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Developmental effects of estrogenic chemicals are predicted by an in vitro assay incorporating modification of cell uptake by serum ☆

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Abstract

Many estrogenic chemicals found in the environment (xenoestrogens) show a lower affinity for plasma estrogen binding proteins relative to the natural estrogens such as estradiol. These binding proteins, which include alphafetoprotein in rats and mice, sex hormone binding globulin in humans, and albumin in all species, regulate estrogen uptake into tissues. Therefore, the in vivo estrogenic potency relative to estradiol of xenoestrogens that show lower binding to these serum proteins will thus be underestimated in assays that compare the potency of xenoestrogens to estradiol and do not take serum binding into account. We have examined the effects of the binding components in serum on the uptake of a number of xenoestrogens into intact MCF-7 human breast cancer cells. Since most estrogenic chemicals are not available in radiolabeled form, their uptake is determined by competition with [^{3}H]estradiol for binding to estrogen receptors (ER) in an 18-h assay. Serum modified access (SMA) of cell uptake of xenoestrogens is calculated as the RBA in serum-free-medium ÷ the RBA in serum, and the bioactive free fraction of xenoestrogen in serum is then also calculated. We predicted the concentration of two xenoestrogens, bisphenol A and octylphenol, required to alter development of the prostate in male mouse fetuses. Whereas octylphenol was predicted to be a more potent estrogen than bisphenol A when tested in serum-free medium, our assay predicted that bisphenol A would be over 500-times more potent than octylphenol in fetal mice. The finding that administration of bisphenol A at a physiologically relevant dose predicted from our in vitro assay to pregnant mice from gestation day 11 to 17 increased adult prostate weight in male offspring relative to controls (similar to the effect of estradiol), while the same doses of octylphenol did not alter prostate development, provided support for our hypothesis.

1. Introduction

It has been suggested that environmentally relevant exposures to chemicals currently present in the environment have the ability to disrupt the endocrine system of humans and wildlife, and these chemicals have been termed 'endocrine disruptors' [1]. An endocrine disruptor is defined by the US Environmental Protection Agency as 'an exogenous substance that changes endocrine function and causes adverse effects at the level of the organism, its progeny, and of populations or organisms' [2]. A number of these chemicals in the environment have estrogenic activity and are referred to as 'xenoestrogens', as they are produced outside of the body. Phytoestrogens are naturally occurring xenoestrogens produced by plants [3–6], however, most xenoestrogens are synthetic compounds produced by man. While some xenoestrogens were designed to be estrogenic, such as ethinyl estradiol which is used in oral contraceptives, many xenoestrogens were designed for other purposes and only coincidentally have estrogenic activity [7–9].

There are more than 75,000 man-made chemicals in the Toxic Substances Control Act Inventory, but only

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a few have been tested for endocrine disrupting effects. At this time about 60 chemicals have been identified as endocrine disruptors. For example, bisphenol A and the alkylphenols, nonylphenol and octylphenol, have been reported to have estrogenic activity [7–11]. Bisphenol A is an essential component of epoxy resins used in the lacquer lining of metal food cans [12], as the monomer used to manufacture polycarbonate plastics [8], in some dental sealants [13], and as a flame retardant (after bromination). Bisphenol A leaches out of products into food or liquids stored in containers made from or lined with bisphenol A [8,13]. Approximately two billion pounds of bisphenol A are produced per year. Some alkylphenols, such as octylphenol and nonylphenol, which are used in a wide variety of detergents and plastics, have been reported to show estrogenic activity as well as environmental persistence [9]. These chemicals are found in rivers near textile mills and have been related to developmental abnormalities in fish living in these rivers [14,15]. Many other synthetic compounds have also been reported to show estrogenic activity [16].

2. The issue of relative potency of xenoestrogens and estradiol

A critical issue with regard to the hypothesis that amounts of endocrine disrupting chemicals being encountered by wildlife and human populations are sufficient to result in adverse effects relates to both the potency of the compounds and the type of effect being examined. Many xenoestrogens have been considered safe because they exhibit very low acute toxicity. However, these same chemicals can show estrogenic activity at concentrations many orders of magnitude below the concentration at which toxicity occurs [17,18]. Furthermore, some xenoestrogens show estrogenic activity in mice and in cell culture systems at concentrations within the range of current human and wildlife exposure to these chemicals [19,20].

Many in vitro assays used to assess potency of xenoestrogens relative to estradiol have not taken into account a number of critical factors that influence the bioactivity of a xenoestrogen in vivo. The bioactivity of a xenoestrogen in the animal is determined by the concentration that reaches intracellular estrogen receptors, and this concentration is influenced by a number of factors. Some of these factors include absorption, metabolism and rate of clearance relative to the route of administration, partitioning, serum transport, affinity for estrogen receptor subtype in the cell, and the interaction of the ligand-receptor complex with tissue-specific transcription factors. Many of these factors are currently being addressed by our laboratory and others. In this paper we summarize how incorporating just one additional factor, serum transport, with the assessment of affinity for the estrogen receptor into a cell culture assay system, significantly improves the prediction of biological activity of some xenoestrogens in fetal mice over in vitro assays that do not incorporate this factor.

A number of investigators have shown that some xenoestrogens which show full activity as estrogen receptor agonists show little or no binding to the specific high affinity estrogen binding plasma proteins sex hormone binding globulin (SHBG) in humans, and alphafetoprotein (AFP) in rats and mice [21–27]. These finding have led to suggestions that the in vivo potency of these xenoestrogens will be increased relative to estradiol, which shows significant binding to these plasma proteins. In contrast to the approach of examining specific components of blood for their interaction with xenoestrogens, we will describe below our cell culture assay system using human MCF-7 breast cancer cells and complete serum. We use this assay system to compare the ability of an xenoestrogen to pass into cells and bind to intracellular estrogen receptors from medium containing 100% human serum, compared to medium containing no serum. This assay system thus takes into account the effects of all components of serum on cell uptake of xenoestrogens. One of the objectives of developing this new assay system was to determine the physiological range of activity of xenoestrogens. Prior to discussing the assay system, we will discuss the issue of physiologically relevant doses, since this is an issue that has been largely ignored in both in vitro and in vivo pharmacological and toxicological studies in which estrogenic chemicals have been examined.

3. Physiological range of activity of estradiol: concordance of in vitro studies with MCF-7 cells and in vivo studies with mice

The mechanism of action for steroidal estrogens has been well characterized, so when examining a xenobiotic's potential for estrogenic activity much is already known about its mechanism of action. However, in many areas of research this information has been largely ignored when designing studies to examine the mechanisms of action of xenobiotics that act through endogenous hormone receptors. For example, estrogen receptor-mediated responses occur at low receptor occupancy, most often between 0.1 and 10% receptor occupancy. This corresponds to a concentration 10 to 1000-fold lower than the dissociation constant (K_d) for hormone receptor complex.

