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Low-dose bioactivity of xenoestrogens in animals: fetal exposure to low doses of methoxychlor and other xenoestrogens increases adult prostate size in mice

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The hormonal activity of natural estrogens is influenced by the degree to which they bind to serum proteins. In the pregnant female and in the fetus, greater than 99% of estradiol may be bound by serum binding proteins. Therefore, even though total serum levels of estradiol appear very high in fetuses, we have found that in rodent fetuses, there is a very low free concentration of estradiol (0.2 pg/ml). Naturally occurring variation in fetal serum estradiol predicts differences in numerous postnatal traits, including prostate size. In addition, when this low level of free estradiol was experimentally increased from 0.2 to 0.3 pg/ml during the last third of fetal life, treated male mice showed an increase in adult prostate weight. Fetal exposure to low doses of xenobiotic estrogens by feeding to pregnant females, including the compounds methoxychlor (20 and 2000 μg/kg body weight), DES (0.02 to 2 μg/kg body weight) and bisphenol A (2 and 20 μg/kg body weight), also led to increased prostate weight in adulthood. In contrast, fetal doses of natural estradiol and DES above the physiological range of estrogenic activity, and within a toxicological dose range, led to the opposite outcome, a reduction in subsequent adult prostate weight. This indicates that it may be impossible to assess endocrine-disrupting activities in response to low doses within a physiological range of activity by using high, toxic doses of xenoestrogens in testing procedures. We have developed approaches *in vitro* to predict the potential estrogenic bioactivity of compounds in the physiologically relevant range in animals and humans. We address the following factors in predicting the final observed endocrine-disrupting effect in the animal: (1) the intrinsic estrogenic activity of a given molecule, (2) the effective free concentration determined by how the molecule is carried in serum, (3) partitioning between aqueous and lipid compartments in body and cell lipids, and (4) absorption and metabolism relative to the route of exposure. The studies and strategies we describe are important in developing criteria for a tiered testing system for the detection of estrogenic chemicals as well as endocrine-disrupting chemicals with different modes of action.

Keywords: *bisphenol A, diethylstilbestrol (DES), dose–response, low-dose effects, methoxychlor, prostate.*

Background

Estrogenic Endocrine-Disrupting Chemicals (EEDC)

Large numbers of chemicals are synthesized for current use in commercial applications and are released into the environment. Some of these chemicals have been found accidentally to have endocrine-disrupting properties. Many are mimics of the natural estrogens, such as 17ß-estradiol, and these chemicals represent an important group of endocrine disruptors (Colborn and Clement, 1992; Colborn et al., 1993). Estrogenic endocrine disruption will be addressed here.

Individual chemicals can act as endocrine disruptors in several different ways. (A) The compound can act as an estrogen, that is, it can bind to estrogen receptors and bring about estrogenic actions. The compound can also act as an antiestrogen and lead to endocrine disruption by blocking normal estrogen actions. Both of these mechanisms are receptor-mediated. (B) Further, some chemicals themselves have little estrogenic or hormonal activity but can be metabolized into chemicals which are very active and which bring about estrogenic responses after exposure to the parent compound. These are termed proestrogens, and the insecticide methoxychlor is an example (Bulger et al., 1978; ATSDR, 1994). (C) In addition to endocrine-disrupting activity by binding to a receptor and activating (or inhibiting) normal hormonal responses, any number of chemicals can also act through other mechanisms, including changing the activities of enzymes which synthesize hormones, or through high-dose acute toxicity on the hormone-secreting, endocrine organs. All of these act to change the normal synthesis or secretion of endogenous hormones. (D) Finally, acute toxicity acting on the final

1. Abbreviations: DES, diethylstilbestrol; EEDC, estrogenic endocrine-disrupting chemical; LOAEL, lowest-observed-adverse-effect level; NOAEL, no-observed-adverse-effect level; POPs, persistent organic pollutants; RBA, relative binding affinity; SERM, selective estrogen receptor modulator; SMA, serum modified access.

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hormone target organs can act to create endocrine disruption as well. However, the largest number of the endocrine disruptors currently known that affect the estrogen response mechanism fall into the categories (A) and (B), chemicals which directly or as precursors act as estrogens (or antiestrogens). It is important to group these compounds together because of the shared receptor-mediated action mechanisms; receptor-mediated effects can occur at lower, more environmentally relevant doses than are required for direct actions on organs, cells, or enzymes, and this is why they are particularly important.

Structures of Chemicals with Estrogenic Activity

Nearly all chemical estrogens contain one or more phenolic hydroxyl groups critical in the binding of the chemical to the estrogen receptor, a property required for (direct) estrogen action by any compound. The phenolic hydroxyl is also a commonly used group in synthetic organic chemistry, and this undoubtedly has contributed accidentally to the large number of chemicals that have been identified to show estrogenic activity. The major steroidal natural estrogen, 17ß-estradiol, as well as other natural and synthetic estrogens, generally have in common the phenolic hydroxyl group on a small lipophilic molecule of approximately 200 to 300 Daltons. At the concentrations at which they show biological effects, these molecules are soluble in both water and lipids. As a consequence of this they can diffuse easily across the plasma membrane to reach the interior of the cell. However, their greater solubility in lipids favors accumulation in body stores of adipose tissue.

Sources of exposures to environmental estrogens include some of the persistent organic pollutants (POPs) originally used as pesticides, and they can be consumed in foods in which they accumulate, including milk (Dillon et al., 1981; Rogan et al., 1987; Jensen and Slorach, 1991; Thomas and Colborn, 1992). Detergents can contain compounds that give rise to free alkylphenols, which are estrogenic. A number of widely used plastics that are important in food handling can release compounds such as bisphenol A and nonylphenol that are estrogenic as well (Soto et al., 1991; White et al., 1994). In addition, we are exposed to a number of plant-derived estrogens (phytoestrogens) in foods such as soy (Welshons et al., 1990).

All Estrogens, Both Natural and Environmental, Act Through Estrogen Receptors

All natural estrogens and environmental estrogens that we know of exert their estrogenic actions by way of the steroid receptors, a family of receptor proteins located predominantly in the nucleus of the cell. These receptors act as ligand-dependent transcription factors (Figure 1), although evidence exists for membrane associated, nongenomic steroid receptors as well (Judy and Welshons, 1998).

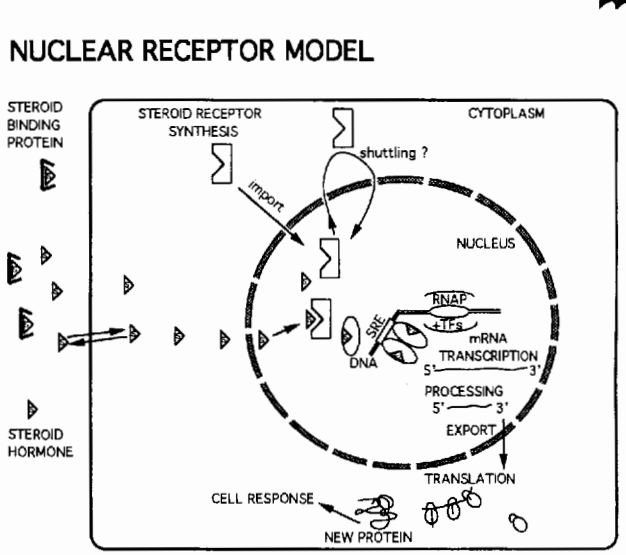

Figure 1. Model of steroid hormone action through receptors of the steroid receptor superfamily. Abbreviations: RNAP, RNA polymerase; SRE, steroid response element; TFs, transcription factors. From Judy and Welshons (1998). Reproduced by copyright permission of Oxford University Press.

