicle.

Vascular permeability changes in response to test treatment. A quantitive index of the permeability of the uterine vasculature 4 hr after sc injection of the test compound was obtained from the leakage of radiolabeled albumin from the circulation (*7,8*). In brief, 3.5 hr after the injection of the test substance, 50 µl 0.5 µCi [^{125}I]-labeled human serum albumin was injected into the jugular vein of mice anesthetised with tribromoethanol. Thirty minutes later, a blood sample was drawn from the suborbital canthal sinus with a heparinized capillary pipette and the animals were killed by cervical dislocation. The blood sample was centrifuged to provide a 100 µl plasma sample. The uteri and a sample of thigh muscle were removed, briefly washed in saline, and weighed. The radioactivity in the uterus, plasma sample, and muscle was determined. Previous studies on the uterus (*8,9,10*) have suggested that the blood volume of the uterus is very much smaller than the albumin space (after a circulation time of 30 min) and that the extravascular albumin space is the major determinant of the total tissue albumin space. The tissue-specific extravascular albumin volume (EAV) was expressed as a ratio of the [^{125}I] counts per minute per milligram of tissue to [^{125}I] counts per minute per microliter of plasma and used as an index of tissue vascular permeability (*7*).

Analyses of results. Results were expressed as mean ± standard error (SE) and analyzed using analysis of variance (ANOVA).

Results

Experiment 1—Dose–response effects of xenobiotic estrogens on uterine vascular permeability. Ovariectomized mice were injected with one of the following in a volume of 0.1 ml saline: control vehicle, E_2 (10^{-12} –10^{-9} mol), or various doses of E_3, coumestrol, genistein, diadzein, formonetin, nonylphenol, octylphenol, bisphenol A, dioctyl phthalate, dibutyl phthalate, and benzyl butyl phthalate; uterine vascular permeability was determined 4 hr later. The results are shown in Figures 1 and 2. E_2 induced a marked increase in uterine vascular permeability at injected doses down to 10^{-10} mol. The highest doses of E_2 produced a fourfold increase in extravascular albumin volumes (>22 µl/mg compared to <4 µl/mg for controls), with about 10^{-9}–10^{-10} mol being required to produce half-maximal (EAV≈13 µl/mg) stimulation. E_3 was also

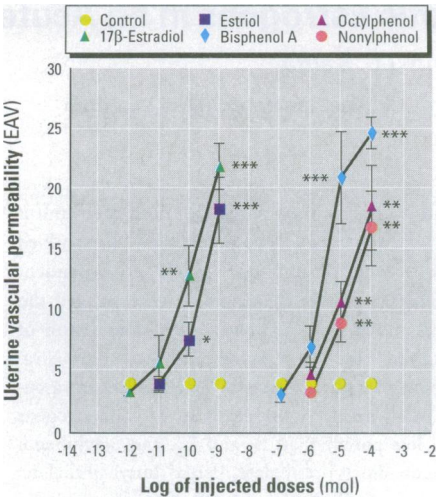

Figure 1. The dose–response effects of 17β-estradiol, estriol, and the environmental estrogens bisphenol A, octylphenol, and nonylphenol on uterine vascular permeability 4 hr after subcutaneous administration in ovariectomized mice. Saline controls were included for each experimental data point. Vascular permeability was determined by the extravascular accumulation of [^{125}I]-albumin and was expressed as extravascular albumin volume (EAV; see Methods). The data are expressed as mean ± standard error (n = 6–12). Where no error bars are shown, the errors were smaller than the symbol. Statistical significance was determined by comparison with the controls.
*p<0.05; **p<0.01; ***p<0.001.