However, many experiments designed to study the estrogen receptor-mediated effects of xenoestrogens are conducted at hormone concentrations far above those

that saturate estrogen receptors, many orders of magnitude above their dissociation constant (K_d). Effects seen at these ligand concentrations cannot be assumed to be mediated through the estrogen receptor, since at very high concentrations, there can be considerable binding of estrogenic chemicals to other steroid receptors, effects on enzyme systems, as well as general toxic effects. The very high doses of xenoestrogens, which are typically used in toxicological studies [28], as well as in studies of estrogen action are thus not within a physiological range of estrogenic activity. In the next section we will describe how we established the physiological range of estrogenic activity in mouse fetuses. The bioassay that we used to detect changes in estrogenic activity during fetal life was the size of the prostate in subsequent adulthood. The physiological range of activity of estradiol and xenoestrogens in this in vivo assay system corresponds closely with findings from studies using cultured human MCF-7 cells.

4. Estrogen action in adults and fetuses

Steroidal estrogens circulate and act in both males and females. However, estrogens have been best characterized with regard to regulating a variety of functions in females, such as stimulation of growth and activity of the mammary gland and endometrium, preparation of the female reproductive tract for spermatozoal transport, and stimulation and maintenance of female secondary sexual characteristics [29]. These actions typically seen in the adult are referred to as activational effects and are reversible, i.e. once the estrogen is removed the response diminishes. However, there can also be irreversible effects of estrogens in adults. At puberty in humans in both males and females, epiphyseal fusion is estrogen dependent and irreversible [30]. In mice and rats during pregnancy, mammary gland differentiation requires estrogen, progesterone, and prolactin [31], and some changes persist in the postpartum mammary gland relative to the prepregnant state. However, most described actions of estrogens in adults are reversible.

In the fetus, however, estrogens have developmental effects in both males and females which are irreversible and permanent (referred to as organizational effects). A considerable amount of work has demonstrated that the perinatal mouse (a period which corresponds to sexual differentiation in the human fetus during the second trimester) is sensitive to the permanent organizational effects of exogenous estrogen exposure, prompting Dr. Howard Bern to coin the term 'fragile fetus' to describe this phenomenon [32]. In addition, evidence suggests that the fetus may in fact be very sensitive to endogenous estrogens [33–35], and this is discussed in more detail below.

In addition to the developmental effects observed from exposure to estrogens in rodents, humans exposed in utero to the synthetic estrogen diethylstilbestrol (DES) throughout the 1950's and 1960's illustrate the disastrous and irreversible effects that exposure to a high dose of a xenoestrogen can have during development. DES was administered to pregnant women in the mistaken belief that this would prevent spontaneous abortion and promote healthy pregnancies [36,37]. 'DES sons and daughters' have experienced an increase in reproductive tract cancers and other abnormalities, as well as subfertility and infertility due to administration of DES to their mothers during the first trimester of pregnancy [36,37]. Our primary interest in xenoestrogen action is centered around potential effects in fetuses during development due primarily to the permanent effects that estrogens can have during this time.

5. The intrauterine position phenomenon: correlation between estradiol and prostate development in fetal mice

Our interest in the relationship between estrogen and prostate development began with an observation which, at first, appeared to contrast with the accepted view that exposure to an increase in estrogen inhibited normal prostate development in rodents [34,38]. We had observed that there were higher serum concentrations of estradiol in female relative to male mouse fetuses, and higher serum testosterone levels in male relative to female mouse fetuses [39–41]. The transport of both estradiol and testosterone between adjacent fetuses within a uterine horn serves as a source of variation in these steroids during fetal life. We refer to this as the intrauterine position phenomenon, which is mediated by steroid transport between fetuses across the placental membranes via the amniotic fluid [42]. As a consequence of steroid transport between fetuses, male mice that develop in utero between two female fetuses (2F males) are exposed to higher blood concentrations of estradiol (about 35% difference) and lower blood concentrations of testosterone (about 30% difference) than are male fetuses that develop between two male fetuses, referred to as 2 M males [41]. There are a wide range of traits that differ as a function of random intrauterine placement in comparisons of both males and females [41]. With regard to the prostate and other reproductive organs, however, a logical prediction would be that 2 M males would have enlarged reproductive organs relative to 2F males. In contrast, the prostate in 2F males was found to be significantly larger (by about 30%) than that in 2 M males. The enlarged prostate in 2F males was associated with a three-fold greater number of prostatic androgen receptors in 2F relative to 2 M males [35].

An important aspect of studies of the relationship between natural variation in gonadal steroid concentrations in fetal blood and subsequent phenotype is that the normal physiological range of activity of these hormones during fetal life was established. Development of a modification of a procedure (centrifugal ultrafiltration dialysis) for determining the very low percent of total estradiol not bound to plasma binding proteins (the free fraction) in rat and mouse fetuses was critical in this regard [43]. Prior studies had only determined total (bound plus free) levels of circulating estradiol during development. However, it is well recognized that the free fraction of circulating steroids has significantly greater predictive value for clinical outcomes relative to information provided by only total concentrations. These experiments provided a basis for comparison of the free concentration of circulating estradiol in rodents and the bioactive concentration of estradiol in cultured human breast cancer cells. In addition, they provided the basis for predictions concerning the consequences of altering blood levels of estradiol during critical periods of organ development in mice.

6. Effects on prostate development of an experimental increase in estradiol in male mouse fetuses

We examined the hypothesis that an increase in estradiol within a physiological range during fetal life would permanently increase prostate size. We experimentally increased serum estradiol levels in male mouse fetuses during the time of fetal prostate development by implanting pregnant females with a Silastic capsule containing estradiol [44]. The dose of estradiol that we chose to administer via Silastic capsule resulted in a 50% increase in free serum estradiol in male mouse fetuses from 0.2 pg/ml (in controls) to 0.3 pg/ml. This 0.1 pg/ml increase in free serum estradiol was associated with an increase in total serum estradiol of 52 pg/ml (from 94 pg/ml in controls to 146 pg/ml; the percent free estradiol in fetal mouse serum was 0.2% in both). This increase in estradiol was chosen since it produced a mean value for serum estradiol in all treated male fetuses that was at the high end of the normal range of estradiol values measured in the serum of individual 2F males (that have the highest levels of circulating estradiol), and thus represented an increase in estradiol that was within the normal physiological range.

The 0.1 pg/ml increase in free serum estradiol increased the number of developing prostate glands by 40%, based on a three-dimensional reconstruction of the prostate collected from male fetuses on gestation day 18, one day after initiation of fetal prostate development [44]. In addition, the developing prostatic glandular ducts were enlarged in estrogen-treated males relative to control males. This effect on the prostate was permanent. In adulthood, males exposed to the 50% increase in estradiol during fetal life had enlarged, hyperplastic prostates (by 40%) that showed a six-fold increase in prostatic androgen receptors relative to prenatally untreated males. The same permanent stimulation of prostate growth in male mice occurred with maternal ingestion of 0.02, 0.2, or 2 μg of DES per kg body weight per day from gestation day 11–17. Males exposed during fetal life to these low doses of DES had significantly enlarged prostates in adulthood relative to control males [44].