Estrogens or xenoestrogens diffuse into the cell and bind to estrogen receptors. It is the occupancy of these receptors that turns on specific responses in target tissues, and it is abnormal or physiologically uncontrolled occupancy of these receptors which brings about endocrine disruption. As noted above, chemicals may not themselves bind to estrogen receptors but may be metabolized into compounds that do. These chemicals are termed 'proestrogens' since they are not themselves estrogenic.

Estrogen Actions and Effects: Activational and Developmental

The natural steroidal estrogens circulate and act in both males and females, although estrogenic effects have been best characterized with regard to regulating reproduction and the reproductive tract in females. These effects include stimulation of growth and activity of the mammary gland and uterine endometrium, preparation of the female reproductive tract for spermatozoal transport, and maintenance of female secondary sexual characteristics (Vom Saal et al., 1994). These actions typical in the adult are reversible and are referred to as activational effects; if the estrogen is withdrawn the response diminishes. However, there are also irreversible effects of estrogens in adults. At puberty in humans in both males and females, epiphyseal fusion is estrogen-dependent and this event is irreversible (Smith et al., 1994). In mice and rats during pregnancy, mammary gland differentiation requires estrogen, progesterone, and prolactin (Imagawa et al., 1994), and some changes persist in the postpartum mammary gland relative to the pre-pregnant state. However, most described actions of estrogens in adults are reversible.

In the fetus, steroid hormones have developmental effects in both males and females which can be irreversible and permanent (referred to as organizational or developmental effects) and which can vary by degree of effect. For example in the development of the normal male, testosterone in fetal circulation plays a major role in the masculinization and defeminization of the female body plan, and these fetal effects are largely irreversible (Vom Saal et al., 1992). The perinatal mouse (at 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 Bern to coin the term 'fragile fetus' to describe this phenomenon (Bern, 1992). In addition, recent evidence indicates that normal development of the male and female reproductive tracts in the rodent fetus is very sensitive to, and in fact dependent on, endogenous estrogens (Nonneman et al., 1992; Santti et al., 1994; Ekbom et al., 1997). The normal role of *circulating* estrogens in development of the reproductive tract in both the male and female fetus, and specific details of these effects, is a relatively new focus in the study of hormonal control of sexual development, but current information can be applied to understand some mechanisms of endocrine disruption by environmental estrogenic compounds, and to understand which chemicals are likely to show endocrine-disrupting effects at environmentally relevant exposures.

Concentration Ranges for 'Low-Dose' Endocrine Disruption Versus 'High-Dose' Acute Toxicity

The consequences of exposure to chemical estrogens can be described at two levels. We use 'high-dose' effects to represent the acute toxicity by the chemical. High-dose effects are typically analyzed in toxicological studies in which an acutely toxic but sublethal dose, termed the 'maximum tolerated dose', is established. For purposes of risk assessment, an experimental dose corresponding to a NOAEL (no-observed-adverse-effect level) or LOAEL (lowest-observed adverse-effect level) is determined and divided by a series of uncertainty factors to calculate the exposure level of the chemical that should be without risk of any acute toxicity to humans or wildlife. This method is based on a linear extrapolation to a dose-axis (*X*-axis) intercept which represents a threshold for the acute toxicity, below which the chemical is assumed to be safe.

In contrast, a completely separate and different group of effects derive specifically from endocrine effects of some chemicals, not as acute toxicants but as mimics of one or more types of hormones. Because hormones are biologically active at very low levels in the blood, chemicals that mimic these hormones may also act at low levels of exposure, and at levels potentially much lower than required for acute toxicity. For the endocrine-disrupting chemicals that show hormonal activity, we use the term 'low dose' to describe effects that occur within a physiological range of estrogenic (or other hormonal or endocrine-disrupting) activity for a specific chemical. The use of the term 'low dose' in the context of a physiologically relevant dose is different from the use of 'low dose' in toxicology, where a dose 10–50 times below the dose that causes acute toxicity (but not death) is referred to as a 'low dose' (Vom Saal and Sheehan, 1998). As will become apparent below, the physiologically relevant dose range may be thousands or even millions of times lower than the ranges used in chronic toxicological studies.

The use of 'low dose' here refers to effects of chemicals acting as xenobiotic hormones. The low-dose range for estrogenic activity of a chemical is the concentration at which the estrogenic activity of the chemical is similar to the activity of the natural estrogens, predominantly estradiol. These levels are constantly regulating the reproductive system in the adult and the development of reproductive tissues and organs in the fetus. Therefore changes in these levels brought about by an exogenous, uncontrolled endocrine disruptor must, to some degree, result in abnormal regulation and/or development. Not only the dose but also the precise timing of exposure to estrogen are critical for normal development, and timing is subject to endocrine disruption as well.

An example of high-dose cytotoxicity for MCF-7 human breast cancer cells is shown in Figure 2 for the compounds estradiol, diethylstilbestrol (DES), octylphenol, and bisphenol A. All four compounds show cytotoxicity in the micromolar concentration range. However, low-dose stimulatory effects of the same compounds on the growth of the cells appear across a much wider range of concentrations, from 0.22 pM for DES to 0.24 μM for bisphenol A. The high-dose cytotoxicity by the compounds clearly does not predict the low-dose estrogenic activity of the compounds, and the low-dose estrogenic activity does not predict their high-dose cytotoxicity.

Like all hormone responses, the responses that occur within the low-dose range of estrogenic actions of endocrine disruptors saturate (they reach a peak or plateau and do not continue to increase; Figure 2). Beyond the point of saturation of response, therefore, the response cannot continue to increase as a function of dose, and the relationship cannot be linear. Further, there is no evidence for the existence of thresholds for this kind of activity (Lucier et al., 1993).