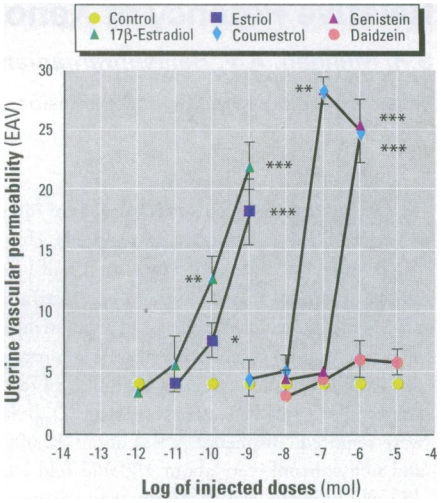

Figure 2. The dose–response effects of 17β-estradiol, estriol, and the phytoestrogens coumestrol, genistein, and daidzein on uterine vascular permeability 4 hr after subcutaneous administration in ovariectomized mice. Saline controls were included for each experimental data point. Vascular permeability was determined by the extravascular accumulation of [^{125}I]-albumin and was expressed as extravascular albumin volume (EAV; see Methods). The data are expressed as mean ± standard error (n = 6–12). Where no error bars are shown, the errors were smaller than the symbol. Statistical significance was determined by comparison with the controls.
*p<0.05; **p<0.01; ***p<0.001.

very effective in stimulating vascular permeability, with a median effective dose (ED_{50}) <10^{-9} mol. All the other industrial compounds were required at considerably higher doses before any increase in uterine vascular permeability was observed (Fig. 1), and some showed no effect at all. Compared to E_2, bisphenol A was about 10,000 times less effective in inducing the uterine vascular response (ED_{50} ~10^{-5} mol), and octylphenol and nonylphenol were about 100,000 times less effective (ED_{50} ~10^{-4} mol). The three phthalates, dioctyl phthalate, benzyl butyl phthalate, and dibutyl phthalate (10^{-4} mol), and trichlorinated biphenol (10^{-8} mol) produced no significant effect on uterine vascular permeability.

Coumestrol was the most potent of the phytoestrogens (ED_{50} between 10^{-6} and 10^{-7} mol), but still required 100–1,000-fold higher doses of the compound than E_2 (Fig. 2). The amount of genistein required to induce the vascular response was about 10-fold higher again, while neither daidzein nor formononetin produced any marked effects, even when given at doses up to 10^{-5} mol.

Experiment 2—The ability of the antiestrogen ICI 182,780 to inhibit estrogen-induced increases in uterine vascular permeability. Ovariectomized mice were subjected to daily sc injections of 100 µg ICI 182,780 for 4 days. One hour after the last injection, the mice were given a single sc injection of one of the following: E_2 (at doses of 10^{-11}, 10^{-10}, and 10^{-9} mol), E_3 (10^{-9} mol), coumestrol (10^{-7} mol), genistein (10^{-6} mol), nonylphenol (10^{-4} mol), octylphenol (10^{-4} mol), and bisphenol A (10^{-5} mol); vascular permeability was determined 4 hr later. Pretreatment with the antiestrogen ICI 182,780 suppressed the increase in vascular permeability induced by the estriol (10^{-9} mol) and the lower dose of estradiol (<10^{-10} mol). The antiestrogen suppressed the vascular responses to all the other substances tested (Table 1). There were no significant effects of the antiestrogen on the vascular permeability in the control tissue.

Experiment 3—The effect of low levels of environmental estrogens on estradiol-stimulated increases in vascular permeability. The possibility that low doses of xenobiotic estrogens (i.e., below the level required to induce an estrogenic response) might act as antiestrogens was investigated by examining the effect of a nonstimulatory dose of the compounds in combination with a stimulatory dose of E_2. Ovariectomized mice were injected sc with E_2 (10^{-10} mol) together with either coumestrol (10^{-8} mol), daidzein (10^{-5} mol), formononetin (10^{-5} mol), nonylphenol (10^{-6} mol), octylphenol (10^{-6} mol), bisphenol A (10^{-7} mol), dioctyl phthalate (10^{-4} mol),

Table 1. The ability of the antiestrogen ICI 182,780 to inhibit estrogen-induced increases in uterine vascular permeability