7. Opposite effects of high (toxicological/ pharmacological) and low (physiological-range) doses of estrogen on the developing prostate in male mice

Using diethylstilbestrol (DES) as an example, we have shown how ignoring the issue of dose can lead to false conclusions about doses of estrogenic chemicals that appear not to produce an effect (referred to as the NO Effect Level or NOEL in toxicological studies). We fed pregnant mice a broad (5-log) range of doses of DES, and we found opposite effects on the prostate in adult mice exposed as fetuses to the highest dose of DES from those seen at much lower doses. Specifically, as described above, the prostate in male offspring was permanently enlarged by feeding pregnant mice (from gestation day 11–17) low doses of DES (0.02, 0.2 and 2.0 μg/kg body weight). In marked contrast to the effects of these low doses of DES, the prostate was permanently reduced in size by a dose of 200 μg DES/kg body weight, which is 10,000-times higher than the 0.02 μg/kg dose that increased prostate size [44]. The possibility of opposite effects occurring at high (toxicological) and low (physiologically relevant) doses has relevance not only in toxicology, but also in endocrinology and in the study of pharmaceuticals and other xenobiotics.

8. Steroid binding proteins and the free fraction of estradiol in serum

Steroidal estrogens circulate in blood associated with serum proteins [46]. This association reduces hepatic metabolism and thus their clearance [47]. In adult women, approximately 2, 4, and 8% of the total estradiol, estrone and estriol is free, respectively [48,49]. The glycoproteins, sex hormone binding globulin (SHBG) in humans and alpha-fetoprotein in mice and rats, are specific estrogen binding proteins

[22,49]. An SHBG homodimer [50] binds both testosterone and estradiol with high affinity ($K_d \approx 1.5$ nM) and circulates at approximately 30 nM in men and 40 nM in women [49]. Albumin binds estradiol with low affinity ($K_d \approx 10$ μM) but with high capacity, circulating at around 560 μM. Albumin thus accounts for 80% of the protein in plasma in both men and women. In women, of the total circulating estradiol, approximately 37% is associated with SHBG, and approximately 61% is associated with albumin [49].

About 1 to 3% of the total estradiol is free or unbound in circulation in adult women and men. The free fraction of circulating steroid is generally accepted to be the bioactive fraction [51], i.e., the fraction that is able to pass from serum, dissolve in and pass through cell membranes, and bind to intracellular receptors where the estrogen receptor complex functions as a ligand-dependent transcription factor. This concept that the free fraction of estradiol is the bioactive fraction is generally referred to as the free hormone hypothesis [51,52]. While there is relatively little information available concerning the free concentration of estradiol in different species and at different physiological and pathological states, it is widely accepted that the clinically-relevant levels of, for example, thyroid hormone and testosterone, are the concentrations of free hormone rather than the total. Serum estrogen-binding proteins thus provide a mechanism to limit the cell-uptake of and response to steroidal estrogens in target issues.

The possibility that SHBG may interact with its plasma membrane receptor to sequester or deliver both estradiol and testosterone to target tissues has also been suggested [53]. The serum proteins SHBG, AFP and albumin have all been shown to be internalized through receptor-mediated endocytosis in MCF-7 cells [54,55]. Many tissues in the reproductive tract, such as the breast and the prostate, contain SHBG and AFP receptors. Two studies with different cell lines have examined the role of the SHBG receptor in determining the bioactive fraction of steroids. Fortunati et al. [56] examined estrogen stimulated proliferation of MCF-7 human breast cancer cells, and Damassa et al. [57] examined the conversion of testosterone to dihydrotestosterone by LNCaP human prostate cancer cells. In both of these studies, SHBG significantly decreased the bioactivity of the steroid. These findings do not support the hypothesis that the bioactive fraction of estradiol may include or be restricted to the SHBG-bound fraction of hormone, but rather provide support for the free hormone hypotheses that albumin as well as SHBG serve to determine the bioactive fraction of testosterone and estradiol in serum.

9. Development of an in vitro assay that provides information concerning the physiological dose range for xenoestrogens in animal studies

We describe here a new approach that we developed to incorporate mechanistic information from an in vitro assay to predict the physiologically relevant range of activity of xenoestrogens in animals based on a comparison of activity of the xenoestrogen with that of estradiol. This approach focuses on the effects of serum in regulating bioactivity of endogenous estrogens and xenoestrogens. The following approaches were used to address the issue of how serum influences the bioactivity of estradiol and xenoestrogens. First, the bioactive fraction of estradiol in adult human male serum was examined (due to ready availability in comparison to fetal serum). Specifically, saturation analysis of estrogen receptors in intact MCF-7 cells was used to compare the whole cell uptake of tritiated estradiol from serum-free medium with the uptake from serum. This approach was then modified to determine the bioactive fraction of xenoestrogens in adult male serum. This was accomplished using a competition assay where the ability of a xenoestrogen to inhibit the binding of tritiated estradiol to estrogen receptors was compared in serum-free medium with its ability in serum. Next, the findings of how serum affected the bioactive fractions of xenoestrogens was used to predict the in vivo estrogenic bioactivity in fetal mice of two xenobiotics, octylphenol and bisphenol A.

Serum is a complex medium which consists not only of estrogen binding proteins but also many other proteins and lipoproteins, and in the animal, estrogens that exert estrogenic activity must travel from serum through cell membranes prior to binding to intracellular estrogen receptors. We used 100% human serum and MCF-7 human breast cancer cells to develop a tissue culture model to measure the bioactive fraction of estradiol and xenoestrogens in serum. MCF-7 cells express not only estrogen receptors but also the plasma membrane receptors for both SHBG and AFP [56,58]. While these receptors have not been found to play a role in the bioactive fraction of estradiol in serum, the interaction between estradiol and serum albumin, AFP and SHBG with their membrane receptors in 100% serum is also assessed in this assay.

9.1. Determination of the bioactive fraction of estradiol in adult human serum

In our assay, we first measured the concentration of [^{3}H]estradiol required in serum-free medium outside of cells to occupy 50% of estrogen receptors inside of cells (which occurs at the dissociation constant or K_d). We note that the K_d of estradiol for the estrogen receptor measured in this assay (0.053 nM) is indistin-