For natural estrogens and for the more potent of the estrogenic endocrine disruptors, these effects can occur at levels that are below the ranges of standard chemical detection by instruments such as HPLC (high performance liquid chromatography) coupled with UV detection. For this reason bioassays of response to the natural hormones and hormone mimics, which do not depend on physical

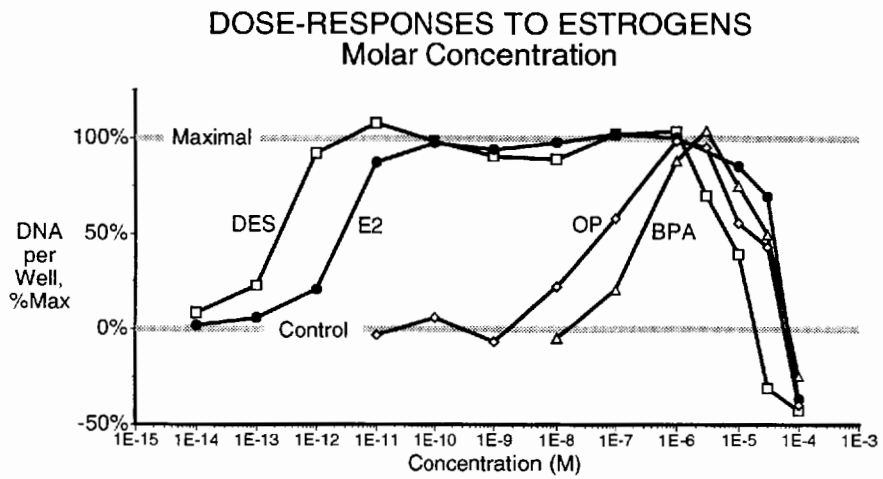

Figure 2. Stimulation of MCF-7 human breast cancer cell proliferation at varied 'low-dose' concentrations of diethylstilbestrol (DES), estradiol (E_2), octylphenol (OP) and bisphenol A (BPA), and cytotoxicity induced by all of the compounds at similar micromolar concentrations. Estrogen-dependent cell proliferation and cytotoxicity were evaluated as described elsewhere (Welshons et al., 1990, 1995; Grady et al., 1991). Half-maximal stimulation of cell proliferation: DES, 0.22 pM; E_2, 2.6 pM; OP, 60 nM; and BPA, 240 nM.

detection of the chemical, are important in the evaluation of estrogenic endocrine disruptors.

Low-Dose, Endocrine Effects are Predictable from Dose Required for Estrogen Receptor Occupancy

To characterize the high-dose acute toxicity of a chemical, the effects must be determined first, and then the doses of the chemical are characterized that bring about those particular effects, which will be specific to each chemical. But for the low-dose endocrine effects of an estrogenic chemical it is a different matter; many of the targets and effects that are subject to disruption by an estrogen are already known. The targets will be the estrogen-responsive tissues and cells of the organism, while the effects will include all effects known to be under direct regulation by estrogen.

The unknown issue is the concentration at which the battery of endocrine effects will be disrupted by the estrogenic endocrine-disrupting chemical (EEDC). This disruption in the targets is predictable from, and in fact requires, a concentration of the EEDC at the target cell (dose at target) that can occupy estrogen receptors. This information can be obtained by comparison to the concentration range of estradiol that brings about normal hormonal signaling, since response varies as a function of receptor occupancy. The exact consequences of exposure to a given EEDC may not be known without experimental determination, since tissue-specific responses to different estrogenic chemicals have been demonstrated, for example by tamoxifen or raloxifene as 'selective estrogen receptor modulators' or SERMs (Jordan and Murphy, 1990; Yang et al., 1996). However, the concentration of an EEDC at the estrogen-sensitive target at which consequences will occur, and in fact must occur, is highly predictable.

Experimental approach to prediction of low-dose activity

1. *Developmental Effects of Estrogen on Rodent Reproductive Tract*

Prior to conducting experiments with estrogenic endocrine-disrupting chemicals, we first described effects of naturally occurring differences in circulating estradiol on the development of the male reproductive tract (Nonneman et al., 1992). In subsequent studies, we determined the levels of total and free (unbound) estradiol that were active during normal development (Montano et al., 1995). Finally, we determined experimental increases of estradiol that disrupted development of the male reproductive system showing both low-dose increases in adult prostate weight, and high-dose acute toxicity for the system (Vom Saal et al., 1997). This dose–response information for estradiol provided the foundation information for predicting the doses of estrogenic endocrine disruptors that would cause the same type of developmental effects in this system.

2. *Evaluation of EEDC to Predict Dose that will be Active in the Developmental Response*

We modeled bioactivity of the EEDC by addressing four key factors that influence the EEDC activity (Nagel et al., 1997, 1998): (1) absorption and metabolism relative to the route of exposure, (2) how the compound partitions between the circulation and body lipid, (3) how the compound is carried in blood and what fraction is delivered free (unbound) to cells (Figure 3), and (4) the intrinsic estrogenic activity of the molecule in interaction with estrogen receptors in the nucleus of the cell. Most assays *in vitro* measure primarily the fourth factor, intrinsic estro-

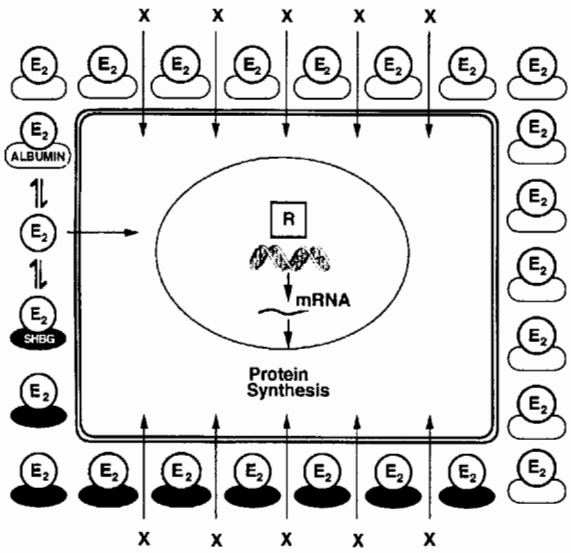

Figure 3. Model of xenoestrogens in blood. In humans, 17ß-estradiol is primarily associated with the serum binding proteins, sex hormone binding globulin (SHBG, dark ovals) and albumin (open ovals), and only a small fraction is unbound or free. Xenoestrogens (X) that escape serum binding will have a greater effective concentration in serum available to reach and bind to estrogen receptors. From Nagel et al. (1998).

genic activity of xenoestrogens, but do not address the other factors systematically. We have developed an *in vitro* assay to assess more of these factors (Nagel et al., 1997), beginning with how xenoestrogens are carried in blood, in order to better understand and predict the levels of EEDC that are disruptive of normal development in the animal.

3. *Prediction of Low-Dose Exposure Range for Several EEDC*

For the synthetic estrogen DES we were able to evaluate all four of the factors listed above, and this accurately predicted the dose of DES administered to pregnant female mice that led to low-dose endocrine disruption of fetuses, measured as increased prostate weight in the subsequent adult (Vom Saal et al., 1997). We also examined the activity of two other EEDC, bisphenol A and octylphenol (Nagel et al., 1997), although we were more limited in terms of information concerning uptake and metabolism for these compounds relative to DES. Nevertheless, there was good correspondence of observed bioactivity in the animal with the prediction of the bioactive low dose in the animal for both octylphenol and bisphenol A.