Compound administered (mol)	Number of mice	Uterine weight (mg)	Uterine vascular permeability ratio (%)	Muscle vascular permeability ratio (%)
Control	12	13.00 ± 1.25	4.26 ± 0.51	0.64 ± 0.08
ICI 182,780	6	8.47 ± 0.01	4.20 ± 0.27	0.47 ± 0.04
17β-Estradiol 10^{-12}	6	16.82 ± 0.02	3.53 ± 0.39	0.5 ± 0.04
17β-Estradiol 10^{-12} + ICI	6	10.05 ± 0.01	3.40 ± 0.12	0.44 ± 0.06
17β-Estradiol 10^{-10}	6	18.72 ± 0.01	17.63 ± 2.04	0.47 ± 0.05
17β-Estradiol 10^{-10} + ICI	6	13.23 ± 1.32	7.83 ± 1.73*	0.61 ± 0.03
17β-Estradiol 10^{-9}	6	17.38 ± 1.83	25.79 ± 1.90	0.42 ± 0.02
17β-Estradiol 10^{-9} + ICI	6	16.12 ± 0.01	22.19 ± 2.50	0.46 ± 0.05
Coumestrol 10^{-7}	6	19.58 ± 1.85	28.40 ± 2.98	0.50 ± 0.01
Coumestrol 10^{-7} + ICI	6	15.10 ± 0.01	4.31 ± 0.36*	0.57 ± 0.04
Genistein 10^{-6}	8	19.56 ± 1.11	25.43 ± 1.92	0.60 ± 0.08
Genistein 10^{-6} + ICI	6	13.16 ± 0.09	5.92 ± 0.54*	0.55 ± 0.04
Estriol 10^{-9}	6	26.5 ± 0.02	18.27 ± 2.70	0.54 ± 0.04
Estriol 10^{-9} + ICI	6	16.32 ± 0.02	12.8 ± 1.46*	0.51 ± 0.04
Nonylphenol 10^{-4}	6	18.80 ± 0.01	16.74 ± 3.06	0.47 ± 0.05
Nonylphenol 10^{-4} + ICI	6	15.08 ± 0.03	4.53 ± 1.17*	0.65 ± 0.11
Octylphenol 10^{-4}	6	12.60 ± 1.52	18.45 ± 3.49	0.53 ± 0.06
Octylphenol 10^{-4} + ICI	6	13.07 ± 0.01	5.7 ± 0.10*	0.44 ± 0.04
Bisphenol A 10^{-5}	6	16.50 ± 1.20	15.18 ± 2.40	0.42 ± 0.02
Bisphenol A 10^{-5} + ICI	6	8.15 ± 0.03	3.67 ± 0.48*	0.45 ± 0.02

Ovariectomized mice were subjected to daily subcutaneous (sc) injections of 100 µg ICI 182,780 for 4 days. One hour after the fourth injection, the test compound was administered sc. Saline controls were included for each experimental data point. Vascular permeability was determined by the extravascular accumulation of [^{125}I]-albumin and was expressed as a ratio (mean ± standard error) of the [^{125}I] counts per minute per milligram of tissue to [^{125}I] counts per minute per microliter of plasma. Thigh muscle was the control tissue.

*Significantly lower than the test compound alone ($p<0.001$).

dibutyl phthalate (10^{-4} mol), or benzyl butyl phthalate (10^{-4} mol), and uterine vascular permeability was determined. There was no evidence that any of the environmental estrogens (at the doses given) produced any significant ($p<0.05$) inhibitory effect on the estradiol-stimulated increase in vascular permeability. The index of vascular permeability was 12.9 ± 2.5 µl/mg (mean ± SE) for E_2 (10^{-10} mol) alone, while the combination of E_2 with the other estrogenic compounds gave values of 10.3 ± 1.5 for nonylphenol and octylphenol (each at a dose of 10^{-6} mol), 14 ± 2.8 for bisphenol A (10^{-8} mol), and 11.4 ± 1.6 for dioctyl phthalate, 12.1 ± 1.8 for dibutyl phthalate, and 14.9 ± 1.1 (µl/mg for benzyl butyl phthalate (all at doses of 10^{-3} mol).

Experiment 4—The effects of combinations of a variety of environmental estrogens on uterine vascular permeability. The effect of low doses of different compounds given in combination was examined to determine whether there was any evidence of synergistic interactions in the induction of increases in uterine vascular permeability. Ovariectomized mice were injected sc with one of the following combinations: 1) nonylphenol (10^{-6} mol), octylphenol (10^{-6} mol), and bisphenol A (10^{-7} mol); and 2) dibutyl phalate (10^{-4} mol), dioctyl phalate (10^{-4} mol), and benzyl butyl phalate (10^{-4} mol). There was no evidence of any significant ($p>0.05$) postive or negative synergy or negative effects between the compounds (at the doses used) in any of the combinations tested in terms of inducing an increase in vascular permeability. In control uteri, the index of vascular permeability was 4.2 ± 0.51 µl/mg (mean ± SE); the combination of nonylphenol with octylphenol (both at 10^{-6} mol) and bisphenol A (10^{-7} mol) produced an index of 2.91 ± 0.27 µl/mg; for dibutyl phthalate with benzyl butyl phthalate and dioctyl phthalate (all at 10^{-3} mol), the index of vascular permeability was 2.79 ± 0.54 µl/mg.