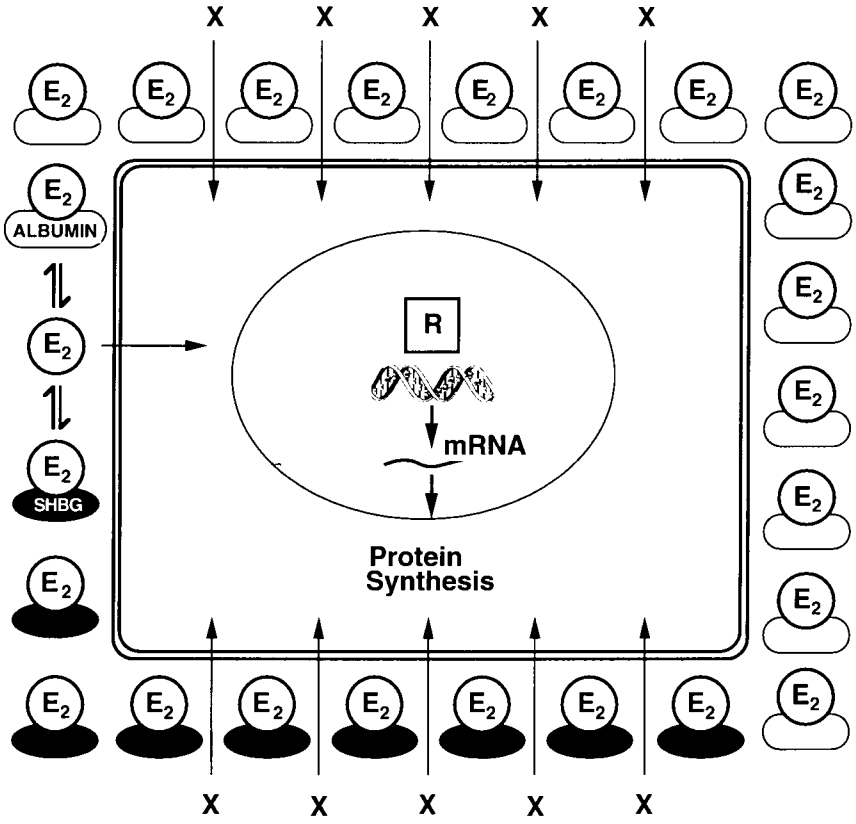

Fig. 1. Model of xenoestrogens in blood. In humans, 17β-estradiol is primarily associated with the serum binding proteins, sex hormone binding globulin (SHBG), and albumin, and only a small fraction is unbound or free. Xenoestrogens (X) that escape serum binding will have a greater effective concentration in serum available to compete with estradiol for estrogen receptors [61].

guishable from the K_d first measured in cell-free receptor extracts of MCF-7 cells [0.051 nM, [59]]. In adjacent wells on the same 24-well plate, the same assay was performed using 100% human serum and an apparent K_d was measured. We have no evidence that serum alters the affinity of estradiol for its receptor, and our assumption is that the K_d for [^{3}H]estradiol is the same whether the [^{3}H]estradiol is added to serum-free medium or serum prior to entering MCF-7 cells in this assay. However, more estradiol must be added to serum to provide enough free estradiol to occupy the same number of estrogen receptors as in serum-free medium, because a large fraction ($\approx$98%) of the estradiol is bound to serum proteins and unable to passively diffuse into cells and occupy estrogen receptors. There is thus the appearance of a shift in K_d. By calculating the ratio of the concentration of [^{3}H]estradiol required to occupy 50% of the receptors (which occurs at the dissociation constant, K_d) in serum-free medium divided by the concentration required to occupy 50% of the receptors in 100% serum (which occurs at the apparent dissociation constant measured in serum) in each assay, the bioactive fraction of estradiol in serum is determined using Eq. 1.

$$\text{Bioactive fraction of tritiated estradiol} = K_d \text{ in SFM} \div \text{apparent } K_d \text{ in serum} \quad (1)$$

Using this equation, we calculated the bioactive fraction of estradiol in adult male serum to be 2.39% $\pm$ 0.17% [19].

Traditionally, the free fraction of estradiol (or any hormone) in serum has been determined using dialysis techniques (for example, ultrafiltration dialysis). In these assays, separation of free from bound estradiol in serum is accomplished by diffusion of [^{3}H]estradiol to equilibrium across a membrane with pore sizes of 10,000 to 30,000 MW. [^{3}H]estradiol associated with SHBG (MW = 90 kDa) or albumin (MW = 70 kDa) is retained, and only the [^{3}H]estradiol that is not associated with these large serum proteins can pass through the membrane. Dialysis techniques provide very accurate measurements of the free fraction of estradiol in a given serum.

However, we measured the bioactive fraction of estradiol in serum by incorporating several additional factors that may determine the fraction of estradiol available to bind to intracellular receptors, not only

serum binding proteins, but also potential cell uptake or exclusion mechanisms and sequestration in serum and cell lipids. The bioactive fraction of estradiol calculated from the whole cell uptake assay (2.39% ± 0.17%) was indistinguishable from the measurement of the free fraction of estradiol using ultrafiltration dialysis (2.46 ± 0.08%), providing support for the hypothesis that the free fraction alone is the bioactive fraction in this cell system [19]. The saturation assay using MCF-7 cells is thus a new method to calculate the biologically active fraction of estradiol in serum. Since MCF-7 cells contain not only estrogen receptors but also progesterone, androgen, and glucocorticoid receptors, this approach could be modified to examine the bioactive fractions of progestins, androgens and glucocorticoids.

9.2. Determination of the bioactive fraction of xenoestrogens in adult human serum

Since serum binding proteins provide a mechanism to limit the cell-uptake of steroidal estrogens, xenoestrogens that do not bind to serum proteins will escape this mechanism (Fig. 1). A number of xenoestrogens show little binding to SHBG, including DES [22], ethinyl estradiol [23], DDT, dieldrin [24], octylphenol [25] and several phytoestrogens [21]. Also, DES, zearalenone, and zearalanol show little binding to the major serum estrogen binding glycoprotein in rodents, alphafetoprotein [22,26,27]. Thus, these xenoestrogens may have greater access to cells from blood than estradiol. This may increase their bioactive concentration relative to estradiol and lead to increased estrogenic activity of these compounds in vivo relative to their predicted activity from cell-free or serum-free assays. The period when this would have the greatest impact would be during fetal life, when the concentration of plasma binding proteins, such as alphafetoprotein in rats and mice, are dramatically higher than in adults.

In order to determine the bioactive fraction of xenoestrogens in human serum, we developed a cell culture assay to assess the degree to which serum modifies the access of xenoestrogens to intracellular estrogen receptors. The objective was to understand how serum contributes to the physiology of delivery of xenoestrogens, and to use this information to be able to predict bioactive doses of xenoestrogens in animals more accurately than do assays that did not incorporate information regarding serum binding.

While many factors determine the final concentration of a xenoestrogen available to interact with intracellular estrogen receptors, they can be summarized in four steps: (1) absorption and metabolism relative to the route of exposure; (2) partitioning between lipid and aqueous compartments; (3) bioactive concentration in circulation determined by how a xenoestrogen is carried in blood; and (4) the intrinsic estrogenic activity of a xenoestrogen or the affinity of an xenoestrogen for the estrogen receptor. We developed the relative binding affinity serum modified access (RBA-SMA) assay to better estimate the biological activity of xenoestrogens by incorporating the intrinsic estrogenic activity of a xenoestrogen with how a xenoestrogen is carried in blood, and partially assessing how a xenoestrogen partitions between aqueous and lipid compartments in serum and cells. Since most xenoestrogens are not available in radiolabeled form, we designed a competition assay to determine the bioactive fraction of a xenoestrogen, similar to the method used to measure the bioactive fraction of estradiol in serum described above.

Relative binding affinity (RBA) assays were performed with intact MCF-7 cells by adding increasing concentrations of unlabeled competitor to a constant concentration of [^{3}H]estradiol in either serum-free medium or in 100% serum. RBAs for xenoestrogen are expressed relative to estradiol by dividing the IC_{50} of estradiol (the concentration of non-radioactive estradiol required to inhibit 50% of [^{3}H]estradiol from binding to estrogen receptors) by the IC_{50} of the xenoestrogen and expressing the ratio as a percent.