In addition, we include here new findings on the current-use pesticide methoxychlor. The major endocrine-disrupting activities of methoxychlor derive not from the parent compound, but from two metabolites formed in the liver by demethylation to mono- and bis-hydroxymethoxychlor. The most potent metabolite is bis-hydroxymethoxychlor, which shows both strong estrogenic activity (Bulger et al., 1978) as well as equally potent antiandrogenic activity (Gray et al., herein). By comparing the estrogenic activity of bis-hydroxymethoxychlor to that of the more fully characterized DES, we can approximate an expected low-dose exposure range for methoxychlor. As described below, this range proved to be close to the actual dose of methoxychlor that caused endocrine-disrupting activity in the prostate of male mouse fetuses when fed to pregnant female mice.

Results supporting the experimental approach to understanding and predicting low-dose effects

1. *Developmental Effects of Estrogen on Rodent Reproductive Tract Development*

In this section we describe fetal developmental effects of normal circulating estrogen on the development of the male reproductive tract. Because developmental effects can lead to permanent alterations in the animal, these effects have high impact on the animal, and the high fetal sensitivity to hormones may make the fetus a particularly sensitive target for exposures to environmental estrogens.

Intrauterine Fetal Position Phenomenon

As a consequence of steroid transport between fetuses during development, male mice that develop *in utero* between two female fetuses (2F males) are exposed to higher concentrations of estradiol (about 35% difference; Figure 4A) and lower concentrations of testosterone (about 30% difference) than are male fetuses that develop between two male fetuses, referred to as 2M males (Vom Saal, 1989). There are a number of traits observed in the adult that differ with intrauterine fetal position in both males and females (Vom Saal, 1989; Vom Saal et al., 1999). Of particular interest, we found that the prostate in 2F males was significantly larger (by about 30%; Figure 4B) than that in 2M males. The enlarged prostate in 2F males was also associated with a threefold greater number of prostatic androgen receptors in 2F relative to 2M males (Figure 4C) (Nonneman et al., 1992). These findings suggested that a slight increase in estradiol during fetal life actually led to prostate enlargement and an increase in the number of prostatic androgen receptors during later adult life. However, comparisons of 2F and 2M animals represent correlational studies that do not allow for conclusions concerning causality, which require experimental manipulations to determine.

Free versus Total Circulating Estradiol: The Free Hormone Hypothesis

The free hormone hypothesis is largely accepted for estrogens and other steroids (Ekins et al., 1982; Mendel, 1989). However, unlike for the assessment of thyroid hormones or

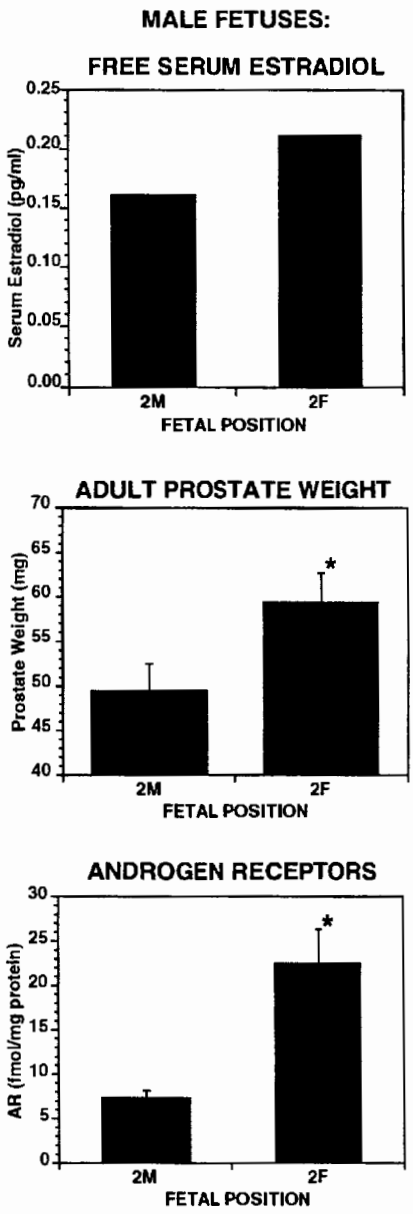

Figure 4. (A) The free serum estradiol concentration (pg/ml) in 2M (between two males *in utero*) and 2F (between two females *in utero*) male mice fetuses. (B) Mean (±SEM) prostate weight (mg) in 2M and 2F adult male mice. (C) Androgen binding by high-salt extracts (occupied receptors) of 2M and 2F adult mouse prostates. * $p < 0.01$; $n = 7$ 2M and 2F prostates. From Nonneman et al. (1992).

testosterone, where the free levels are monitored routinely in clinical determinations, free estrogens are not often evaluated, in part because of the difficulty in detecting the very low levels of circulating estrogen that are free. We developed methods for measuring the very low free level of estradiol in rodent fetuses, and detailed methods are described elsewhere (Montano et al., 1995). This is especially important for analysis of chemical estrogens because individual chemicals may not bind to the extent as natural estrogens to the serum binding proteins that maintain a low, stabilized level of natural estrogens during fetal development (Figure 3). We, and others, have reported that a characteristic of some EEDC is that they circumvent the fetal protection mechanism that serum binding provides, and this can be evaluated by focusing on the levels of free circulating estrogens instead of the total values (free plus bound) in the serum (Skalsky and Guthrie, 1978; Sheehan and Young, 1979; Nagel et al., 1997, 1998).

Experimental Low-Dose Increase in Estradiol and DES

The changes in prostate development described above due to a male developing in an intrauterine position between female fetuses were subsequently found to be induced by an experimental increase in serum estradiol. Specifically, we experimentally increased serum estradiol levels in male mouse fetuses during the initial phase of fetal prostate development by implanting pregnant females with a Silastic capsule containing estradiol (Vom Saal et al., 1997). The dose of estradiol that we administered 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 groups). This increase in estradiol 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 three-dimensional reconstruction of the prostate collected from male fetuses on gestation day 18, one day after initiation of fetal prostate development (Vom Saal et al., 1997). In addition, the developing prostatic epithelial glandular ducts were enlarged in estrogen-treated males relative to control males. These changes in prostate development were permanent. In adulthood (8 months), males exposed to the 50% increase in estradiol during fetal life had enlarged prostates (by 40%; Figure 5) that showed a sixfold increase in prostatic androgen receptors relative to prenatally untreated males (Vom Saal et al., 1997).

The same developmental increase in 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 during gestation days 11–17 (Figure 6). Males exposed during fetal life to these low doses of DES had significantly enlarged prostates in adulthood relative to control males (Vom Saal et al., 1997). The doses which modified the development of the prostate to yield a larger adult organ were thousands of fold lower than the doses examined in

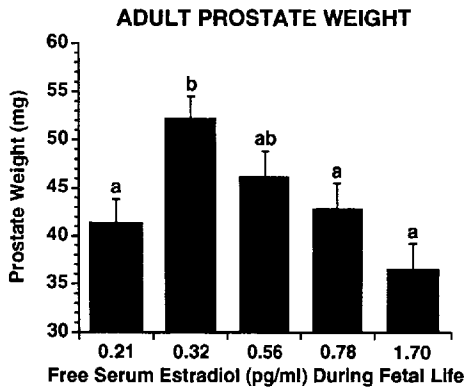

Figure 5. Mean (± SEM) prostate weight (mg) in 8-month-old male mice produced by mothers implanted s.c. with Silastic capsules containing 0, 25, 100, 200, or 300 μg of estradiol from day 13 to 19 of pregnancy. The free serum estradiol concentration (in pg/ml) in male fetuses on gestation day 18 in response to these doses of estradiol (controls = 0.21 pg/ml) is shown in relation to adult prostate weight. Group means that differed significantly are indicated by different letters, while groups with the same letter did not differ significantly. From Vom Saal et al. (1997). Copyright (1997) National Academy of Sciences, U.S.A.

prior studies of DES and reproductive tract cancers (McLachlan et al., 1975; Santti et al., 1994).