Discussion

The availability of very sensitive *in vitro* bioassays for estrogens has led to the identification of estrogenic activity in many industrial (*11,12*) and plant-derived compounds (*13*). However, even *in vitro* assays, the estrogenic activity of these compounds is usually less than 1/1,000–1/100,000 that of E_2 (*1,4,11–13*). Our data is in general agreement with published data on the *in vitro* potency of the xenobiotic estrogens, emphasizing that these compounds can only exert significant estrogenic activity when present in very large quantities. Bisphenol A appears to be one of the more potent xenobiotic estrogens (*14,15*); a recent *in vivo* study in rats examining prolactin secretion (*14*) suggested that bisphenol A was only 100–500-fold less active than E_2, while bisphenol A potency *in vitro* was 1,000–5,000-fold less than E_2. In the present study, bisphenol A was about 10,000-fold less potent than E_2. These observations, coupled with the present observations, suggest that *in vitro* potencies of xenobiotic estrogens may not be an accurate guide to *in vivo* potencies, and estimates of *in vivo* potency will depend on the assay used.

The *in vivo* estrogenic potency of xenobiotic estrogens has received rather superficial attention, with results largely based on uterine growth after daily sc injections (*16*) or after administration by gavage (*17*). However, the relative potency of estrogenic compounds depends on a number of factors, including the route of administration and the nature of the response monitored. The particular problem of using uterine growth responses to assess estrogenic potency is well illustrated by comparing the effects of E_2 and E_3; while single sc injections of either of these hormones are very effective in inducing rapid uterine responses (that occur within a few hours of administration), E_3 is unable to mimic E_2 in terms of the uterine growth response measured at 24 hr after injection (*5*). Such differences in estrogenic responses led to E_3 being described as a weak estrogen (*18*). However, the apparent low potency of single injections of E_3 to induce uterine growth hides the biological reality of the estrogenic response. E_3 is fully effective in producing the complete range of estrogenic responses when it is given as rapidly repeated injections (*5*) or as slow-release implants (*19*). These apparent differences between E_3 and E_2 in terms of their potency to induce acute and sustained responses highlight the difficulty of interpreting assessments of estrogenic potency by just single *in vivo* assay methods. In contrast to the long-term nature of uterine growth assays, the increase in uterine vascular permeability is an acute, rapid, easily monitored response of the uterus to estrogenic stimulation (*7*). The estrogen receptor-mediated nature of this response was confirmed in the present study by the ability of the pure antiestrogen ICI 182,780 to block the responses induced by E_2, E_3, and other compounds. The present study provides a direct indication of the acute *in vivo* potencies of a variety of dietary and industrial estrogenic compounds on an important target site for estrogens in female mammals. The results indicate that, even when measured in terms of their ability to induce acute responses, the activity of such compounds is very low in comparison with established estrogens like E_2 and E_3.

The relative potency of any of the environmental estrogens compared to E_3 and E_2 in this acute assay is particularly interesting. While E_3 is a relatively weak estrogen in terms of any long-term growth responses to acute exposures, it is almost as effective as E_2 in inducing rapid estrogenic effects in the uterus. However, even in this rapid *in*

vivo assay that allows the estrogenic activity of the relatively weak E_3 to be exposed, the potency of all the weak environmental estrogens was considerably lower. This would tend to emphasize that prolonged *in vivo* estrogenic responses to such compounds are unlikely to occur unless the exposures are both considerable and extended; this is currently under investigation. However, the possibility that acute exposures to these compounds may induce significant, although short-term, estrogenic responses should not be underemphasized. Rapid responses to estrogens are an important feature of a number of reproductive processes, such as control of the cell cycle (*5*) and the sensitivity of the uterus to an implanting blastocyst (*20*); exogenous estrogenic agents may be able to either mimic endogenous estrogens and/or provide a disturbance to the sequence of endogenous estrogenic stimulation essential for the normal pattern of uterine responses.

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