We used the data from the RBA assays to calculate the inhibition constant (K_i) from these competition assays for each xenoestrogen in serum-free medium and in serum, since the K_d for an unlabeled xenoestrogen cannot be measured directly. The K_i was calculated as described by Bylund et al. [60] and is shown in Eq. (2).

$$K_i = IC_{50} \div (1 + F \div K_d) \quad (2)$$

The IC_{50} is the concentration of unlabeled competitor required to inhibit 50% of [^{3}H]estradiol binding, F is the concentration of [^{3}H]estradiol in the medium or serum, and K_d is the dissociation constant in serum-free medium or the apparent dissociation constant in serum. The bioactive fraction of a xenoestrogen was calculated using Eq. (3).

$$\text{Bioactive fraction of unlabeled estrogen} = K_i \text{ in SFM} \div K_i \text{ in serum} \quad (3)$$

Thus, using the same technique described above for assessing the bioactive fraction of estradiol in serum, the RBA assay is performed in both serum-free medium and with 100% serum. This is done to determine how serum modifies the access of the xenoestrogen to intracellular estrogen receptors in a manner similar to the effects of serum on estradiol. These results are quantified by calculating a serum modified access (SMA) value as shown in Eq. (4).

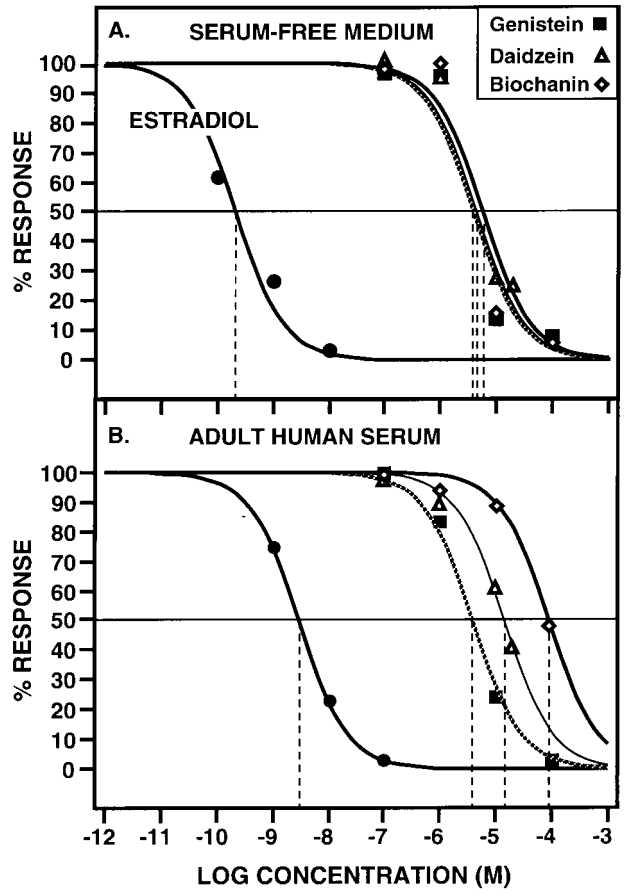

Fig. 2. Relative Binding Affinity–Serum Modified Access (RBA–SMA) assay. Relative binding affinity (RBA) analysis conducted in serum-free medium (SFM) in (A) or in 100% male serum in (B). Unlabeled reference estradiol (—●—), genistein (—■—), daidzein (—△—) and biochanin A (—◇—) competed with 1 nm [^{3}H]estradiol in SFM or 10 nM [^{3}H]estradiol in 100% adult male serum [61].

$$\text{Serum Modified Access (SMA)} = \text{RBA in serum} \div \text{RBA in SFM} \quad (4)$$

We have compared the RBAs for phytoestrogens in serum-free medium and in serum from adult men. For the three phytoestrogens (Fig. 2), the RBAs were similar when examined using MCF-7 cells in serum-free medium. However, in adult male serum their inhibition profiles separated. For example, genistein's competition profile was closer to that of estradiol in serum than in SFM, revealing that a greater proportion of the genistein added to serum was biologically active (48%) in comparison to estradiol (approximately 4%). The in vivo potency of genistein (calculated as an RBA) will thus be underestimated in assay systems in which the effects of serum are not taken into account. A summary of the K_i (inhibition constant) values for a number of xenoestrogens and their calculated bioactive fractions in 100% adult male serum is shown in Table 1.

We note here that we have recently made some changes in the procedures for conducting these assays that have resulted in slightly different findings from those initially reported. Specifically, the initial assays were conducted without rocking and at high cell density. These changes in recent experiments have resulted in the bioactive fraction of estradiol being somewhat lower (2.39%, [19]) than initially reported (4%, [61]), however the relationship between the bioactive fraction of estradiol and xenoestrogens is the same.

10. Relative binding affinity-serum modified access (RBA–SMA) assay predicts in vivo xenoestrogen bioactivity

We next incorporated the information concerning the bioactive fraction of xenoestrogen with its ability to bind to the estrogen receptor in order to predict the estrogenic activity of two industrial chemicals in vivo. The in vivo bioassay we used to assess the estrogenic activity of these chemicals (prostate size) was based on our prior finding that estrogen exposure during fetal life results in enlargement of the adult prostate in male mice. Our approach was to determine the amount of xenoestrogen delivered in serum to fetal cells as free (unbound) xenoestrogen that would supplement the endogenous free estradiol to lead to net increased free (unbound) estrogenic activity that would equal the high end of the physiological range. As noted above, this level of total free estrogenic activity, that from endogenous estradiol plus that from free xenoestrogen, would be predicted to lead to developmental disruption that we would measure as prostate enlargement. In this experiment the chemicals were fed to pregnant female mice. Since at the time this experiment was conducted we did not have information concerning the absorption, fetal uptake and rate of clearance of these chemicals, we viewed this experiment as providing a rough initial estimate of the in vivo bioactivity of the chemicals.

The environmental estrogens we used in these studies, bisphenol A and octylphenol, like most endocrine disruptors, were not available in radiolabeled form for direct determination of free levels in serum. Due to the low binding affinity of these chemicals for estrogen receptors, this would require a very high specific activity to accomplish. Therefore, we used the RBA–SMA assay to estimate the free fraction of these xenobiotics in fetal serum to use in predicting a bioactive dose of these chemicals to administer to pregnant mice.