2. *Evaluation of EEDC to Predict Dose that will be Active in the Developmental Response*

In this section we asked whether the range of activity of EEDC could be understood by reference to the experimental levels of estradiol that acted on the development of the prostate. We modeled bioactivity of several environmental estrogens by addressing four key factors that influence the EEDC activity: (1) absorption and metabolism relative to the route of exposure, (2) how the compound partitions between the circulation and body lipid, (3) how the compound is carried in blood and what fraction is delivered free (unbound) to cells, and (4) the intrinsic estrogenic activity of the molecule in interaction with estrogen receptors in the nucleus of the cell. The factors are addressed in reverse of the numerical order listed above.

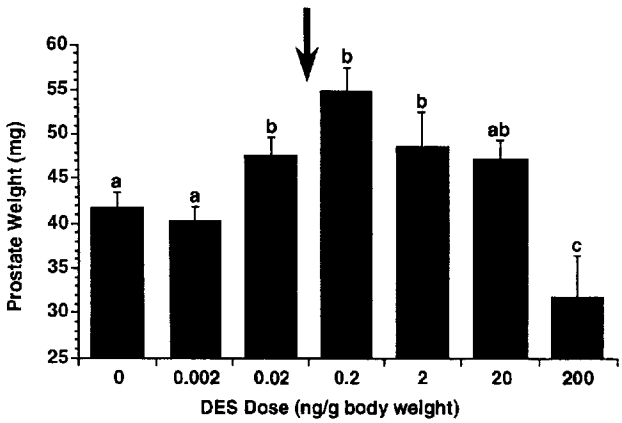

Figure 6. Mean (± SEM) prostate weight (mg) in 8-month-old CF-1 male mice produced by females fed different doses of DES from day 11 to 17 of pregnancy. Group means that differed significantly are indicated by different letters. Arrow = the dose of DES predicted to be active by our approach. From Vom Saal et al. (1997). Copyright (1997) National Academy of Sciences, U.S.A.

Factor 4: Intrinsic Estrogenic Activity of the Chemical

There are many *in vitro* assays of intrinsic estrogenic activity that are capable of determining the estrogenic activity or the affinity for estrogen receptors, relative to the activities or affinity of estradiol. We and others have used the estrogen-dependent proliferation of human breast cancer-derived MCF-7 cells (Welshons and Jordan, 1987; Welshons et al., 1987; Soto et al., 1995) in prior studies. One of the reasons for using this MCF-7 cell assay is that it is sensitive to estrogen (as free, unbound estradiol) in the same range that is present during prostate development in the mouse. The half-maximal response of the MCF-7 cell proliferation assay is approximately 0.2 pg free estradiol/ml medium (Table 1), while the midrange value of free estradiol in male mouse fetuses during the initial critical period for prostate development is 0.21 pg/ml (Vom Saal et al., 1997). Therefore, the sensitivity of MCF-7 cells corresponds very closely to the sensitivity of the mouse prostate cells, whose development is modeled by the activity of EEDC in MCF-7 cells. Not all assays of estrogenic activity are capable of detecting estrogen in the low, physiological range.

Factor 3: How the Compound is Carried in Serum

To evaluate the role of serum in the access of environmental estrogens for intracellular estrogen receptors and its importance in the bioactivity of EEDC, we developed the relative binding affinity–serum modified access (RBA–SMA) assay (Nagel et al., 1997). This assay is based on determining the relative binding affinity (RBA) of the test

Table 1. Sensitivities of mouse fetal prostate development and MCF-7 cell proliferation assay.

	Free estradiol (pg/ml)
Fetal mouse prostate size, mid-physiological range	0.21
MCF-7 cell proliferation, 50% maximal response	0.2

Calculated from total estradiol of 94 pg/ml, and free (unbound) fraction of estradiol at 0.23% in fetal mouse serum in the 1 MF male (male between 1 male and 1 female fetus *in utero*) (Vom Saal et al., 1997); and from total estradiol of 2.6 pM (0.71 pg/ml) at 50% maximal proliferative response (from Figure 2, legend), and free estradiol of approximately 30% in MCF-7 cell medium (Welshons et al., unpublished observations).

compound in 100% serum (affinity of the EEDC relative to estradiol, which serves as the reference compound), compared to the RBA of the EEDC determined in serum-free medium. By examining the uptake into MCF-7 cells of EEDC with and without serum present, we determined that serum substantially affected the measurement and interpretation of the relative estrogenic activities of several EEDC (Nagel et al., 1997, 1998), including bisphenol A and octylphenol (Figure 7), and a number of additional estrogenic drugs, industrial chemicals, and pesticides (Nagel et al., 1998).

Factors 1 and 2: Absorption, Metabolism, and Partitioning in the Body

Evaluation of absorption, metabolism, and partitioning in the body with *in vitro* assays is complex and we have no *in vitro* approach for them at this time. However, if the estrogenic chemical is available in tritium-labeled form, the final result of these factors can be determined directly, by evaluating in one step how an administered dose is recovered in serum. For example, this permitted the evaluation of how a dose of tritiated DES administered to the pregnant mouse is recovered in the serum of the fetus (Shah and McLachlan, 1976). The RBA–SMA assay described above can then assess estrogenic activity from the serum to the intracellular receptors. Unfortunately, even

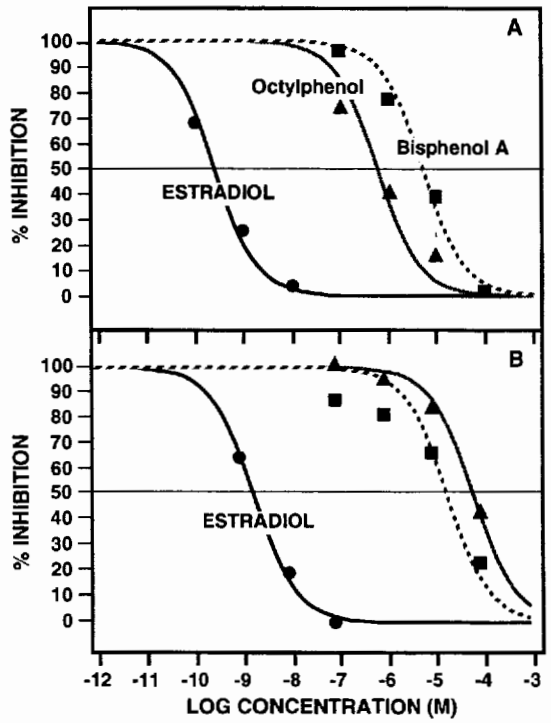

Figure 7. Relative binding affinity–serum modified access (RBA–SMA) assay. Unlabeled reference estradiol, octylphenol, or bisphenol A competed with (A) 1 nM [^{3}H]estradiol in serum-free media or (B) 10 nM [^{3}H]estradiol in 100% adult male serum. From Nagel et al. (1997).

for levels in fetal serum following maternal dosing, there have been few EEDC that have been examined.