Relative binding affinity-serum modified access analysis was conducted using bisphenol A and octylphenol. As indicated above, these chemicals are used in a wide variety of products, and exposure of wildlife

Table 1
Calculations of the bioactive fraction of xenoestrogens in human serum[a]

Compound	N	K_i (M) in SFM ± SEM[a]	K_i (M) in serum ± SEM[a]	Bioactive in serum % ± SEM
Estradiol	9	3.99E-11 ± 0.17	8.80E-10 ± 0.44	4.06 ± 0.19
Phytoestrogens				
Coumestrol	3	2.86E-7 ± 0.83	5.77E-7 ± 0.88	47.8 ± 8.10
Equol	3	2.94E-7 ± 1.65	5.89E-7 ± 2.71	49.7 ± 9.82
Genistein	3	1.76E-7 ± 0.24	3.83E-7 ± 0.42	45.8 ± 1.33
Daidzein	3	2.19E-7 ± 0.10	1.25E-6 ± 0.24	18.7 ± 2.99
Biochanin A	3	2.66E-7 ± 0.72	1.19E-5 ± 0.12	2.42 ± 0.93
Formononetin[b]	3	4.94E-7 ± 0.96		
Synthetic estrogens				
Diethylstilbestrol	5	2.12E-10 ± 0.40	9.02E-10 ± 1.07	26.9 ± 7.53
Moxestrol	3	6.92E-11 ± 0.88	3.00E-10 ± 1.43	33.6 ± 13.48
Dienestrol	3	2.52E-11 ± 0.96	4.92E-9 ± 1.59	0.51 ± 0.06
Hexestrol	3	7.58E-12 ± 2.15	1.61E-9 ± 0.61	0.57 ± 0.17
Antiestrogens				
Raloxifene	3	1.39E-10 ± 0.29	2.10E-9 ± 0.50	6.81 ± 0.59
Tamoxifen	3	2.91E-8 ± 0.18	3.53E-6 ± 0.85	0.94 ± 0.25
Commercial additives [a,b],				
Bisphenol A	3	3.70E-7 ± 0.57	6.64E-6 ± 3.32	7.84 ± 2.27
Nonylphenol	3	1.14E-7 ± 0.52	2.57E-5 ± 1.85	1.34 ± 0.92
Octylphenol	3	2.73E-7 ± 2.47	8.02E-5 ± 4.14	0.31 ± 0.14

[a] Abbreviations: N = number of assays, K_i = inhibition constant, SFM = serum-free medium, SEM = standard error of the mean.
[a] Numbers are in exponential notation; 3.99E-11 = 3.99×10^{-11}.
[b] Solubility limited the measurement of an IC_{50} in serum [61].

and humans to these chemicals occurs as a result. Competition profiles for bisphenol A and octylphenol from a single experiment conducted in serum-free medium and in 100% human adult serum are shown in Fig. 3. In serum-free medium (Fig. 3A), octylphenol exhibited an RBA that was approximately 10-fold greater than the RBA of bisphenol A, consistent with prior reports of the relative estrogenic activities of these compounds [8,9,62]. However, when the competing compounds were tested in 100% serum, the relationship of the competition profiles was in fact reversed, and the RBA of bisphenol A became approximately four-fold greater than that of octylphenol (Fig. 3B).

A summary of results and calculations from RBA–SMA assays of bisphenol A and octylphenol is shown beginning in Table 2. Table 2 includes the RBA measured in serum-free medium, the RBA measured in 100% adult serum, and the calculated serum modified access (SMA), which is the ratio of the RBA in serum to the RBA in serum-free medium. The RBA of bisphenol A in serum was 1.7-fold higher than that measured in serum-free medium (Table 2, SMA = 1.7 for bisphenol A), indicating that its biological impact relative to estradiol would be greater in serum than that which would be estimated by this factor in serum-free assays. In contrast, octylphenol showed an SMA of substantially less than one (Table 2, SMA = 0.045).

Table 2
Relative Binding Affinity–Serum Modified Access (RBA–SMA) assay. Relative Binding Affinity (RBA) analysis conducted in serum-free medium (SFM) and in 100% adult male serum. Serum modified access (SMA) values are the mean of three independent RBA–SMA assays. Bisphenol A showed enhanced access from serum while octylphenol and nonylphenol showed decreased access from serum relative to estradiol [18]

Compound	N	RBA in SFM ± SEM[a] (%)	RBA in serum ± SEM[a] (%)	SMA[b] ± SEM[a]
Estradiol	3	100	100	1.00
Bisphenol A	3	0.0060 ± 0.0009	0.0100 ± 0.0012	1.70 ± 0.29
Nonylphenol	3	0.026 ± 0.0069	0.0039 ± 0.0011	0.20 ± 0.11
Octylphenol	3	0.072 ± 0.0152	0.0029 ± 0.0005	0.045 ± 0.013

[a] SEM = standard error of the mean.
[b] SMA (Serum Modified Access) = RBA in serum ÷ RBA in serum-free medium.

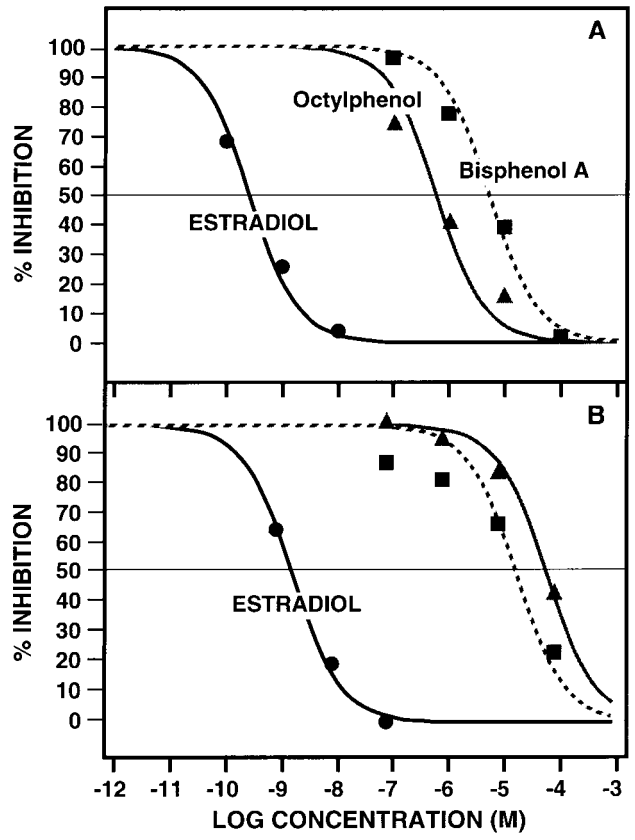

Fig. 3. Relative Binding Affinity–Serum Modified Access (RBA–SMA) assay. Relative binding affinity (RBA) analysis conducted in serum-free medium (SFM) in A or conducted in 100% male serum in B. Unlabeled reference estradiol (—●—), octylphenol (—▲—) or bisphenol A (—■—) competed with 1 nM [^{3}H]estradiol in SFM or 10 nM [^{3}H]estradiol in 100% adult male serum [18].

Hence, its activity would be overestimated in serum-free assays. We chose these chemicals to examine for their in vivo bioactivity as a result of this finding and after screening a number of chemicals for the possibility that they would be influenced in opposite ways by serum.

The results obtained for the effects of serum on the activity of bisphenol A and octylphenol were used to estimate what the relative bioactivities would be in fetal mice, and what maternal doses would result in the developmental changes in the prostate described above. Ideally, the effects of serum modified access on the estrogenic activity of these compounds would have been calculated directly from RBA values determined in fetal mouse serum. However, the volumes of serum required for these measurements were not available from fetal mice. In the absence of fetal serum data, we extrapolated the SMA calculations obtained in adult male serum, to what would be expected in fetal mouse serum.

The free fraction of estradiol in fetal mouse serum

Table 3
Predicted serum modified access (SMA) in fetal mouse serum estimated by extrapolation from SMA in human serum (shown in A), and predicted estrogenic activity calculated from the predicted SMA (shown in B), for bisphenol A and octylphenol [18]

	Serum-free (100% free E_2)	Adult human (4% free E_2)	Estimated fetal mouse (0.2% free E_2)	Predicted SMA in fetal mouse
A. Serum Modified Access[a] (SMA): Extrapolation from adult human to fetal mouse serum				
Bisphenol A	1.0	1.7	$(1.7-1)\times 20^b+1$ =	15
Octylphenol	1.0	0.045	$0.045\div 20^b$ =	0.0022

	Predicted SMA in fetal mouse		RBA in Serum-free medium		Predicted bioactivity[d]	Activity[e]	Relative
B. Predicted fetal acitivity[c] (% of E_2)							
Bisphenol A	15	×	0.006%	=	0.09%	563	
Octylphenol	0.0022	×	0.072%	=	0.00016%		1

[a] SMA = RBAin serum ÷ RBA in serum-free medium.
[b] [4% ÷ 0.2% = 20-fold].
[c] Predicted SMA × RBA in serum-free medium = predicted RBA in fetal mouse serum.
[d] Predicted estrogenic activity in Fetal Mouse Serum.
[e] Predicted bioactivity of bisphenol A/octylphenol.

Table 4
Reference Dose for the xenoestrogens bisphenol A and octylphenol that would result in adult prostate enlargement in the offspring, based on the observation that an elevation of 23 pg/ml serum estradiol(=84 pM) in fetal mice results in adult prostate enlargement. The calculation of reference dose used the predicted bioactivities in fetal mouse serum from Table 3B (SERUM), the results from the RBA–SMA assay. For comparison, reference doses were also calculated using the RBAs that we determined in serum-free medium (Serum–free) [18].

Calculation of Xenoestrogen Reference Doses

	Estradiol reference (pM)[a]	Predicted Bioactivity (%)[b]	Equivalent estr. act. (μ)[c]	MW, μg/μmol	Reference dose[d] (μg/kg)
Serum					
Bisphenol A	84	÷ 0.09	= 0.093	× 228.3	= 21
Octylphenol	84	÷ 0.00016	= 52.5	× 208.3	= 10,900
Serum-free					
Bisphenol A	84	÷ 0.006	= 1.400	× 228.3	= 320
Octylphenol	84	÷ 0.072	= 0.117	× 208.3	= 24

[a] Fetal estradiol elevation that resulted in adult prostate enlargement: 84 pM = 23 pg/ml [3,4].
[b] From Table 3B, activity relative to estradiol, based on relative molar concentrations; the predicted bioactivities Serum-free are the RBAs measured in serum-free medium.
[c] Equivalent estrogenic activity; molar concentration of xenoestrogen predicted to equal the estrogen activity of the estradiol reference.
[d] Equivalent estrogenic activity in mole/litter converted to mass, μg/liter; value then expressed as μg/kg for reference dose; MW of bisphenol A = 228.3; MW of octylphenol = 208.3.

during the time of sexual differentiation and initiation of prostate development is 0.2% which is 20-fold lower than the 4% bioactive free estradiol initially measured in adult male serum in the RBA–SMA assay [19,44]. We have observed a linear relationship between the percent free estradiol in serum and the SMA when the RBA assay was conducted with serial dilutions of adult serum [19]. Therefore, we estimated that the effects of fetal mouse serum on the access of xenoestrogens to estrogen receptors would be approximately 20-fold greater than the effects we had measured in adult serum. The deviations in the SMA from the value of 1.0 in adult serum were thus altered by a factor of 20 for both bisphenol A and octylphenol in order to predict the SMA in fetal serum, and this calculation for conversion of the SMA in adult serum (4% free estradiol) to the estimated SMA in fetal serum (0.2% free estradiol) is shown in Table 3A. The calculation step (multiplication of division) involving the correction factor (20-fold) depends on whether the SMA value showed enhancement (SMA > 1) or inhibition (SMA < 1) of cell-uptake. If the SMA > 1, the degree of enhancement of cell uptake was determined by multiplying the SMA in adult serum by the correction factor; hence, the enhancement will be greater relative to estradiol. If the SMA < 1, the assumption is that in fetal serum the cell-uptake would be even less than estradiol, and the SMA value was divided by the correction factor.

The SMA values for bisphenol A and octylphenol predicted for fetal mouse serum were then applied to adjust the activity of the compounds measured in serum-free medium to the bioactivity predicted in fetal mouse serum (Table 3B). The predicted bioactivity in fetal mouse serum was calculated by multiplying the RBA measured in serum-free medium by the predicted SMA in fetal serum. These calculations predicted that in the presence of fetal mouse serum, bisphenol A would exhibit over 500-fold greater bioactivity than would octylphenol in mouse fetuses, despite the higher estrogenic activity of octylphenol (RBA = 0.072% for octylphenol vs 0.006% for bisphenol A) that was observed before taking serum into account [18].

Finally, the results of the RBA–SMA assay were used to calculate the circulating concentration or 'Reference Dose' in pregnant mice and their fetuses that we expected would produce an increase in prostate weight in male offspring. Calculation of reference dose was based on two factors. (1) The initial study described above in which male mice that had developed as fetuses between female fetuses (2F males) were found to have enlarged prostates relative to males located in utero between other male fetuses (2 M males), and this was associated with an increase in total serum estradiol of 23 pg/ml (84 pM) in 2F male fetuses relative to 2 M male fetuses [35]. (2) The predicted bioactivity, relative to estradiol, of bisphenol A in serum of fetal mice calculated in Table 3B (predicted bioactivity = 0.09%). This prediction of bioactivity for bisphenol A was determined based on the hypothesis that the free concentration of estrogen in serum, not the total circulating concentration, would determine bioactivity in the animal. With these two pieces of information, reference doses of bisphenol A and octylphenol were calculated that would be equivalent to an increase in serum estradiol in male fetuses of 23 pg/ml (84 pM), and would thus be predicted to produce prostate enlargement [18].

This calculation (Table 4) predicted that bisphenol A at 21 ng per g would lead to an increase in prostate

weight in male mice. Strikingly, this dose is thousands of fold lower than the levels which had previously been thought to be without biological effects in humans or animals (see NOEL dose, see below). We administered 20 μg/kg bisphenol A per day (the approximate reference dose) and 2 μg/kg bisphenol A per day to pregnant mice on days 11 to 17 of pregnancy. Both of these doses are within the range of reported daily human exposure [12,13]. Our expectation was that if the RBA–SMA assay correctly predicted estrogenic bioactivity in the animal, then the 20 μg/kg does of bisphenol A would result in enlargement of the prostate in adult male offspring. Because octylphenol was predicted to be over 500-fold less estrogenic than bisphenol A, neither of the two doses we administered (2 and 20 μg/kg body weight) was predicted to produce an increase in prostate weight in males exposed to octylphenol (Table 4).