3. *Prediction of Low-Dose Exposure Range for Several EEDC*

Diethylstilbestrol For DES, all of the key factors could be evaluated in studies with pregnant mice. Intrinsic estrogenic activity of DES and effects of delivery to target cells by serum were evaluated with the RBA–SMA assay with male human serum (estradiol free fraction of 4%) and then extrapolated to the conditions of mouse fetal serum (estradiol free fraction of 0.2%). The factors of absorption, metabolism, and distribution to the fetus following maternal dosing were evaluated from the work of Shah and McLachlan (1976) with tritiated DES. In that report, the authors concluded that approximately 3% of the maternal dose was subsequently recovered in and significantly retained in the fetal circulation.

Calculations for evaluating the estrogenic endocrine-disrupting activity of DES by the RBA–SMA assay, and incorporation of information on metabolism and fetal distribution, are shown in Table 2, A–E. These calculations, which are discussed in greater detail elsewhere (Nagel et al., 1997), yielded a dose of DES predicted to be active in the prostate endocrine disruption model of 0.077 μg DES per kg maternal body weight per day (Table 2E). With data for all key factors available, the dose of DES predicted to fall within the low-dose endocrine disruption range that was observed with feeding of DES to pregnant mice (Figure 6) was essentially identical with the dose predicted by our approach (Figure 6, arrow). Prediction of an active dose *without* considering delivery of DES by fetal serum and uptake into the developing reproductive tract yielded 8 μg/kg (Table 2E), over 100 times higher than the dose predicted by taking all relevant information into account. The implications of dose–response relationships in endocrine disruption are further discussed elsewhere (Vom Saal et al., 1997, 1998; Vom Saal and Sheehan, 1998). However, accurate prediction of fetal dose is clearly crucial in evaluating low-dose endocrine disruption. In the experiment of Figure 6, testing only at or above the 100 times higher dose predicted, without considering delivery by serum, might well have been unable to detect an increase in prostate weight by DES.

Bisphenol A and Octylphenol

Most known environmental estrogens are not available in radiolabeled form, and we could directly evaluate only two of the key factors described above: serum binding and intrinsic activity. For the two EEDC bisphenol A and octylphenol, we applied the RBA–SMA analysis to predict relative estrogenic activities (Nagel et al., 1997, 1998) and

Table 2.

A. Relative binding affinity–serum modified access (RBA–SMA) assay: RBA analysis was conducted in serum-free medium (SFM) and in 100% adult male serum

Compound	*N*	RBA in SFM (%)	SEM[a]	RBA in serum (%)	SEM[a]	SMA[b]	SEM[a]
Estradiol	7	100		100		1.00	
DES	7	9.38	± 9.24	51.15	± 3.79	6.18	± 1.13

B. Serum modified access (SMA): extrapolation of SMA[c] from adult human serum to fetal mouse serum for diethylstilbestrol (DES)

	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
DES	1.0	6.18	$(6.18-1) \times 20^{d} + 1$	=	104.6

C. Predicted fetal bioactivity[e] (% of E_2): Predicted estrogenic activity (relative to estradiol), calculated from the predicted SMA from (B), for DES

	Predicted SMA in fetal mouse		RBA in serum-free medium (%)		Predicted bioactivity[f] (%)
DES	104.6	×	9.38	=	981

D. Calculation of reference doses for DES (see note for D below)

DES	Estradiol reference[g]		Predicted bioactivity[h] (%)		Equivalent estrogenic activity[i] (pM)		MW (μg/umol)		Reference dose[j] (μg/kg)
SERUM	84 pM	÷	981	=	8.56	×	268.4	=	0.0023
SERUM-FREE	84 pM	÷	9.38	=	896	×	268.4	=	0.24

E. Calculation of final reference dose by accounting for fetal distribution of maternal dosing of DES: Predicted dose to the mother that will lead to circulating fetal DES equivalent to estradiol at 84 pM, a circulating concentration of estradiol that leads to prostate enlargement

DES	Reference dose[k] (μg/kg)		Fetal distribution of maternal dose[l] (%)		Final reference dose[m] (μg/kg)
SERUM	0.0023	÷	3	=	0.077
SERUM-FREE	0.24	÷	3	=	8.0

[a] SEM = standard error of the mean.
[b] SMA (Serum Modified Access) = RBA in serum ÷ RBA in serum-free medium.
[c] SMA = RBA in serum ÷ RBA in serum-free medium.
[d] [4% ÷ 0.2% = 20-fold].
[e] Predicted SMA × RBA in serum-free medium = predicted RBA in fetal mouse serum.
[f] Predicted estrogenic activity in fetal mouse serum.
Note for D: The reference dose for the xenoestrogen DES is the concentration (in units of μg/kg) of DES in fetal circulation that would result in 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 (B) (SERUM), the results of the RBA–SMA assay. For comparison, the reference dose was also calculated from the RBAs that were determined in serum-free medium (SERUM-FREE); these relative activities are representative of the values that have been obtained with assays which do not take the effects of serum into account when measuring xenoestrogen activity.
[g] Fetal estradiol elevation that resulted in adult prostate enlargement: 84 pM [= 23 pg/ml (Vom Saal, 1989; Nonneman et al., 1992)].
[h] From (C), activity relative to estradiol, based on relative molar concentrations; the predicted bioactivity SERUM-FREE is the RBA measured in serum-free medium.
[i] Equivalent estrogenic activity; molar concentration of DES predicted to equal the estrogenic activity of the estradiol reference.
[j] Equivalent estrogenic activity in mol/L converted to mass, μg/L; value then expressed as μg/kg for reference dose; molecular weight (MW) of DES = 268.4.
[k] Reference dose from (D) = the circulating level of DES predicted to be as estrogenic as 84 pM estradiol in fetal mouse circulation.
[l] Distribution of maternal dose of [^{3}H]DES to fetal circulation in the mouse; approximately 3% of maternal dose reaches fetal circulation (Shah and McLachlan, 1976).
[m] Final reference dose = the dose of DES administered to the pregnant mouse that will lead to a circulating level of DES in the fetus that is predicted to be as estrogenic as 84 pM estradiol in fetal mouse circulation.

estimated actual active doses with less information than was available for DES. The effects of delivery of these EEDC to target cells by serum and its binding proteins changed the relative estrogenic activities of these two

compounds and predicted that bisphenol A would be more than 500-fold more active than octylphenol in endocrine disruption. This contrasted sharply with prior published results that octylphenol was approximately tenfold more estrogenically active than bisphenol A.