Prostates were collected from six month old males whose mother had been fed vehicle, bisphenol A or octylphenol at 2 or 20 μg/kg body weight per day from gestation day 11–17. Males exposed to either does of bisphenol A had significantly heavier prostates than control males (Fig. 4); the 2 and 20 μg doses of bisphenol A resulted in prostate weights that were 30 and 35% greater than control prostates, respectively [18]. Body weight was not related to prostate weight, and thus we did not correct prostate weight for body weight. Interestingly, however, body weight was reduced by about 10% by bisphenol A, and the magnitude of the effect of bisphenol A on prostate enlargement would have appeared larger had we corrected for body weight. As predicted, prostates from males exposed prenatally to either the 2 or 20 μg doses of octylphenol were not significantly different from the prostates from control animals (Fig. 4). Based on the doses that we administered in this preliminary experiment, bisphenol A appeared to be at least 100-fold more estrogenic than octylphenol, which is consistent with the prediction derived from the calculations from the RBA–SMA assay above.

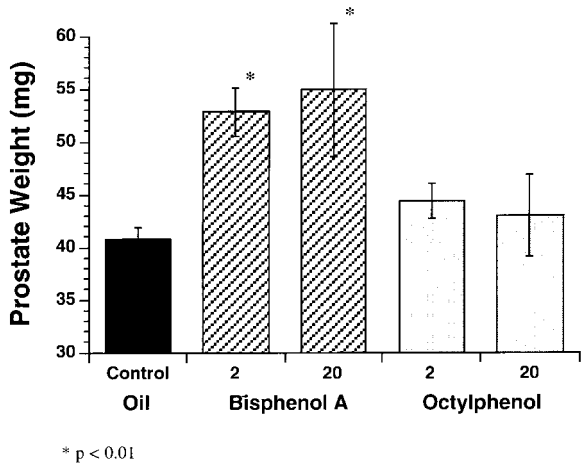

Fig. 4. Prostate weight in adult male mice (six months old) exposed as fetuses to bisphenol A or octylphenol. Mothers were fed 2 or 20 μg/kg body weight/day bisphenol A or octylphenol from days 11 to 17 of pregnancy. Error bars are the SEM, $n=7$ for bisphenol A and octylphenol, and $n=11$ for unexposed controls [18].

Since bisphenol A exhibited estrogenic effects at the lowest dose we administered (2 μg/kg), and since the calculation of reference dose above did not include the effects of metabolism and pharmacokinetics, bisphenol A appeared to be even more estrogenic in vivo than predicted. In addition, the estrogenic activity of bisphenol A was only 100-fold lower (or less) than that of DES when using the prostate as a bioassay; a dose of 0.02 μg/kg DES enlarged the prostate to a similar degree [44], again indicating that bisphenol A appeared to be more estrogenic in vivo than predicted.

Several possible mechanisms could explain this. (1) Our predictions were based on the estrogen receptors in MCF-7 cells which are ER-alpha, while, after our work was completed, a novel estrogen receptor, ER-beta, was cloned from the rodent prostate [63]. This estrogen receptor has been reported to show eight-fold greater affinity for bisphenol A than does the classical ER-alpha present in MCF-7 cells we used [64], and this could account for increased biological activity of bisphenol A in the mouse prostate. (2) Two- to four-fold increased bioaccumulation of labeled DES in fetal circulation has been reported in the mouse fetus after administration to the mother [45]. Similar or greater fetal bioaccumulation of bisphenol A could increase its bioactivity by another several fold. (3) Alternatively, bisphenol A may be bioactivated in vivo to a more estrogenic metabolite, either in maternal or in fetal circulation [65]. (4) There are several reports that some xenoestrogens can act cumulatively and/or synergistically and have several-fold greater (e.g., three-fold greater) activity in mixtures than predicted when the individual compounds are assayed alone [66]. It is possible that bisphenol A acts synergistically or addictively with the phytoestrogens including genistein known to be present on soy-based mouse chow. (5) Finally, bisphenol A may bind even less to the serum proteins in fetal mouse serum than to those in adult human serum, thus having greater activity and greater serum modified access in fetal mouse serum than was predicted by extrapolation from results obtained in adult human serum. One or more of these mechanisms may be sufficient, with the effects of serum on the physiology of xenoestrogen delivery to fetal cells, to explain the high bioactivity of bisphenol A in these experiments.

11. Implications for endocrine disruptors

In our study a maternal dose of only 2 μg/kg per day bisphenol A enlarged the prostate in male offspring. This dose is equivalent to a daily dose of 50 μg for a 25 kg child or 150 μg for a 75 kg adult. In this regard, Olea et al., have shown that after a 50 mg dental sealant treatment, the saliva measured in a one hour collection after the application of the sealant from human subjects contained from 90 to 931 μg of bisphenol A [13]. Brotons et al., analyzed vegetables packaged in food cans with lacquer coating and found that as much as 23 μg of bisphenol A was recovered from 50 ml of the liquid portion of one food can [12]. An important aspect of our study is the proximity of the maternal doses of bisphenol A administered to pregnant mice and the reported ranges of human exposure to this chemical.

Our findings reveal that fetal exposure to bisphenol A can alter the reproductive tract in rodents at much lower doses than previously thought to be active. The predicted NOEL (no observed effect level) for bisphenol A has been estimated at 50 mg/kg body weight/day [17], however, bisphenol A was active in rodents at 2 to 20 μg/kg/day, which lies near or within the reported ranges of current human exposure to this chemical [12,13,67]. Until additional information showing actual safety at these exposures is obtained, default risk assessment applied to bisphenol A would indicate that far lower doses of human exposure to bisphenol A would be prudent [68], particularly during pregnancy, but also during childhood development.

12. Conclusions

We have described a new approach to estimate low doses of xenoestrogens that are expected to exhibit estrogenic effects in developing mice. Initially we conducted a series of studies to characterize the effects of estrogens at physiologically relevant concentrations in fetal mice. We have shown that a 50% elevation of estradiol during fetal life results in mice with enlarged prostates in adulthood, and that prenatal exposure to diethylstilbestrol results in the same effect at physiologically relevant concentrations (low doses), while opposite effects on prostate weight occur at high doses [44]. These studies led to the development of an in vitro assay to estimate concentrations of xenoestrogens expected to have estrogenic effects. The relative binding affinity–serum modified access (RBA–SMA) assay incorporates the affinity of xenoestrogens for the estrogen receptor and the effects of serum on their bioactivity. Results from this assay indicated that serum can dramatically alter the ability of a xenoestrogen to enter cells and bind to estrogen receptors, and thus alter their estrogenic bioactivity. By incorporating this information with the developmental effects of estradiol on fetuses described above, we improved the ability to predict in vivo estrogenic activity from in vitro results, and accurately predicted the low dose activities of the xenoestrogens bisphenol A and octylphenol.

Importantly, we have shown that: (1) under physiological conditions estradiol meditates developmental effects in mice; (2) xenoestrogens can mediate these same effects at low doses; (3) the potency of xenoestrogens can be dramatically altered by how they are carried in blood; (4) by incorporating this information from our cell culture assay, physiologically relevant doses of xenoestrogens can be accurately predicted; and (5) these effects can be seen at environmentally relevant exposure levels.

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