The calculations are detailed in Table 3 and are discussed in detail elsewhere (Nagel et al., 1997). The analysis will not be explained here other than to indicate that the results predicted (1) that bisphenol A would be far more active than octylphenol (Table 3C), and (2) bisphenol A

Table 3.

A. Relative binding affinity–serum modified access (RBA–SMA) assay: RBA analysis was conducted in serum-free medium (SFM) and in 100% adult male serum

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

B. Serum modified access (SMA): extrapolation from adult human to fetal mouse serum

	Serum-free (100% free E_2)	Adult human (4% free E_2)	Estimated fetal mouse (0.2% free E_2)		Predicted SMA[c] in fetal mouse
Bisphenol A	1.0	1.7	$(1.7-1) \times 20^d + 1$	=	15
Octylphenol	1.0	0.045	$0.045 \div 20^d$	=	0.0022

C. Predicted fetal activity[e] (% of E_2)

	Predicted SMA in fetal mouse		RBA in serum-free medium		Predicted bioactivity[f] (%)	Relative activity[g]
Bisphenol A	15	×	0.006	=	0.09	563
Octylphenol	0.0022	×	0.072	=	0.00016	1

D. Calculation of xenoestrogen reference doses (see note for D below)

	Estradiol reference[h] (pM)		Predicted bioactivity[i] (%)		Equivalent estrogenic activity[j] (μM)		MW (μg/μmol)		Reference dose[k] (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] SEM = standard error of the mean.
[b] SMA (serum modified access) = RBA in serum ÷ RBA in serum-free medium.
[c] SMA = RBA in serum ÷ RBA in serum-free medium.
[d] [4% ÷ 0.2% = 20-fold].
[e] Predicted SMA × RBA in serum-free medium = predicted RBA in fetal mouse serum.
[f] Predicted estrogenic activity in fetal mouse serum.
[g] Predicted bioactivity of bisphenol A/octylphenol.
Note for D: 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 (C) (SERUM). For comparison, reference doses were also calculated using the RBAs that we determined in serum-free medium (SERUM-FREE); these relative activities are representative of the values that have been obtained with assays that do not take the effects of serum into account when measuring xenoestrogen activity.
[h] Fetal estradiol elevation that resulted in adult prostate enlargement: 84 pM (= 23 pg/ml (Vom Saal, 1989; Nonneman et al., 1992)).
[i] From (C), activity relative to estradiol, based on relative molar concentrations; the predicted bioactivities SERUM-FREE are the RBAs measured in serum-free medium.
[j] Equivalent estrogenic activity; molar concentration of xenoestrogen predicted to equal the estrogenic activity of the estradiol reference.
[k] Equivalent estrogenic activity in mol/l converted to mass, μg/l; value then expressed as μg/kg for reference dose; MW of bisphenol A = 228.3; molecular weight (MW) of octylphenol = 208.3.
From Nagel et al., 1997.

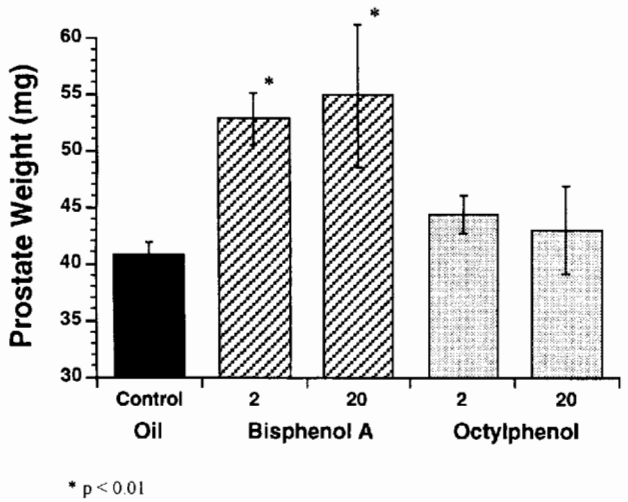

Figure 8. Prostate weight in adult male mice (6 months old) exposed as fetuses to bisphenol A or octylphenol. Mothers were fed 2 or 20 μg/kg body weight/day bisphenol A or octylphenol from days 11 to 17 of pregnancy. Error bars are the standard error; $n = 7$ for bisphenol A and octylphenol, and $n = 11$ for unexposed controls; * $p < 0.01$. From Nagel et al. (1997).

could be active in endocrine disruption at a dose of approximately 20 μg/kg (Table 3D), a dose thousands of times lower than the no-effect or NOAEL for bisphenol of 50 mg/kg previously reported by the Society of the Plastics Industry, which had been used to establish an acceptable daily intake dose for this chemical by government regulators (Society of the Plastics Industry, 1996). When bisphenol A and octylphenol were fed to pregnant mice at both 2 and 20 μg/kg body weight/day from day 11–17 of pregnancy, the prediction that the 20 μg/kg dose of bisphenol A would be bioactive, while octylphenol would not be bioactive in terms of an effect on prostate development, was confirmed (Figure 8). Importantly, even without full information on absorption, metabolism and fetal distribution of these two compounds, our approach to estimating a low-dose-active exposure range from the RBA–SMA assay yielded information that permitted a prediction of the low-dose exposure range for these EEDC that was orders of magnitude more accurate than had been predicted from prior studies that used only high doses to examine acute toxicity (Nagel et al., 1997; Vom Saal et al., 1998).

Estrogenic effects of the current-use pesticide methoxychlor on development of the prostate in male mice

We report here for the first time results on low-dose endocrine disruption by methoxychlor in the part per billion (ppb) range of maternal exposure. Methoxychlor is an insecticide that provides an example of a proestrogen; the compound is metabolized by liver enzymes to at least two estrogenic compounds that circulate at significant levels, mono- and bis-hydroxymethoxychlor (Bulger et al., 1978). The estrogenic activity of bis-hydroxymethoxychlor is within tenfold of the estrogenic activity of estradiol and DES, indicating that this current-use pesticide might be quite active as an endocrine disruptor despite the requirement for metabolic activation.

Methods

In this as in all previously described studies, we used CF-1 mice (*Mus domesticus*). Adult females were time-mated. Methoxychlor was dissolved in tocopherol-stripped corn oil (Cat# 901415, ICN, Aurora, OH). Each pregnant female received daily administration of 30 μl of corn oil (with or without a chemical) from day 11 to day 17 of pregnancy (day 0 = mating). An electronic micropipetter (Rainin) enabled delivery of an accurate volume of corn oil into the mouth of an animal. Mice readily consume corn oil, and this procedure did not result in the stress associated with gavage.

On day 11 of pregnancy, females were randomly assigned to treatment groups. Females were administered corn oil alone (vehicle controls), and methoxychlor in two doses: 20 and 2000 μg/kg maternal body weight. Females were allowed to deliver naturally, and pups were weaned on postnatal day 23. Males were housed three per cage with other male siblings. Only one male from each litter was randomly selected for examination of prostate weight to control for litter (maternal) effects. When males were adults (8.5 months old), a randomly selected male from each litter was individually housed for 4 weeks to eliminate any effects of having been housed with other males. At the end of this time, the individually housed males were killed by CO_2 asphyxiation and cervical dislocation, and the prostate, seminal vesicles, preputial glands, liver, and adrenals were removed and weighed. The seminal vesicles and preputial glands were squeezed to remove fluid. The prostate, seminal vesicles, and preputial glands were removed by one researcher to minimize variability in organ weight due to differences in tissue collection. In addition, animals were coded so that persons removing tissues were unaware of experimental treatment.

Results

When fed to pregnant female mice, we found that methoxychlor was active in endocrine disruption of the prostate and seminal vesicles of male offspring (Table 4). Specifically, fetal exposure to methoxychlor (by feeding to the mother) resulted in an increase in adult prostate weight at doses of 20 and 2000 μg/kg/day and appeared to be only 100-fold weaker than DES (based on dose administered to pregnant females). Quantitatively this could be

Table 4. Mean weights (± SEM) of organs in 9.5-month-old CF-1 male mice exposed prenatally (Day 11–17 of pregnancy) to methoxychlor (MXC).

	Body weight (g)	Prostate (mg)	Seminal vesicles (mg)	Preputials (mg)	Testes (mg)	Liver (g)	Adrenals (mg)
Control	39.2 ± 1.1	40.0 ± 3.0	66.3 ± 3.7	41.2 ± 2.9	242 ± 6.6	2.26 ± 0.03	4.2 ± 1.3
MXC 20	38.6 ± 1.3	64.5 ± 3.7**	77.3 ± 4.5	42.2 ± 3.8	249 ± 6.9	2.15 ± 0.04*	5.0 ± 1.5
MXC 2000	37.4 ± 1.5	60.3 ± 4.1**	79.5 ± 5.0*	46.2 ± 3.8	244 ± 7.3	2.12 ± 0.04*	4.7 ± 0.6

Only one male from each litter was examined to control for litter effects. Seminal vesicles and preputial glands were blotted to remove fluid prior to weighing. Vehicle controls ($n = 9$); methoxychlor 20 μg/kg ($n = 6$) and 2000 μg/kg ($n = 5$). Body weight accounted for a significant portion of the variance for the seminal vesicles ($p < 0.05$) and liver ($p < 0.001$), and was marginally related to prostate weight ($p = 0.06$) and testis weight ($p = 0.07$), and means presented for these organs are adjusted for the effect of body weight by analysis of covariance. Planned comparisons of adjusted means were conducted using the LS means test on SAS (GLM procedure). Body weight was unrelated to adrenal weight ($p > 0.1$) and preputial gland weight ($p > 0.1$), and these organs were compared by ANOVA without correction for body weight.
* $p < 0.05$; ** $p < 0.001$; analysis of covariance.

interpreted as requiring a higher exposure (feeding) dose due to the requirement for synthesis of bis-hydroxy-methoxychlor from the inactive parent compound.

Overall, our approach provided a basis for understanding the endocrine-disrupting activity by methoxychlor at only 20 μg/kg, which is below the current reference dose (the dose predicted to not increase risk of adverse effects due to consumption over a lifetime) (ATSDR, 1994). We have previously reported that this low, 20 μg/kg dose also resulted in an increase in territorial urine marking behavior in male siblings of the males used for the prostate measurement in this study. The 2000 μg/kg dose also resulted in an increase in territorial behavior (Vom Saal et al., 1995).

Methoxychlor Acts through Multiple Receptor-Mediated Mechanisms

An unexpected aspect of these results is that the prostate enlargement produced by methoxychlor appeared to be greater than the enlargement brought about by fetal exposure to estradiol, DES, or bisphenol A in prior studies. The increase in prostate weight due to prenatal exposure to methoxychlor was approximately 60% greater than controls. In addition, in previous studies the seminal vesicles have been found to be smaller in males exposed to a slight elevation in natural estrogens or xenoestrogens during fetal life (Nonneman et al., 1992; Vom Saal et al., 1998). In contrast, the 2000 μg/kg dose of methoxychlor significantly increased seminal vesicle weight relative to control males. Another unexpected finding was that at both the 20 and 2000 μg/kg doses, methoxychlor resulted in a smaller liver relative to controls.

Other information in this journal edition presented by Gray et al. suggests that the bis-hydroxy metabolite of methoxychlor acts through multiple mechanisms. The metabolite binds to estrogen receptors and mimics the activity of estradiol, and also binds to androgen receptors and inhibits the activity of androgens. However, its effects appear to be highly tissue specific and this suggests that methoxychlor may also act *via* additional, as yet unknown, mechanisms, as suggested initially by Lubahn (Lubahn et al., 1998). One intriguing hypothesis is that the separate endocrine-disrupting activity by bis-hydroxymethoxychlor as a potent *androgen antagonist* interacts with its activity as a potent *estrogen agonist*, and leads to endocrine disruption of prostate development through these two receptor mechanisms crucial in the development of the male reproductive tract. It may be that somehow this interaction results in a greater effect on the prostate (in terms of permanent enlargement) than exposure to a low dose of estrogen alone. This hypothesis is currently being examined.

Conclusions

High fetal sensitivity to endocrine disruptors is due in part to chemicals which are able to circumvent the normal serum binding protection mechanisms. Our approach based on the evaluation of free (unbound) EEDC is able to predict low-dose exposures to EEDC which result in permanent, developmental effects on the offspring of exposed mothers.

Low-dose predictions through the RBA–SMA assay yielded unexpected results, particularly for bisphenol A and octylphenol, but these predictions were confirmed in exposure tests in animals. The low-dose prediction was essentially exact for DES, where data on metabolism and maternal-to-fetal distribution are available.

Testing a low, physiologically-relevant dose of methoxychlor (20 μg/kg) by feeding it to pregnant mice permanently increased the weight of the prostate in male offspring. This dose had been deemed 'safe' for human consumption (below the reference dose) based on traditional 'high dose' studies with this chemical. A higher dose of methoxychlor, 2000 μg/kg, permanently increased both prostate and seminal vesicle weight. Neither dose of methoxychlor altered body weight.

The studies and strategies we describe are important in developing criteria for a tiered testing system for the

detection of estrogenic chemicals as well as endocrine-disrupting chemicals with different modes of action. In this regard it seems unlikely that any single assay, *in vitro* or in animals, would ever be sufficient alone to predict human or ecological effects at different doses. However, a panel of appropriate *in vitro* assays based on the use of human or other animal cells can contribute greatly to an understanding of mechanisms of action and can provide critical information regarding the physiologically relevant dose range to use in animal studies. This information is necessary to assess both human and ecological relevance but has not previously been incorporated into the design of toxicological studies used in risk assessment to establish acceptable levels of exposure. Using our approach, we were able with reasonable accuracy to predict doses of xenobiotic estrogens that mimic the effects of low doses of estradiol when administered during fetal development. Our findings reveal that the impact of xenoestrogens is substantially affected by their specific access to cells from serum, and that *in vitro* assays that evaluate the effective free concentration and other factors substantially improve the prediction of the bioactivity of endocrine disruptors.

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